LETTER

Different Gene Networks Are Disturbed by Zika Virus Infection in A Mouse Microcephaly Model

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Abstract The association of Zika virus (ZIKV) infection with microcephaly has raised alarm worldwide. Their causal link has been confirmed in different animal models infected by ZIKV. However, the molecular mechanisms underlying ZIKV pathogenesis are far from clear. Hence, we performed global gene expression analysis of ZIKV-infected mouse brains to unveil the biological and molecular networks underpinning microcephaly. We found significant dysregulation of the sub-networks associated with brain development, immune response, cell death, microglial cell activation, and autophagy amongst others. We provided detailed analysis of the related complicated gene networks and the links between them. Additionally, we analyzed the signaling pathways that were likely to be involved. This report provides systemic insights into not only the pathogenesis, but also a path to the development of prophylactic and therapeutic strategies against ZIKV infection.

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Introduction

Zika virus (ZIKV) is a global concern as a result of its association with severe birth defects, including microcephaly and other congenital malformations. The Brazilian Ministry of Health reported a nearly 20-fold increase of neonatal microcephaly cases in 2016 [1]. As a result, the WHO declared a public health emergency of international concern on Feb 1, 2016. Although the outbreak has been largely controlled in American countries, the possibility of a new epidemic still exists. In October 2018, a Zika outbreak in Rajasthan, India, which affected at least 117 people, was reported by the Rajasthan health department and in The Hindu.

Disturbance of normal neurogenesis, including proliferation/self-renewal and differentiation of neural progenitor cells (NPCs), and neural development processes would cause developmental brain disorders including microcephaly [2–4]. Mammalian brains consist of 50%–90% glial cells, including oligodendrocytes, astrocytes, and microglia [5–8]. Therefore, normal glial cell development is also essential for normal brain size and brain function.

The causal link between ZIKV infection and microcephaly has been demonstrated in multiple mouse models [9–14]. Importantly, ZIKV has been shown to replicate efficiently in mouse embryonic brain by infecting NPCs directly in the early stages of neural development, thus disturbing the developing of various neuronal lineages [10,12,14,15]. The infection leads to deregulation of NPC proliferation and differentiation, as well as neuronal death. ZIKV infection also leads to the microglial hyperplasia, reactive gliosis, delayed myelination, and corpus callosum hypoplasia in humans [16–18]. Glial cell develops during the late stages of brain development and this process continues after birth [6,19]. Recently, we generated a murine fetal brain ZIKV infection model of microcephaly, which recapitulates most of the symptoms of the congenital ZIKV syndrome in humans [15]. These animals are able to survive after birth and phenocopy the progressive neuronal cell death, microglia activation, and astrogliosis found in humans. In addition, disturbance of glial cell development including the abolishment of oligodendrocyte development was also detected [15]. ZIKV infection has been hypothesized as a trigger for the immune response and aberrant expression of genes associated with cell proliferation, differentiation, and death, as well as neurogenesis and microcephaly [10,12,14,20]. However, whether ZIKV infection dysregulates expression of genes related to glial cell development, microglia activation, and other brain development associated processes is not known. More importantly, detailed analyses of the ZIKV infection-induced gene network disruption, the associated pathological processes, and the relationships between the affected pathways are still lacking.

Although there have been multiple studies of ZIKV infection in cultured neuronal progenitors and cerebral organoids [21–24], the intensive immune response and perturbations in a number of biological processes reported in animal models were not observed in these studies [10,12–15]. This is likely due to the lack of microglia and other glial cells in cultured systems. Thus, we carried out high-quality global transcriptome analyses (RNA-seq) of mouse brains cultivated from a fetal brain ZIKV infection model of microcephaly [15]. At the biological network level, global gene expression analysis revealed significant dysregulation of gene sub-networks related to brain development and several other biological processes that may be involved in the disruption of brain development. Importantly, we provide a detailed analysis of the cross-talk between different sub-networks/processes and the signaling pathways involved.

Results

ZIKV infection represses the sub-networks of neural development

To investigate the global impact of ZIKV infection on brain development at the molecular level, we carried out RNA-seq analyses of mouse brains infected with ZIKV. ZIKV infection at embryonic day 15.5 (E15.5) caused severe microcephaly in newborn mice [15]. The production of prenatal neurons peaks at around E15.5, whereas gliogenesis and generation of neuronal networks remain very active till postnatal day 3 (P3). We thus chose to perform RNA-seq analyses on 3 pairs of P3 brains which were infected with ZIKV or culture medium at E15.5 as previously described [15]. Genome-wide analysis identified 3327 strict differentially-expressed genes (DEGs) with 2535 significantly upregulated and 792 significantly downregulated [fold change (FC) > 2 or FC < 0.5, false discovery rate (FDR) < 0.05] (Table S1; Figure S1A and B). The significantly enriched Gene Ontology (GO) biological process terms of top 500 DEGs were shown in Figure S1C. In order to obtain more GO biological process terms of interest, we defined 7112 loose DEGs with 3490 upregulated and 3622 downregulated (FC > 1.5 or FC < 0.75, FDR < 0.05) (Table S2).

Brain development starts with neurogenesis followed by the generation of axons and dendrites, as well as the formation of synapses. These processes have been shown to be affected by ZIKV infection during the early and later stages of brain development in multiple animal models [10,13–15]. GO analysis of 3622 loosely downregulated DEGs in the ZIKV-infected brains revealed an enrichment of genes associated with brain development, including the development of different regions or parts of the brain (Figure 1A; Table S3). Importantly, expression of genes associated with neuronal development, including axon and dendrite development, synapse structure or activity, neuronal differentiation, and neural migration, was downregulated in the viral infected brains (Figure 1A; Table S3). Of note, many of these genes were shared amongst the different processes. For example, 22 loosely downregulated DEGs were involved in axon and dendrite development, as well as synapse structure or activity (Figure 1B).

We also found that, in infected brains, expression of 40 genes involved in neurogenesis was downregulated significantly, including Epha4, Epha7, Hes5, Dlx1, and Fgf13 (Figure 1C; Table S4). We went on to analyze in more detail the genes involved in neuronal development. As shown in Figure 1D and Table S4, expression of 80, 38, and 40 genes involved in axon development, dendrite development, and synapse assembly, respectively, was significantly downregulated with ZIKV infection. Many of these genes were also shared between these pathways. For example, Reln, Ephb2, Cacna1a, and Slitrk5 were involved in axon development, dendrite development, and synapse assembly; Mapt, Slitrk1, and Epha7 were involved in both axon development and synapse assembly.
assembly; 15 genes were involved in the development of both axons and dendrites.

In support of these observations, most of the signaling pathways involved in neurogenesis and neuronal development including semaphorin-plexin, ephrin receptor, fibroblast growth factor receptor (FGFR), Wnt, Notch, insulin-like growth factor receptor (IGFR), vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR), retinoic acid receptor, platelet-derived growth factor receptor (PDGFR),
ZIKV infection induces neuronal cell death

Massive cell death, especially in neurons, has been detected in aborted ZIKV-infected human fetus as well as in various animal models [1,10,13–15]. Enrichment analysis of GO biological process terms revealed the enrichment of strictly upregulated DEGs related to cell killing, apoptosis, and necroptosis processes (Figure 2A and B, Figure S2; Table S5). Interestingly, the expression of 57 genes, which were involved in neuronal cell death including \textit{Fas, Fasl, Il-6, and Tnfrsf1a}, was also strictly upregulated by ZIKV infection (Figure 2A and C; Table S5). Some genes were found in more than one of these processes (Figure 2A).

The JNK signaling pathway has been shown to play an important role in neuronal cell death, while the PI3K, Akt, and ERK signaling pathways usually play a role in neuronal survival [25–30]. We found that the expression of 33 genes involved in the JNK pathway, including \textit{Map3k9, Tnf, Tnfrsf11a, and Gadd45a}, was strictly upregulated in ZIKV-infected brains, while only 4 genes showed strictly downregulated expression (Figure 2D; Table S6). Meanwhile, many genes involved in the PI3K, Akt, and ERK cascades showed downregulated expression (Figures S1C and S3; Table S3). These results indicate that the activation of the JNK pathway and/or the suppression of the PI3K, Akt, and ERK pathways may play crucial roles in ZIKV-mediated neuronal cell death.

ZIKV infection of human fetal neural stem cells has been shown to inhibit the Akt-mTOR pathway, which may lead to the activation of autophagy and cell death [31]. Accordingly, we found that the expression of 20 genes associated with autophagy was strictly upregulated in ZIKV-infected brains (Figure S4A; Table S5). Meanwhile, 24 genes involved in TOR signaling pathway showed loosely downregulated expression (Figure S4B; Table S3).

ZIKV infection deregulates the sub-networks of glial cell development

Glial cells represent at least 50% of cells in the brain [6,19]. Most glial cells are oligodendrocytes (around 75.6%), while the rest are mainly astrocytes (around 17.3%) and microglia (around 6.5%) [8]. ZIKV infection has been shown to cause reactive gliosis, microglial cell activation, and delayed myelination, indicating gliogenesis disruption and microglial cell activation [16–18]. Enrichment analysis showed that many of the loosely downregulated DEGs were involved in the glial cell related processes (Figure 1A). In addition, many of the strictly dysregulated DEGs have shared functions in gliogenesis, cell fate commitment, glial cell differentiation, glial cell migration, and microglial cell activation (Figure 3A; Table S6). Interestingly, \textit{Tlr2, Tlr4, and Clu} were shared by microglial cell activation, gliogenesis, and glial cell differentiation, while most genes were shared among the different stages of glial cell development including gliogenesis, glial cell differentiation, and glial cell migration (Figure 3A). This indicates that genes involved in glial cell development may not play an exclusive role in a single biological process, but instead, may function together in multiple processes. To further pinpoint such an interconnected gene network, we performed integrative analysis by combining our RNA-seq data with available protein interaction data [32]. A complex network was identified and shown in Figure 3B. The cross-talk among gliogenesis, glial cell fate commitment, glial cell differentiation, glial cell migration, and microglial cell activation suggests that ZIKV may affect the different stages of glial cell development.

Oligodendrogenesis including oligodendrocyte precursor cell (OPC) proliferation and differentiation has been shown to be disrupted by ZIKV infection in the brain [15]. We found that the expression of 18 genes involved in oligodendrogenesis was loosely downregulated in ZIKV-infected brains (Figure 3C; Table S3). A recent study has identified some genes especially enriched in OPCs [33]. They are recognized as the potential markers for OPCs. Remarkably, the expression of 20 out of the top 40 enriched genes in OPCs, as well as the well-known markers \textit{Olig1} and \textit{Olig2}, was significantly downregulated in brains infected with ZIKV (Figure 3D).

ZIKV infection upregulates gene networks associated with immune response

GO analysis of strictly upregulated DEGs revealed an enrichment of genes associated with viral infection and immune response (Figure 4A; Table S5). Microglial cells are specialized macrophages in the brain and play an essential role in immune response [4,5]. As expected, the expression of 14 genes involved in microglial cell activation was strictly upregulated in ZIKV-infected brains (Figure 4B; Table S6). In support of this observation, the expression of 58 genes involved in phagocytosis, the major function of microglia, was also strictly upregulated (Figure S4C; Table S6).

Among the strict DEGs, genes involved in cytokine production and cellular responses to cytokines (277 upregulated vs. 18 downregulated DEGs) were the most significantly enriched, indicating that cytokines may play a crucial role in the pathogenesis of ZIKV infection (Figure 4C; Table S6). Interestingly, 42 of these genes were involved in both cytokine production and cellular responses to cytokines (Figure 4C; Table S6). Accordingly, genes involved in the production of tumor necrosis factor (TNF) and interferons (IFN-α, β, and γ), as well as the cellular responses to these cytokines, are highly enriched (Figure 4A; Table S5). In addition, genes involved in the production of different interleukins (ILs) showed strictly upregulated expression following ZIKV infection (Figure 4A; Table S5). GO enrichment analysis indicated that dozens of signaling pathways involved in the immune response were significantly upregulated (Table S5). These results are consistent with the recent reports that ZIKV infection induces the immune response and upregulates different cytokines in various systems [22,34].
Cytokines are a family of signaling effectors, which includes IFNs, ILs, and TNFs. IFNs belong to a large class of cytokines and are named for their capability to "interfere" with viral replication and protect cells from viral infection [35]. GO analysis revealed that there is a special enrichment of strict DEGs (144 upregulated and 3 downregulated) related to production and response to IFNs (Figure 5A; Table S6). Although expression of many of these genes was dysregulated specifically for the production or response to one type of IFNs, some of them have multiple roles (Figure 5A). For example, Tlr3, Tlr4, Tlr7–Tlr9, Havcr2, and Ptpn22 are involved in the production of different IFNs including IFN-α, β, and γ, while Txk,
Xcl1, Il12rb1, Slc11a1, Irf8, and Il12b have roles in both the production and cellular response to IFN-γ (Figure 5A).

TNF-α is involved in systemic inflammation and is one of the cytokines that makes up the acute phase reaction [36]. Originally observed in leukocytes, ILs as an important group of cytokines are found in a wide variety of cells including astrocytes and microglia cells in the brain [37]. GO analysis of the strict DEGs revealed an enrichment of genes (143 upregulated and 5 downregulated) involved in the production of TNF-α and different ILs including IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-17, especially those involved in TNF-α and IL-6 production (Figure 5B; Table S6). It is interesting to note that many of these genes have multiple functions. For example, Tlr2–Tlr4, Tlr9, Acp5, Irap3, Pycard, Lep, and Bcl3 play roles in the production of different cytokines of TNF-α, IL-1, IL-6, IL-8, IL-10, and IL-12 (Figure 5B). The number of overlapping genes in these pathways is indicative of the underlying complexity of the system and the
Figure 4  ZIKV infection induces strong immune response
A. Significantly enriched GO biological process terms of strictly upregulated DEGs. B. Heat map of strict DEGs related to microglial cell activation. C. The network of cytokine related strict DEGs grouped by GO term annotation for biological process. The blue nodes denote DEGs. The lines indicate interactions between DEGs and corresponding GO terms. D. Real-time PCR validation of the expression level of Cxcl10 in the ZIKV-infected mouse brain samples. \( P \) value was calculated using unpaired Student’s \( t \)-test. ***, \( P < 0.001 \).
Figure 5  The gene networks of IFN, IL and TNF-α related DEGs

A. The network of IFN-related strict DEGs grouped by GO term annotation for biological process. The purple nodes denote strict DEGs. The colorful fonts denote different GO terms that associated with IFNs and lines indicate interactions between DEGs and corresponding GO terms.

B. The network of IL and TNF-α related strict DEGs grouped by GO term annotation for biological process. The purple nodes denote strict DEGs. The red fonts denote different GO terms. The lines indicate interactions between DEGs and corresponding GO terms.

IFN, interferon; IL, interleukin; TNF-α, tumor necrosis factor alpha.
complex interactions between these networks with ZIKV infection in the brain (Figure 5B).

The induction of many cytokines would activate different signaling pathways. As predicted, most of the signaling pathways mediated by cytokines were also significantly enriched according to the GO analysis, such as different toll-like receptor (TLR) signaling pathways, IFN-mediated signaling pathways, and IL-mediated signaling pathways (Table S5).

Several of the dysregulated genes related to immune response in the ZIKV-infected brains, including Tnf-α, Ifn-α, Il10, Il2, and Mcp1/Ccl2, have been validated by real-time PCR previously based on the analysis before [14]. In addition, the strong induction of Il6 was also confirmed (manuscript in preparation). Importantly, we have validated one of the genes induced most significantly in our RNA-seq data, Cxcl10. Real-time PCR validation indicates that expression of Cxcl10 is induced by nearly 700 folds in ZIKV-infected brains (Figure 4D).

Discussion

In this study, we adopted a fetal brain ZIKV infection animal model of microcephaly, which phenocopied most of the pathologies found in congenital ZIKV syndrome in humans [15]. Using this model, we were able to carry out global transcriptome analysis. We have found that the sub-networks involved in neurogenesis and neuronal development, (neuronal) cell death, glial cell development, microglial cell activation, immune response, and various related signaling pathways were dysregulated in infected brains (Figure 6). Therefore, our global transcriptome analysis provides insight into most of the pathologies detected in brains during ZIKV infection as well as the underlying mechanisms involved in congenital ZIKV syndrome, especially brain development associated disorders.

ZIKV infection has been reported to lead to a strong immune response in the brain [10,12,13]. Our GO analysis disclosed the enrichment of genes associated with the viral and immune responses, with cytokine production and cytokine-mediated signaling experiencing the most significant enrichment. This indicates the critical role of the cytokines in the pathogenesis of ZIKV infection. Interestingly, although the networks for the production of most cytokines were induced, TNF-α and IL-6 were the most prominent ones in the analysis, followed by various IFNs. This may explain why some of the symptoms experienced during the acute phases of infection could be alleviated by TNF-α inhibition [38]. It would be interesting to explore the possibility of inhibiting IL-6, which plays a critical role in the pathogenesis of inflammatory disorders [39,40], for the relief of ZIKV infection.

Brain development starts with neurogenesis followed by the generation of axons and dendrites, as well as the formation of synapses [41,42]. These processes have been reported to be affected by ZIKV infection during different stages of brain development [10,13–15]. In infected brains, we found that all the sub-networks involved in these processes were disturbed. Of note, many downregulated genes involved in various processes had a shared function between many networks. Whether downregulation of the genes involved in brain development including neuronal morphogenesis is the direct effect of ZIKV infection, or indirect effect caused by immune response or cell death or both, remains to be investigated in the future.

ZIKV infection has been shown to induce neuronal cell death especially in the brain, which contributes to the smaller size of the brain [10,12,14,15]. We found that the expression of nearly 60 genes associated with neuronal cell death was significantly induced in the infected brains, indicating that a complicated network is involved in neuronal cell death. Our results indicate that the activation of JNK signaling, together with the suppression of PI3K, Akt, and ERK signaling, might play
a role in ZIKV incurred neuronal cell death. Interestingly, the expression of nearly 40 genes involved in the JNK pathway was significantly dysregulated in the infected brains. In addition, many of the significantly induced genes were involved in cytokine production or in response to cytokine such as TNF, Il1b, Tlr3, Tlr4, Tlr6, Tlr9, Ripk1, and Ripk2. This suggests that cytokines may play a role in JNK activation as well as ZIKV-incurred neuronal cell death.

ZIKV infection of human fetal NPCs has been shown to inhibit the Akt-mTOR pathway, leading to defect in neurogenesis and abnormal activation of autophagy [31]. We found the downregulation of neurogenesis associated genes and upregulation of autophagy associated genes, as well as the inhibition of the Akt, PI3K, and TOR signaling pathways in the ZIKV-infected brains. This would not only support the previous in vitro findings but also provide more insight into the potential molecular mechanisms.

Half or more of cells in the brain are glial cells [6,19]. We show here that a significant number of genes associated with different stages of gliogenesis showed strictly dysregulated expression. Furthermore, the induced expression of 14 genes associated with microglial activation and 58 genes involved in phagocytosis may provide some clues regarding how microglial cells are activated for phagocytosis. Oligodendrocytes are the major glial cell type in the brain. Oligodendrogensis and myelination have been shown to be disrupted by ZIKV infection in the brain [14,15]. Further study of the 18 loosely downregulated DEGs involved in oligodendrogenesis and the 20 loosely downregulated DEGs enriched in OPCs would be helpful for the future study of the mechanism of ZIKV induced disruption of oligodendrogenesis. The development of hippocampus and cerebellum as well as glial cells occurs in late embryonic stage and after birth. Although we have shown previously that the neuronal progenitors of cortical neuron might not be affected significantly when the brain was infected at E15.5, we could not exclude the possibility that glial cell development, which also occurs in late embryonic stage and after birth, is secondary to the effects on proliferation and cell survival of neural progenitors.

In summary, our findings are in agreement with other studies and support the application of this animal model. Furthermore, our findings also support the hypothesis that ZIKV infection dysregulates the gene networks involved in neurogenesis, neuronal development, glial cell development (especially oligodendrogenesis), microglia activation, immune response, and cell death. ZIKV infection induces an extensive immune response, including the production and response to cytokines, which ultimately leads to the activation of astrocytes and microglia, as well as neuron loss. ZIKV infection affects the development of neurons and glial cells at different stages directly or indirectly through the immune response and dysregulation of different signaling pathways. Our global transcriptome dataset would provide valuable resources for the exploration of detailed molecular mechanisms of ZIKV infection as well as the treatment of ZIKV-related pathological effects.

Materials and methods

ZIKV infection of animals and sample preparation for RNA-seq analysis

The pregnant mice were bought from Beijing Vital River Laboratory Animal Technology (Beijing, China). For the infection experiments, 1 μl of ZIKV SZ01 (GenBank: KU866423) virus stock (6.5 × 10⁵ PFU/ml) [43] or culture medium (RPMI medium 1640 basic + 2% FBS) was injected into one side of the lateral ventricle (LV) of E15.5 ICR mouse brains as described previously [10]. Virus or medium was injected at the same side of fetal brains in each pregnant dam. Half of the brains (injected side) from both groups (3 for each group) at P3 were used for global transcriptome analysis, which was performed by CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences.

RNA-seq data analysis

Raw reads were preprocessed by means of the standard Illumina pipeline to segregate multiplexed reads. Sequence quality was inspected with FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Data were preprocessed with BBDuk v37.25 (Brian Bushnell within Geneious 10.2.3), and then aligned to the mouse reference genome (version mm10) using HISAT2 v2.0.4 [44]. The alignment results were sorted using SAMtools v1.3.1 [45]. All fragment quantifications were computed using featureCounts v1.5.1 [46]. In total, 20,660 genes were sequenced and aligned, and 4021 genes with expression detected in less than two samples were not considered in the further analysis. The R package DESeq2 v1.18.1 [47] was used to perform differential expression analyses. The read count-based units for gene expression were normalized based on negative binomial GLM fitting by DESeq2. Altogether, 7112 genes were identified as loose DEGs with FC > 1.5 or < 0.75, and FDR (Benjamini–Hochberg adjusted) < 0.05. Furthermore, 3327 genes were identified as strict DEGs with FC > 2 or < 0.5, and FDR < 0.05.

GO biological process enrichment analysis and heat map visualization

DEGs were analyzed using the R package clusterProfiler v3.8.1 [48] to identify enriched functions (GO biological process terms). Heat map of DEGs selected from related GO terms was generated using gplots R package [49].

Network visualization and analysis

The interactions between proteins encoded by the identified DEGs among different functions (GO terms) were visualized using Cytoscape v3.6.1 [50]. To investigate the interactions of
proteins encoded by the identified DEGs in glial cell development, we mapped the associated genes onto STRING v10.5 [32] (high confidence > 0.7) network of Mus musculus. The final output interaction network of glial cell development was then analyzed and displayed with Cytoscape.

**RNA extraction and real-time PCR**

The RNA extraction was carried out with PureLink® RNA Mini Kit (Catalog No. 12183018A, ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol. Real-time PCR was performed as described previously [51]. The primers used for detecting Cxcl10 were 5'-TCAGCACCAGAAGCCAAACG-3' (F) and 5'-CTATGGCCCTATTCTCAGTG-3' (R).

**Ethical statement**

All mouse experiments were carried out in line with protocols approved by the Institutional Animal Care and Use Committee at Beijing Institute of Microbiology and Epidemiology, China.

**Data availability**

All data that support the findings of this study are available upon reasonable request. The RNA-seq data have been deposited in the Genome Sequence Archive [52] at the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences / China National Center for Bioinformation (GSA: CRA002644) and are publicly accessible at http://bigd.big.ac.cn/gsa. These data were also deposited in Sequence Read Archive (SRA:SRP095873) which are publicly accessible at https://www.ncbi.nlm.nih.gov/sra.

**CRediT author statement**

**Yafei Chang:** Formal analysis, Writing - original draft, Writing - review & editing. **Yisheng Jiang:** Formal analysis. **Cui Li:** Formal analysis. **Qin Wang:** Validation. **Feng Zhang:** Validation. **Cheng-Feng Qin:** Resources. **Qing-Feng Wu:** Formal analysis. **Jing Li:** Writing - review & editing, Funding acquisition. **Zhiheng Xu:** Writing - review & editing. **Conceptualization, Supervision, Funding acquisition,** All authors read and approved the final manuscript.

**Competing interests**

The authors have declared no competing interests.

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**Supplementary material**

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