1 Research Article submitted to *bioRxiv* 2019

2

3 **Trimester-specific Zika virus infection affects placental responses in**
4 **women**

5

6 Fok-Moon Lum¹, Vipin Narang¹, Susan Hue², Jie Chen³, Naomi McGovern⁴,
7 Ravisankar Rajarethinam², Jeslin J.L. Tan¹, Siti Naqiah Amrun¹, Yi-Hao
8 Chan^{1,5}, Cheryl Y.P. Lee^{1,5}, Tze-Kwang Chua¹, Wearn-Xin Yee¹, Nicholas
9 K.W. Yeo¹, Thiam-Chye Tan⁶, Xuan Liu^{7,8}, Sam Haldenby⁷, Yee-sin
10 Leo^{9,10,11,12}, Florent Ginhoux¹, Jerry K.Y. Chan^{1,13,14,15}, Julian Hiscox^{1,8,16},
11 Chia-Yin Chong^{17,18,19,20}, Lisa F.P. Ng^{1,8,16,21*}.

12

13 ¹Singapore Immunology Network, Agency for Science, Technology and
14 Research, Singapore 138648, Singapore.

15 ²Institute of Molecular and Cell Biology, Agency for Science, Technology and
16 Research, Singapore 138673, Singapore.

17 ³KK Women's and Children's Hospital, Singapore 229899, Singapore.

18 ⁴Department of Pathology, University of Cambridge, Cambridge CB2 1QP,
19 UK.

20 ⁵NUS Graduate School for Integrative Sciences and Engineering, National
21 University of Singapore, Singapore 119077, Singapore.

22 ⁶Department of O&G, KK Women's and Children's Hospital, Singapore
23 229899, Singapore.

24 ⁷Centre for Genomic Research, Institute of Integrative Biology, University of
25 Liverpool, Liverpool L69 7ZB, UK.

- 26 ⁸NIHR Health Protection Research Unit in Emerging and Zoonotic Infections,
27 University of Liverpool, Liverpool L69 3GL, UK.
- 28 ⁹Communicable Diseases Centre, Institute of Infectious Diseases and
29 Epidemiology, Tan Tock Seng Hospital, Singapore 308433, Singapore.
- 30 ¹⁰National Centre for Infectious Diseases, Singapore 308433, Singapore.
- 31 ¹¹Saw Swee Hock School of Public Health, National University of Singapore,
32 Singapore 117549, Singapore.
- 33 ¹²Lee Kong Chian School of Medicine, Nanyang Technological University,
34 Singapore 636921, Singapore.
- 35 ¹³Department of Reproductive Medicine, KK Women's and Children's
36 Hospital, Singapore 229899, Singapore.
- 37 ¹⁴KK Research Centre, KK Women's and Children's Hospital, Singapore
38 229899, Singapore.
- 39 ¹⁵Cancer and Stem Cell Program, Duke–NUS Medical School, Singapore
40 169857, Singapore.
- 41 ¹⁶Institute of Infection and Global Health, University of Liverpool, Liverpool
42 L69 7BE, UK.
- 43 ¹⁷Department of Paediatrics, KK Women's and Children's Hospital, Singapore
44 229899, Singapore.
- 45 ¹⁸Lee Kong Chian School of Medicine, Nanyang Technological University,
46 Singapore 636921, Singapore.
- 47 ¹⁹Yong Loo Lin School of Medicine, National University of Singapore,
48 Singapore 117597, Singapore
- 49 ²⁰Graduate Medical School, Duke–NUS Medical School, Singapore 169857,
50 Singapore.

51 ²¹Department of Biochemistry, Yong Loo Lin School of Medicine, National
52 University of Singapore, Singapore 117597, Singapore.

53

54 **Running title:** ZIKV infection in pregnant women

55

56 **Word count (abstract):** 129 words

57 **Word count (main text):** 3373 words

58

59 *Address correspondence to Prof Lisa F.P. Ng ([lisa_ng@immunol.a-](mailto:lisa_ng@immunol.a-star.edu.sg)
60 [star.edu.sg](mailto:lisa_ng@immunol.a-star.edu.sg)).

61

62 **Footnote Page**

63

64 **Financial support**

65 This work was supported by the Singapore Biomedical Research Council
 66 (BMRC; core research grants to the Singapore Immunology Network (SIgN))
 67 and the BMRC and Singapore Agency for Science, Technology, and
 68 Research-led Zika Virus Consortium Fund (project 15/1/82/27/001). SIgN
 69 immunomonitoring and Flow Cytometry platforms are supported by the
 70 Immunomonitoring Service Platform (ISP) grant, NRF2017_SISFP09 and
 71 BMRC transition funds #H16/99/b0/011. This work was also partly funded by
 72 the National Institute for Health Research Health Protection Research Unit
 73 (NIHR HPRU) in Emerging and Zoonotic Infections at University of Liverpool
 74 in partnership with Public Health England (PHE), in collaboration with
 75 Liverpool School of Tropical Medicine.

76

77 **Potential conflicts of interest**

78 All authors: no conflicts.

79

80 **Requests for reprints.**

81 Prof Lisa F.P. Ng (lisa_ng@immunol.a-star.edu.sg)

82

83

84 **Abstract (129 words)**

85 Zika virus (ZIKV) infection during pregnancy is associated with neurologic
 86 birth defects, but the effects on placental development are unclear. Full-term
 87 placentas from three women, each infected with ZIKV during specific
 88 pregnancy trimesters, were harvested for anatomic, immunologic and
 89 transcriptomic analysis. In this study, each woman exhibited a unique immune
 90 response, but they collectively diverged from healthy controls with raised IL-
 91 1RA, IP-10, EGF and RANTES expression, and neutrophil numbers during
 92 the acute infection phase. Although ZIKV NS3 antigens co-localized to
 93 placental Hofbauer cells, the placentas showed no anatomical defects.
 94 Transcriptomic analysis of samples from the placentas revealed that infection
 95 during trimester 1 caused a disparate cellular response centered on
 96 differential eIF2 signaling, mitochondrial dysfunction and oxidative
 97 phosphorylation. These findings should translate to improve clinical prenatal
 98 screening procedures for virus-infected pregnant patients.

99

100 **Keywords:** Zika virus, placenta, RNAseq, transcriptomics, infection, histology

101

102 **Background**

103 Zika virus (ZIKV) caused numerous outbreaks of infection worldwide [1] and
 104 the scale of these outbreaks highlighted several features of ZIKV infection that
 105 had previously been unrecognized or under reported. One such feature
 106 included congenital fetal growth-associated anomalies as a result of ZIKV
 107 infection during pregnancy, termed Congenital Zika Syndrome (CZS) [2, 3].
 108 Pregnancy is divided into three trimesters, based on the series of
 109 developmental changes that occur in the fetus and the physiological changes
 110 that occur in the mother. Understanding the nature of the ZIKV–host
 111 interactions at each trimester is required to determine the consequences of
 112 maternal–fetal ZIKV transmission and its impact on CZS development.
 113 Important physiological changes occur in women during pregnancy, including
 114 immune responses [4]. The unique immunological state experienced during
 115 pregnancy prevents the mother from rejecting the fetus [5], but also increases
 116 susceptibility to infection [6].

117 The placenta develops from trophoblasts surrounding the blastocyst
 118 and rapidly grows after implantation to establish the fetal life support system
 119 before the rapid growth of the fetus in the second half of pregnancy. The
 120 placenta is the physical barrier between the mother and her fetus. It is the site
 121 of transport of oxygen nutrients, antibodies and waste products between
 122 mother and baby [7]. Importantly, the placenta also protects the fetus from
 123 infections, exhibiting a robust innate and adaptive immune response to
 124 pathogens [8]. Hofbauer cells, are macrophages located within the stroma of
 125 the placenta and can be isolated from the placenta and be infected by ZIKV
 126 experimentally [9]. Positive ZIKV infection has been detected in placental

127 tissue [10] and fetuses [3] of women who have terminated their pregnancies,
 128 suggesting the virus can evade placental immune defense mechanisms. As
 129 the risk of congenital anomalies, including microcephaly, is largely associated
 130 with ZIKV infection during the first trimester of pregnancy [11], it is also
 131 possible that ZIKV infection perturbs placental development. Such
 132 perturbations could also upset the placental immune function. The
 133 mechanisms underlying how ZIKV infection in the three trimesters affects the
 134 placental cellular responses, especially in pregnancies without any congenital
 135 complications, are currently unknown. Understanding these provide an
 136 alternate perspective regarding ZIKV pathogenesis in asymptomatic pregnant
 137 patients.

138 Here, the placental cellular responses in ZIKV-infected pregnant
 139 women were probed and the study delineated how ZIKV infection affected
 140 placental development in each trimester. Three pregnant women were
 141 recruited to the study who had been infected with ZIKV during the first,
 142 second or third trimesters. After successful, full-term delivery of healthy
 143 infants, the placentas were harvested and separated into the placental discs
 144 (chorionic villi) and fetal membranes (chorion and amnion) to investigate the
 145 cellular responses in these anatomically distinct structures. Histology and
 146 immunofluorescent analyses were used to identify any tissue parenchymal
 147 abnormalities and immune-cell changes. This was combined with RNA-
 148 sequencing (RNA-seq) to determine the transcriptomic profiles of these
 149 samples. Although the number of ZIKV-infected pregnant women recruited
 150 was low and may not represent the entire population, it still provides insightful

151 data on the possible placental responses during trimester-specific ZIKV

152 infection.

153

154 **Methods**

155 **Ethics approval and consent to participate**

156 Written informed consent was obtained from all participants in accordance
157 with the Declaration of Helsinki for human research. The study protocol was
158 approved by the SingHealth Centralized Institutional Review Board (CIRB
159 Ref: 2016/2219). Blood samples were collected from healthy donors who had
160 provided written consent, in accordance with the guidelines of the Health
161 Sciences Authority of Singapore (study approval number: NUS IRB: 10-250).

162

163 **Patients and sample collection**

164 Three pregnant women were enrolled into the study upon admission to the KK
165 Women's and Children's Hospital, Singapore. Upon admission, patients were
166 confirmed to be infected via qRT-PCR detection of ZIKV RNA in their urine.
167 Whole blood samples were collected at two time points: 0-7 days post illness
168 onset (PIO) and 10-14 days PIO, representing the acute and convalescent
169 phases, respectively. Whole blood was collected in EDTA Vacutainer tubes
170 (Becton Dickinson) after peripheral venipuncture. Each sample was first used
171 for blood count, whole blood staining and viral load quantification and then
172 centrifuged at 256 g for 10 min to collect plasma for storage at -80°C. Whole
173 blood samples were also collected from three healthy women and pre-
174 screened for ZIKV viral RNA and ZIKV-specific antibodies using in-house
175 protocols [12]. Placentas from two healthy individuals were obtained as
176 control. All healthy donors were non-febrile and had no signs of acute illness
177 during recruitment.

178

179 **Placenta processing**

180 After successful full-term delivery, the placenta was maintained on ice and
181 transported to the Singapore Immunology Network for immediate downstream
182 processing within 2 h of expulsion. The term placenta was first separated into
183 the fetal membrane (chorion and amnion) and placenta disc (chorionic villi)
184 layers. A small section of the tissue was removed and placed in 10% neutral
185 buffered formalin (NBF; Sigma Aldrich) for histology and immunofluorescence
186 staining. The remaining tissues were finely diced in a digestion medium
187 containing collagenase type 4 (0.2mg/ml) and DNase (0.05mg/ml) prepared in
188 Roswell Park Memorial Institute medium (Hyclone) containing 10% Fetal
189 Bovine Serum (Gibco). Refer to supplemental materials for more information.

190 The fetal membranes were incubated in digestion medium for 3 h in a
191 37°C incubator supplemented with 5% CO₂. The fetal cells were then
192 centrifuged (400 g, 5 min) and repeatedly washed with PBS to remove traces
193 of digestion medium before red blood cell (RBC) lysis. Lysis was done with in-
194 house RBC lysing solution containing 155 mM NH₄Cl (Sigma Aldrich), 10 mM
195 KHCO₃ (Sigma Aldrich) and 0.1 mM EDTA (Life Tech). Lysed cells were then
196 enumerated and used for downstream procedures.

197 The placenta disc were incubated in digestion medium for 1 h, and
198 then the digestion medium was carefully overlaid on Ficoll-Paque Plus (GE
199 Healthcare) at a ratio of 2:1, respectively. The samples were then centrifuged
200 at 1,600 g for 20 min (with minimal acceleration and deceleration) to isolate
201 the “buffy coat” containing leucocytes. The leucocytes were carefully removed
202 and traces of RBCs were subsequently lysed. The cells were enumerated and
203 used for downstream procedures.

204 In both cases, the digestion medium was filtered through a 100µM filter
205 unit after digestion to remove any undigested tissues. The volume of medium
206 used was adjusted according to the size of the tissue being digested.

207

208 **Histology and immunofluorescence**

209 Placental tissues were first fixed in 10% NBF (Sigma Aldrich) at room
210 temperature for 24 h before being processed for routine histologic evaluation.
211 Briefly, isolated tissues were embedded in paraffin wax, cut into 5 µM thick
212 sections, deparaffinized and then stained with Hematoxylin and Eosin (H&E).
213 The stained sections were viewed under an Olympus BX53 upright
214 microscope (Olympus Life Science, Japan), and images were captured with
215 an Olympus DP71 digital camera using an Olympus DP controller and DP
216 manager software. All images were evaluated by a certified pathologist.

217 Tissue sections were stained by standard immunofluorescence
218 technique. In brief, antigen-retrieval was performed after deparaffinisation,
219 using DAKO target retrieval solution, pH9. After washing with TBS-T, the
220 tissues were then treated with 10% goat serum for 30 min to block the
221 nonspecific reaction. The tissues were then incubated overnight with various
222 combinations of a ZIKV in-house antibody [13] (1:100) and CD 163
223 (Thermofisher; 1:50) at 4°C. The slides were incubated with secondary
224 antibodies (1:500) such as Alexa Fluor® 488 goat anti rabbit IgG (Life
225 technologies) and Alexa Fluor® 594 goat anti mouse IgG (Invitrogen) for 30
226 min in the dark, and the nuclei were counter stained with Vectashield™ Hard
227 Set mounting medium with DAPI. The stained slides were examined under
228 Nikon eclipse 90i fluorescence microscope (Nikon, Japan) and images were

229 captured with microscopic camera, DS-Fi3 (Nikon, Japan) using NIS
230 Elements Imaging software, (Nikon, Japan).

231

232 **Blood count**

233 A complete blood count (CBC) was determined using an Ac-T diff hematology
234 analyser (Beckman Coulter), according to manufacturer's instructions.
235 Beckman Coulter 4C[®] Plus Tri-Pack Cell Controls (Beckman Coulter) were
236 used to confirm instrument accuracy and precision.

237

238 **Whole blood labeling and flow cytometry**

239 Whole blood (100 μ L) was labeled as previously described [13]. Antibodies
240 were used to identify CD45⁺ leucocytes (mouse anti-human CD45,
241 Biolegend), high SSC-A CD16⁺ neutrophils (mouse anti-human CD16,
242 Biolegend), CD14⁺ monocytes (mouse anti-human CD14, BD Biosciences),
243 CD3⁺ T cells (mouse anti-human CD3, BD Biosciences), CD4⁺ T helper cells
244 (mouse anti-human CD4, eBioscience), CD56⁺ NK cells (mouse anti-human
245 CD56, Miltenyi Biotec) and CD19⁺ B cells (mouse anti-human CD19,
246 eBioscience). Cell fixation and RBC lysis was performed using 1X FACS
247 lysing solution (BD Biosciences), and permeabilization using 1X FACS
248 permeabilization solution 2 (BD Biosciences) before staining with a ZIKV NS3
249 protein-specific rabbit polyclonal antibody [13]. The labeled cells were
250 counter-stained with a fluorophore-tagged secondary goat anti-rabbit IgG
251 (H+L) antibody (Invitrogen), before acquisition on a LSR Fortessa (BD
252 Biosciences). Dead cells were excluded using a Live/Dead[™] Fixable Aqua
253 Dead Cell Stain Kit (ThermoFisher Scientific). The frequencies of peripheral

254 blood immune subsets were obtained with the following formula: Percentage
255 of specific immune subset (obtained from immune-phenotyping) × total
256 leucocyte number (obtained from CBC) = cellular number of specific immune
257 subset.

258

259 **Multiplex microbead immunoassay for cytokine quantification**

260 Cytokine and chemokine levels in plasma from ZIKV-infected patients were
261 measured simultaneously using a multiplex microbead-based immunoassay,
262 ProcartaPlex Human Cytokine/Chemokine/Growth Factor Panel 1 (Thermo
263 Scientific), as previously described [14]. Plasma samples and reagents were
264 prepared, and immunoassay procedures were performed according to the
265 manufacturers' instructions.

266

267 **ZIKV virion-based ELISA**

268 The presence and titers of ZIKV-specific antibodies in the plasma of the
269 infected pregnant women at both acute and convalescent phases were
270 determined using a ZIKV virion-based ELISA, as previously described [12].
271 Plasma samples were tested at 1:200 dilutions.

272

273 **RNA sequencing (RNA-seq)**

274 RNA samples, extracted from the fractions of isolated placental cells, were
275 treated with DNase using an Ambion Turbo DNA-free Kit (Ambion), and then
276 purified using Ampure XP beads (Agencourt). The DNase-treated RNA (2 µg)
277 was treated with Ribo-Zero using an Epicentre Ribo-Zero Gold Kit
278 (Human/Rat/Mouse) (Epicentre) and re-purified on Ampure XP beads.

279 Successful RNA depletion was verified using a Qubit (Thermo Fisher
280 Scientific) and an Agilent 2100 Bioanalyzer (Agilent) and all depleted RNA
281 was used as input material for the ScriptSeq v2 RNA-Seq Library Preparation
282 protocol. RNA was amplified for 14 cycles and the libraries were purified on
283 Ampure XP beads. Each library was quantified using a Qubit and the size
284 distribution was assessed using an AATI Fragment Analyser (Advanced
285 Analytical); the final libraries were pooled in equimolar amounts. The quantity
286 and quality of each pool was assessed using a Fragment Analyser and by
287 qPCR using an Illumina Library Quantification Kit (KAPA Biosystems) on a
288 Light Cycler LC480II (Roche), according to manufacturer's instructions. The
289 template DNA was denatured according to the protocol described in the
290 Illumina cBot User guide and loaded at a 12 pM concentration. Sequencing
291 was carried out on three lanes of an Illumina HiSeq 2500 with version 4
292 chemistry, generating 2 × 125 bp paired-end reads.

293

294 **RNA-seq data analysis**

295 Quality assessment of the RNA sequencing reads was performed using
296 FASTQC (version 0.11.5;
297 <http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc>). Cutadapt (version
298 1.2.1) [15] was used to remove Illumina adapter sequences with option -O 3
299 so that the 3' end of any reads that matched the adapter sequence for ≥3 bp
300 were trimmed. The reads were further trimmed using Sickle (version 1.20;
301 <https://github.com/najoshi/sickle>) with a minimum window quality score of 20.
302 Reads <10 bp after trimming were removed. The resulting clean, paired-end
303 reads were mapped to human transcript sequences obtained from Gencode

(version 27) [16] using Salmon (version 0.9.1) [17]. Transcript abundances quantified by Salmon were summarized to gene-level counts and normalized gene-level abundances in transcript per million (TPM) units using the tximport R/Bioconductor package (version 1.2.0) [18].

Principal component analysis (PCA) was performed on all samples using genes with TPM > 1 in at least one sample. The TPM values were $\log_2$ transformed after adding a pseudocount of 1.0 to prevent negative values, and used for PCA in FactoMiner R package (version 1.33) [19] with default scaling transformation.

Differential gene expression (DGE) analysis was performed using edgeR R/Bioconductor package (version 3.16.5) [20]. Gene-level counts for samples relevant to the conditions being compared were loaded into edgeR and a negative binomial (NB) generalized linear model (GLM) extended with quasi-likelihood (QL) methods was fitted to the counts data. As recommended for gene-level counts summarized using tximport, the transcript abundances and average transcript lengths of genes were used to specify the offset term in the edgeR model in order to correct for potential differences in transcript usage between samples [18]. Low expressed genes with <5 counts in all samples were removed. The trended NB dispersion was estimated using the estimateDisp function and the QL dispersion was estimated using the glmQLFit function. Differential gene expression was determined using a quasi-likelihood F-test (glmQLFTest function), which provides a conservative and rigorous type I error rate control [20]. Genes with a Benjamini-Hochberg multiple testing corrected p-value <0.05 were considered differentially expressed. Differentially expressed gene lists were analyzed with DAVID

gene ontology analysis (version 6.8) [21], Ingenuity Pathway Analysis® (IPA) and Gene Set Enrichment Analysis (GSEA) (version 3.0) [22] to identify associated biological conditions and pathways. The curated gene sets used for GSEA included the hallmark (H) and immunologic signatures (C7) gene sets downloaded from Broad Institute's Molecular Signatures Database that accompanies the GSEA software.

Multiple factor analysis

Data from immunophenotyping of whole blood and cytokine quantification in plasma were analyzed together using multiple factor analysis (MFA) [23]; MFA permits several groups of variables to be studied on the same set of individuals. Analysis was performed with nine individual samples, including the three ZIKV-infected patients in the acute and convalescent phases of infection, and the three healthy controls. The immunophenotyping and cytokine measurements were specified as two different groups of variables consisting of log₁₀ transformed cell counts per µl of whole blood and log₁₀ transformed cytokine levels in pg/ml units. The analysis was carried out using R package FactoMineR (version 1.33) [19] with the default parameters. The data were plotted using custom R scripts. Data from placenta immunophenotyping and RNA sequencing were also analyzed using MFA. Data acquired from placenta disc and fetal membrane fractions were analyzed separately. The individuals in this analysis included the three ZIKV-infected patients and two healthy controls. Cellular fractions were expressed as a percentage of the parent gate in FACS analysis and RNA-seq measurements were expressed in log₂ TPM units.

354

355

356 **Results**

357 **Pregnant women in different trimesters exhibited differential** 358 **susceptibility to ZIKV infection**

359 Three ZIKV-positive pregnant women were recruited to the study, who had
360 been infected with ZIKV in different trimesters (Table 1). Blood samples were
361 taken during the acute (5-6 days post-illness onset (PIO)) and convalescent
362 (9-14 days PIO) phases of infection, and analyzed for the presence of ZIKV
363 NS3 antigens.

364 CD14+ monocytes are the predominant cellular target of ZIKV [13].
365 Interestingly, each patient showed a different ZIKV NS3 antigen profile in
366 CD14+ monocytes. 15% of Patient 1's CD14+ monocytes were positive for
367 ZIKV NS3 antigens, which persisted to a lesser degree in the convalescent
368 phase (7.61%). In Patient 2, ZIKV NS3 antigens were barely detectable in
369 CD14+ monocytes at both time-points (0.19-0.44%). Lastly, Patient 3 exhibited
370 a "delayed" infectious response, in which 2.66% of CD14+ monocytes were
371 positive for ZIKV NS3 antigen levels during the acute phase, which increased
372 to 10.1% of total CD14+ monocytes during the convalescent phase (Table 1
373 and Supplementary Figure 1A and 1B). The different infection profiles
374 exhibited by the three patients were corroborated by variations observed in
375 their blood immune-cell numbers (Table 1 and Supplementary Figure 2A and
376 2B) and ZIKV-specific antibody titers (Table 1 and Supplementary Figure 1C
377 and 1D). Interestingly, ZIKV-specific antibody production was weak in Patient
378 1, perhaps indicating a direct consequence of ZIKV-infection on the host
379 humoral immune response during the first trimester. Titers of ZIKV-specific

antibodies (both IgM and IgG) did not change during the convalescent phase in both Patients 2 and 3 (Table 1 and Supplementary Figure 1C and 1D).

ZIKV antigen localized to placental Hofbauer cells and persisted until birth

All three patients successfully carried infants to term with no microcephaly or CZS and infants were delivered via normal vaginal deliveries with no intrapartum complications. Once delivered, the full-term placenta was separated into placental discs and fetal membranes, and a series of histological analyses were performed. By H&E staining of the placenta discs, villous maturity within the normal limits of a term placenta was observed in all three cases. The chorionic villi of all three placental discs showed patchy dilatation featuring stromal oedema and prominent aggregates of vacuolated cells. These vacuolated stromal cells have the morphologic features of villous stromal macrophages i.e. Hofbauer cells. No features of ongoing or remote acute or chronic villitis, intervillitis, villous necrosis or fibrosis, was observed (Figure 1A). This indicated that ZIKV infection did not induce any overt adverse placenta pathology. The fetal membranes of all three placentas also showed several reactive changes, including increased numbers of vacuolated mononuclear cells within the subamniotic connective tissue, in keeping with increased infiltration of macrophages. In addition, the amniotic epithelial cells exhibited columnar cell metaplasia, being more pronounced in placentas infected in the first and second trimesters. There are no signs of acute chorioamnionitis (Figure 1B).

Immunofluorescence staining of the placental discs confirmed the presence of CD163+ Hofbauer cells, which exhibit functions similar to those of M2-like macrophages [24]. Counter-staining with a ZIKV NS3-specific antibody [13] indicated that ZIKV protein co-localized to Hofbauer cells (Figure 1C), in line with previous reports [9]. Staining of placental discs with secondary antibody alone, did not result in any signals (Supplementary Figure 3). Thus, this ruled out autofluorescence and indicated positive infection of the placenta, regardless of the pregnancy trimester in which ZIKV infection occurred. Notably, these data showed that ZIKV proteins were present in the placenta up to delivery, without causing any physical harm to the newborn infant. However, it is important to continue monitoring the newborn as anomalies may manifest at a later time [25].

The ZIKV immune response correlated with the level of ZIKV infection in CD14+ monocytes

The immunological response to ZIKV infection in the three pregnant women was compared between the acute and convalescence time-points, which will provide important insights on how the host response is affected by the infection in different trimesters. Blood plasma was assayed for 42 different immune mediators, including pro-inflammatory and anti-inflammatory cytokines, chemokines and growth factors. Plasma samples from healthy, non-pregnant females were used as controls. To segregate the patients based on immunological response, the derived data was processed by multiple factor analysis (MFA) [23], which collectively analysed different

428 measurements made from the same set of individuals. Both datasets were
429 specified as two different groups of measurements to the algorithm.

430 The unsupervised data analysis clearly segregated the samples
431 according to healthy controls and ZIKV patients as well as to convalescent
432 and acute phases of infection along the first principal component (PC1) of the
433 data. PC1 accounted for 42.5% of the variation in the measurements across
434 the samples (Figure 2A). The second and third principal components (PC2,
435 PC3), together accounted for 39.7% of the variation between measurements,
436 and highlighted the differences between individuals. Along these axes, the
437 patient samples from the convalescent phase of infection were closely
438 grouped together compared to those from the acute phase of infection.

439 Correlation of measurements with PC1, which separated ZIKV patients
440 and healthy controls, showed that a subset of immune mediators (IL-1RA,
441 EGF, RANTES, IP-10) and neutrophils were associated with acute ZIKV
442 infection (Figure 2B and 2C). Noteworthy, neutrophil numbers in the
443 peripheral blood positively correlated with the percentage of ZIKV-infected
444 (ZIKV NS3+) monocytes (Figure 2D and Table 1). Overall, the results
445 obtained suggested that ZIKV infection during trimester 1 could lead to a more
446 active pro-inflammatory response as observed in Patient 1.

447

448 ZIKV infection during the first trimester of pregnancy triggered disparate **449 eIF2 signaling and oxidative phosphorylation in the placenta**

450 Transcriptome profiling of the placenta discs and fetal membranes of the
451 placentas was used to investigate the cellular host response during ZIKV
452 infection. Both tissues were digested to obtain a single-cell suspension: the

453 placenta disc was further separated into CD45+ immune and CD45- non-
 454 immune fractions, to define the drivers of ZIKV-induced cellular changes.
 455 Tissue samples were collected in triplicate to account for tissue heterogeneity,
 456 except those obtained from Patient 3, where the sample was processed as a
 457 whole, due to technical difficulties. Placental samples from two healthy
 458 women were included as controls.

459 Over 517 million paired-end RNA-seq reads were obtained with a
 460 median of >15 million paired-end reads per sample mapped to the human
 461 transcriptome and 29,613 genes had a detectable expression level of >1
 462 transcript per million (TPM) per sample. Principal component analysis (PCA)
 463 of the gene expression profiles segregated the samples by their origin
 464 (placenta disc or fetal membrane), regardless of whether they were derived
 465 from patients or controls (Figure 3A). Patient 1 was the only exception, as the
 466 gene expression data all clustered together (Figure 3A, cluster 4). This effect
 467 was further elaborated when PCA was performed separately for each tissue
 468 type (Figure 3B), where samples from Patient 1 segregated from the rest,
 469 indicating that the placenta from Patient 1 bore a distinct gene expression
 470 profile.

471 To determine the cellular differences in the placenta of Patient 1, the
 472 unique differential gene expression signature was compared to healthy
 473 controls. A large number of differentially expressed genes (DEGs) were
 474 identified between the placentas of Patient 1 and those from healthy controls
 475 (Placenta disc CD45+, n = 674; Placenta disc CD45-, n = 2,780; Fetal
 476 membrane, n = 2,467). Conversely, few to no DEGs were identified between
 477 the placentas derived from Patient 2 and healthy controls (Placenta disc

478 CD45+, n = 0; Placenta disc CD45-, n = 51, Fetal membrane, n = 1)
 479 (Supplementary Figure 4A). Unfortunately, samples from Patient 3 could not
 480 be analyzed due to an insufficient number of replicates. Nevertheless, these
 481 findings in DEGs agree with the observations from PCA analysis, where
 482 samples from Patient 1 were distinct from the rest.

483 The potential functions of the DEGs in placental tissue from Patient 1
 484 were analyzed using gene ontology (GO), pathway enrichment and gene set
 485 enrichment analysis (GSEA) to provide a global overview. In the CD45-
 486 fraction of the placenta disc from Patient 1, GO analysis identified several
 487 DEGs as ribonucleoproteins, mitochondrial genes and histones (Figure 4 and
 488 Supplementary Table 1). Ingenuity Pathway Analysis (IPA) identified the role
 489 of eIF2 signaling, as the main canonical pathway associated with these DEGs
 490 (Supplementary Figure 4B). The eIF2 pathway regulates global protein
 491 synthesis and translation in response to stress. Oxidative phosphorylation
 492 was also significantly modulated, where 90% of genes involved in this
 493 pathway showed a reduced expression by >2-fold compared to healthy
 494 controls. An increased abundance of histone transcripts during viral infection
 495 has been previously reported [26, 27] and was also observed in Patient 1
 496 (Figure 4). The data also suggested potential modulation of oxidative
 497 phosphorylation and eIF2 signaling in both the CD45+ cells of the placenta
 498 disc and fetal membrane in the placenta from Patient 1 (Figure 4,
 499 Supplementary Figure 4B and Supplementary Table 2).

500 To determine the percentages of various immune populations,
 501 immunophenotyping of the isolated CD45+ cells was performed (Figure 5A
 502 and Supplementary Figure 5). Correlating this immunophenotyping data with

503 the first two principal components of the gene expression data (Figure 5B)
 504 corroborated that the increase in neutrophil numbers (Figure 5C) was clearly
 505 associated with gene expression changes observed in the placenta from
 506 Patient 1 compared to healthy placentas. In the fetal membrane of the
 507 placenta, several transcripts encoded by inflammatory response genes and
 508 pathways were increased in abundance, including NFKB1, IL1B, TNF, TLR2,
 509 TLR4, CXCL8, NLRP3 and NLRP6 (Supplementary Figure 4B and
 510 Supplementary Table 3). Taken together, these data suggest that ZIKV
 511 infection during the first trimester of pregnancy could have led to an
 512 upregulated abundance of transcripts targeting eIF2 signaling, oxidative
 513 phosphorylation and neutrophil numbers, as observed in Patient 1.

514

515 **Discussion**

516 The emergence of ZIKV in recent years came as a surprise and the apparent
 517 association of ZIKV infection with neurological complications in developing
 518 fetuses, namely congenital microcephaly, further alarmed the world. ZIKV is
 519 capable of infecting neural cells [28-33], providing a direct causative role of
 520 the virus in causing neurological impairment. In animal studies, ZIKV infection
 521 of immuno-compromised pregnant female mice resulted in premature fetal
 522 death [34]; ZIKV was found in the placenta in these mice, leading to active
 523 placental infection, apoptosis and vascular damage [34]. In humans, it is
 524 worth noting that the estimated prevalence of microcephaly in babies born to
 525 ZIKV-infected pregnant women was ~2.3% [35]. This estimate, however,
 526 considers infections that have occurred in any of the three trimesters [35]. The
 527 likelihood of microcephaly is thought to be higher if ZIKV infection occurs
 528 during the first trimester [11].

529 This study recruited three pregnant women who were infected with
 530 ZIKV during different trimesters of pregnancy. Despite the low number of
 531 patients recruited (one patient per trimester), this remains an excellent
 532 opportunity to obtain an insight into the impact of ZIKV infection during
 533 different trimesters on host cellular responses. Nevertheless, a series of
 534 histologic, immunologic and transcriptomic analyses were used to determine
 535 the impact of ZIKV infection on pregnancy progression and the cellular
 536 responses of the host towards the infection. All three babies were born at term
 537 with no observable birth defects, and all of the ZIKV-infected mothers were
 538 clinically well, as with majority of ZIKV patients [14]. However, different
 539 degrees of infection were apparent in the level of CD14+ monocytes between

the three patients. This difference led to a disparate immune response exhibited by the patients. As confirmed by MFA, several immune mediators were associated with the acute phase of ZIKV infection, including IL-1RA, EGF, RANTES and IP-10 [14]. Interestingly, EGF and IP-10 are typically expressed at higher levels in women carrying a fetus with developmental anomalies [36]. The association of neutrophils with the acute phase should also be given more attention since most studies have focused on the role of monocytes during ZIKV infection thus far [37, 38]. Neutrophils have been shown to infiltrate the central nervous system of ZIKV-infected immunocompromised mice [39], and may therefore be a causative element in Zika-induced neurological damage.

ZIKV NS3 antigen was detected in placental tissues of all three patients at full term, indicating a strong tropism of the virus to the placenta regardless of the trimester of infection. The ZIKV NS3 antigen was detected in Hofbauer cells, placental macrophages [9], but not in trophoblasts. The susceptibility of trophoblastic cells to ZIKV infection is still uncertain, with reports indicating either permissive or resistance towards ZIKV infection [9, 40, 41]. Interestingly, the localization of the ZIKV NS3 antigen to CD163+ Hofbauer cells did not corroborate with positive detection of ZIKV RNA load in the placental tissue. This finding may indicate that the virus has been cleared and the NS3 antigens are merely phagocytosed, non-infectious viral remnants. This observation was further supported by the lack of any pathological insults (villitis, intervillitis, chorioamnionitis, or funisitis) to the placental tissues in any of the three patients as well as the lack of detectable viral RNA by RNAseq, performed at the resolution sequenced. This

565 observation also indicated an absence of overt inflammation, which permitted
566 the pregnancy to progress to term without complications. This result was
567 further confirmed by the absence of Hofbauer cell-mediated hyperplasia in the
568 chorionic villi, which has been previously associated with progression of
569 congenital ZIKV infection [41].

570 The transcriptomic profiling of placental cells showed that the cellular
571 responses in Patient 1 (infected during the first trimester) were quite different
572 from the other two patients and healthy controls for all three cell types
573 analyzed: placenta disc CD45+, placenta disc CD45- and fetal membrane
574 cells. GSEA revealed that a subset of genes in the CD45+ cells of Patient 1
575 (Figure 5D) resembled those documented in the bacterium *Anaplasma*
576 *phagocytophilum*-stimulated human primary neutrophils [42]. This finding is
577 not surprising, given that isolated CD45+ cells comprise a high proportion of
578 neutrophils and thus would contribute the most to the transcriptome. More
579 importantly, this finding also indicates the vast number of neutrophils present
580 in the placenta during pregnancy. Their participation should not be taken
581 lightly as this cell type may be involved in infertility, preeclampsia and even
582 fetal loss. Neutrophils also participate in implantation, angiogenesis, spiral
583 artery modification and parturition [43].

584 The DEGs in the placenta disc, both CD45+ and CD45- fractions, and
585 fetal membrane of Patient 1 were strongly associated with eIF2 signaling,
586 oxidative phosphorylation and mitochondrial dysfunction. eIF2 signaling is
587 crucial in regulating translational initiation leading to protein synthesis, and
588 participates in cellular stress responses, erythropoiesis and immune
589 responses to viral infection [44]. Perturbed eIF2 signaling in placental

590 development has been associated with intrauterine growth restriction of
 591 developing fetuses [45]. In rats, eIF2 signaling was also shown to be heavily
 592 involved in the regulation of placental and fetal development [46]. Likewise,
 593 oxidative phosphorylation and mitochondrial dysfunction have been
 594 associated with a range of gestational disorders [47].

595 These findings described herein can be translated into the clinical
 596 prenatal screening procedures in ZIKV-infected or any arbovirus-infected
 597 pregnant patients. In such circumstances, abnormalities in the numbers of
 598 leukocytes [48, 49] as well as biomarkers indicative of ongoing inflammation
 599 [50] or oxidative stress [51] and presence of any microbial infections [52]
 600 should be screened as advised by the clinic. Likewise, RNA from the amniotic
 601 fluid or amniocytes, can be profiled to determine the transcript abundance of
 602 molecules involved in important biological pathways [53]. Importantly, fetal
 603 development (e.g. blood flow, heart rate, head circumference) should be
 604 closely monitored by ultrasound scanning [54] to detect for unwanted
 605 anomalies that may surface in the later part of life [25]. Adopting these steps
 606 will potentially improve the clinical care administered to affected patients, by
 607 identifying potential danger signs associated with ZIKV infection.

608

609 **Acknowledgements**

610 We would like to thank Ivy Low, Seri Mustafah, and Anis Larbi (SIgN flow
611 cytometry team) and the SIgN Immunomonitoring Group, for their assistance.
612 We are also grateful to Fatima Yturriaga and Emmerie Wong Phaik Yen of
613 KKH for the patient recruitment, Kai-Er Eng and Guillaume Carissimo of SIgN
614 and Jessica Tamanini of Insight Editing London for editing the manuscript
615 prior to submission. We would also like to thank all study participants for their
616 participation in this study.

617

References

1. Korzeniewski, K., D. Juszczak, and E. Zwolinska, *Zika - another threat on the epidemiological map of the world*. Int Marit Health, 2016. **67**(1): p. 31-7.
2. Brasil, P., et al., *Zika Virus Infection in Pregnant Women in Rio de Janeiro - Preliminary Report*. N Engl J Med, 2016.
3. Mlakar, J., et al., *Zika Virus Associated with Microcephaly*. N Engl J Med, 2016. **374**(10): p. 951-8.
4. Mor, G. and I. Cardenas, *The immune system in pregnancy: a unique complexity*. Am J Reprod Immunol, 2010. **63**(6): p. 425-33.
5. Warning, J.C., S.A. McCracken, and J.M. Morris, *A balancing act: mechanisms by which the fetus avoids rejection by the maternal immune system*. Reproduction, 2011. **141**(6): p. 715-24.
6. Sappenfield, E., D.J. Jamieson, and A.P. Kourtis, *Pregnancy and susceptibility to infectious diseases*. Infect Dis Obstet Gynecol, 2013. **2013**: p. 752852.
7. Burton, G.J. and E. Jauniaux, *What is the placenta?* Am J Obstet Gynecol, 2015. **213**(4 Suppl): p. S6 e1, S6-8.
8. Robbins, J.R. and A.I. Bakardjiev, *Pathogens and the placental fortress*. Curr Opin Microbiol, 2012. **15**(1): p. 36-43.
9. Quicke, K.M., et al., *Zika Virus Infects Human Placental Macrophages*. Cell Host Microbe, 2016. **20**(1): p. 83-90.
10. Eppes, C., et al., *Testing for Zika virus infection in pregnancy: key concepts to deal with an emerging epidemic*. Am J Obstet Gynecol, 2017. **216**(3): p. 209-225.

- 642 11. Johansson, M.A., et al., *Zika and the Risk of Microcephaly*. N Engl J
643 Med, 2016. **375**(1): p. 1-4.
- 644 12. Kam, Y.W., et al., *Cross-reactive dengue human monoclonal antibody*
645 *prevents severe pathologies and death from Zika virus infections*. JCI Insight,
646 2017. **2**(8).
- 647 13. Lum, F.M., et al., *Sensitive detection of Zika virus antigen in patients'*
648 *whole blood as an alternative diagnostic approach*. J Infect Dis, 2017. **216**(2):
649 p. 182-190.
- 650 14. Lum, F.M., et al., *Longitudinal study of cellular and systemic cytokines*
651 *signatures define the dynamics of a balanced immune environment in disease*
652 *manifestation in Zika virus-infected patients*. J Infect Dis, 2018. **218**(5): p. 814-
653 824.
- 654 15. Martin, M., *Cutadapt removes adapter sequences from high-throughput*
655 *sequencing reads*. EMBnet. journal, 2011. **17**(1): p. pp-10.
- 656 16. Harrow, J., et al., *GENCODE: the reference human genome annotation*
657 *for The ENCODE Project*. Genome Res, 2012. **22**(9): p. 1760-74.
- 658 17. Patro, R., et al., *Salmon provides fast and bias-aware quantification of*
659 *transcript expression*. Nat Methods, 2017. **14**(4): p. 417-419.
- 660 18. Soneson, C., M.I. Love, and M.D. Robinson, *Differential analyses for*
661 *RNA-seq: transcript-level estimates improve gene-level inferences*.
662 F1000Res, 2015. **4**: p. 1521.
- 663 19. Lê, S., J. Josse, and F. Husson, *FactoMineR: an R package for*
664 *multivariate analysis*. Journal of statistical software, 2008. **25**(1): p. 18.

- 665 20. Lun, A.T., Y. Chen, and G.K. Smyth, *It's DE-licious: A Recipe for*
666 *Differential Expression Analyses of RNA-seq Experiments Using Quasi-*
667 *Likelihood Methods in edgeR*. Methods Mol Biol, 2016. **1418**: p. 391-416.
- 668 21. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and*
669 *integrative analysis of large gene lists using DAVID bioinformatics resources*.
670 Nat Protoc, 2009. **4**(1): p. 44-57.
- 671 22. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-*
672 *based approach for interpreting genome-wide expression profiles*. Proc Natl
673 Acad Sci U S A, 2005. **102**(43): p. 15545-50.
- 674 23. Escofier, B. and J. Pagès, *Multiple factor analysis (AFMULT package)*.
675 Comput Stat Data Anal, 1994. **18**(1): p. 121-140.
- 676 24. Svensson, J., et al., *Macrophages at the fetal-maternal interface*
677 *express markers of alternative activation and are induced by M-CSF and IL-*
678 *10*. J Immunol, 2011. **187**(7): p. 3671-82.
- 679 25. Nielsen-Saines, K., et al., *Delayed childhood neurodevelopment and*
680 *neurosensory alterations in the second year of life in a prospective cohort of*
681 *ZIKV-exposed children*. Nat Med, 2019.
- 682 26. Guerra, S., et al., *Microarray analysis reveals characteristic changes of*
683 *host cell gene expression in response to attenuated modified vaccinia virus*
684 *Ankara infection of human HeLa cells*. J Virol, 2004. **78**(11): p. 5820-34.
- 685 27. Hoover, S.E., et al., *Downregulation of varicella-zoster virus (VZV)*
686 *immediate-early ORF62 transcription by VZV ORF63 correlates with virus*
687 *replication in vitro and with latency*. J Virol, 2006. **80**(7): p. 3459-68.

- 688 28. Lanko, K., et al., *Replication of the Zika virus in different iPSC-derived*
689 *neuronal cells and implications to assess efficacy of antivirals*. Antiviral Res,
690 2017. **145**: p. 82-86.
- 691 29. Lum, F.M., et al., *Zika Virus Infects Human Fetal Brain Microglia and*
692 *Induces Inflammation*. Clin Infect Dis, 2017. **64**(7): p. 914-920.
- 693 30. McGrath, E.L., et al., *Differential Responses of Human Fetal Brain*
694 *Neural Stem Cells to Zika Virus Infection*. Stem Cell Reports, 2017. **8**(3): p.
695 715-727.
- 696 31. Olmo, I.G., et al., *Zika Virus Promotes Neuronal Cell Death in a Non-*
697 *Cell Autonomous Manner by Triggering the Release of Neurotoxic Factors*.
698 Front Immunol, 2017. **8**: p. 1016.
- 699 32. Simonin, Y., et al., *Zika Virus Strains Potentially Display Different*
700 *Infectious Profiles in Human Neural Cells*. EBioMedicine, 2016. **12**: p. 161-
701 169.
- 702 33. Tang, H., et al., *Zika Virus Infects Human Cortical Neural Progenitors*
703 *and Attenuates Their Growth*. Cell Stem Cell, 2016. **18**(5): p. 587-90.
- 704 34. Miner, J.J., et al., *Zika Virus Infection during Pregnancy in Mice*
705 *Causes Placental Damage and Fetal Demise*. Cell, 2016. **165**(5): p. 1081-91.
- 706 35. Coelho, A.V.C. and S. Crovella, *Microcephaly Prevalence in Infants*
707 *Born to Zika Virus-Infected Women: A Systematic Review and Meta-Analysis*.
708 Int J Mol Sci, 2017. **18**(8).
- 709 36. Kam, Y.W., et al., *Specific Biomarkers Associated With Neurological*
710 *Complications and Congenital Central Nervous System Abnormalities From*
711 *Zika Virus-Infected Patients in Brazil*. J Infect Dis, 2017. **216**(2): p. 172-181.

- 712 37. Foo, S.S., et al., *Asian Zika virus strains target CD14+ blood*
713 *monocytes and induce M2-skewed immunosuppression during pregnancy.*
714 *Nat Microbiol*, 2017.
- 715 38. Lum, F.M., et al., *Zika virus infection preferentially counterbalances*
716 *human peripheral monocyte and/or NK-cell activity.* *bioRxiv*, 2017.
- 717 39. Manangeeswaran, M., D.D. Ireland, and D. Verthelyi, *Zika*
718 *(PRVABC59) Infection Is Associated with T cell Infiltration and*
719 *Neurodegeneration in CNS of Immunocompetent Neonatal C57Bl/6 Mice.*
720 *PLoS Pathog*, 2016. **12**(11): p. e1006004.
- 721 40. Bayer, A., et al., *Type III Interferons Produced by Human Placental*
722 *Trophoblasts Confer Protection against Zika Virus Infection.* *Cell Host*
723 *Microbe*, 2016. **19**(5): p. 705-12.
- 724 41. Schwartz, D.A., *Viral infection, proliferation, and hyperplasia of*
725 *Hofbauer cells and absence of inflammation characterize the placental*
726 *pathology of fetuses with congenital Zika virus infection.* *Arch Gynecol Obstet*,
727 2017. **295**(6): p. 1361-1368.
- 728 42. Borjesson, D.L., et al., *Insights into pathogen immune evasion*
729 *mechanisms: Anaplasma phagocytophilum fails to induce an apoptosis*
730 *differentiation program in human neutrophils.* *J Immunol*, 2005. **174**(10): p.
731 6364-72.
- 732 43. Giaglis, S., et al., *Neutrophil migration into the placenta: Good, bad or*
733 *deadly?* *Cell Adh Migr*, 2016. **10**(1-2): p. 208-25.
- 734 44. Wek, R.C., *Role of eIF2alpha Kinases in Translational Control and*
735 *Adaptation to Cellular Stress.* *Cold Spring Harb Perspect Biol*, 2018. **10**(7).

- 736 45. Yung, H.W., et al., *Evidence of placental translation inhibition and*
737 *endoplasmic reticulum stress in the etiology of human intrauterine growth*
738 *restriction*. Am J Pathol, 2008. **173**(2): p. 451-62.
- 739 46. Gaccioli, F., et al., *Maternal overweight induced by a diet with high*
740 *content of saturated fat activates placental mTOR and eIF2alpha signaling*
741 *and increases fetal growth in rats*. Biol Reprod, 2013. **89**(4): p. 96.
- 742 47. Holland, O., et al., *Review: Placental mitochondrial function and*
743 *structure in gestational disorders*. Placenta, 2017. **54**: p. 2-9.
- 744 48. Park, C.W., et al., *The frequency and clinical significance of intra-*
745 *amniotic inflammation defined as an elevated amniotic fluid matrix*
746 *metalloproteinase-8 in patients with preterm labor and low amniotic fluid white*
747 *blood cell counts*. Obstet Gynecol Sci, 2013. **56**(3): p. 167-75.
- 748 49. Romero, R., et al., *Amniotic fluid white blood cell count: a rapid and*
749 *simple test to diagnose microbial invasion of the amniotic cavity and predict*
750 *preterm delivery*. Am J Obstet Gynecol, 1991. **165**(4 Pt 1): p. 821-30.
- 751 50. Abdallah, M.W., et al., *Amniotic fluid inflammatory cytokines: potential*
752 *markers of immunologic dysfunction in autism spectrum disorders*. World J
753 Biol Psychiatry, 2013. **14**(7): p. 528-38.
- 754 51. Kacerovsky, M., et al., *Amniotic fluid markers of oxidative stress in*
755 *pregnancies complicated by preterm prelabor rupture of membranes*. J
756 Matern Fetal Neonatal Med, 2014: p. 1-10.
- 757 52. Hayat, M., et al., *A very unusual complication of amniocentesis*. Clin
758 Case Rep, 2015. **3**(6): p. 345-8.

- 759 53. Kang, J.H., et al., *Comparative Transcriptome Analysis of Cell-Free*
760 *Fetal RNA from Amniotic Fluid and RNA from Amniocytes in Uncomplicated*
761 *Pregnancies*. PLoS One, 2015. **10**(7): p. e0132955.
- 762 54. Rayburn, W.F., J.A. Jolley, and L.L. Simpson, *Advances in ultrasound*
763 *imaging for congenital malformations during early gestation*. Birth Defects Res
764 A Clin Mol Teratol, 2015. **103**(4): p. 260-8.
- 765

Table 1. Profiles of ZIKV-infected pregnant patients

	Acute phase			Convalescent phase			
	Patient 1	Patient 2	Patient 3	Patient 1	Patient 2	Patient 3	Healthy ^g
Samples collection (PIO) ^a	5	7	6	14	13	9	-
Age (years)	33	34	28	-	-	-	-
Presence of symptom ^b							
<i>Fever</i>	No	No	Yes	-	-	-	-
<i>Headache</i>	Yes	No	Yes	-	-	-	-
<i>Rash</i>	Yes	No	No	-	-	-	-
<i>Pain around eye area</i>	Yes	No	No	-	-	-	-
Presence of ZIKV RNA in urine ^c	Yes	Yes	Yes	-	-	-	-
Percentage ZIKV antigen in blood	15	0.44	2.66	7.61	0.19	10.1	
(Infection level) ^d	(moderate)	(low)	(low)	(low)	(low)	(moderate)	-
Cell counts (numbers/ μ l blood) ^e							

<i>Neutrophils</i>	9378	4915	4868	8934	3668	5936	3784 ± 428
<i>Monocytes</i>	243	257	37	323	239	99	253 ± 96
<i>CD4 T cells</i>	301	954	393	501	491	407	993 ± 105
<i>CD8 T cells</i>	129	341	125	237	236	154	358 ± 83
<i>DNT cells</i>	41	30	27	132	29	25	93 ± 30
<i>NK cells</i>	57	72	126	232	103	115	163 ± 75
<i>NKT cells</i>	81	53	79	264	50	69	87 ± 39
<i>CD56hi cells</i>	16	13	15	28	12	15	11 ± 1
<i>CD14+CD56+ cells</i>	30	43	9	41	43	29	31 ± 13
<i>B cells</i>	74	309	85	105	114	82	211 ± 100
Antibody titer (fold increase) ^f							
<i>IgM</i>	1.29	1.91	2.72	0.92	1.79	2.68	-
<i>IgG</i>	1.10	3.79	3.14	0.88	3.85	2.86	-

^a Samples were collected during both acute (1-7 days post-illness onset (PIO)) and convalescent (8-14 days PIO) phases. Patient 1, 2 and 3 were infected during the first, second and third trimester, respectively.

- ^b Presence of any symptoms were recorded during the patients' first visit to the hospital
- ^c Presence of ZIKV RNA in urine samples was determined by qRT-PCR during the patients' first visit to the hospital.
- ^d Infection is determined by the percentage of ZIKV Ag positive CD14+ monocytes and the level of infection can be classified as moderate (>10%) or low (<10%)
- ^e Cellular numbers of the peripheral blood immune subset were calculated with the following formula: [Percentages of specific immune subset × total leucocyte numbers = cellular numbers of specific immune subset]. Percentages of specific immune subset were obtained with immune-phenotyping. Total leucocyte numbers were obtained with a hematology analyzer.
- ^f Antibody titer is expressed as fold change increase relative to levels obtained in healthy controls
- ^g Average numbers of peripheral blood immune cells obtained from healthy control are shown as mean ± SD

824 **Figure legends**

825 **Figure 1. ZIKV NS3 antigen in CD163-positive macrophages impacts**
 826 **stromal changes in full-term placentas.** Representative hematoxylin and
 827 eosin (H&E)-stained sections showing the placenta disc (chorionic villi) and
 828 fetal membranes of three patients infected by ZIKV at different gestational
 829 age. (A) Aggregates of vacuolated cells morphologically in keeping with
 830 Hofbauer cells are observed in the chorionic villi of all three infected patients.
 831 Areas highlighted by the black boxes are magnified in the lower panel. (B)
 832 Increased stromal and mononuclear cells are noted in the sub-amniotic
 833 connective tissue of the fetal membranes of the infected patients. All images
 834 were captured at 40X magnification. (C) Immunofluorescence microscopy was
 835 used to visualize ZIKV NS3 antigen (green) and CD163 protein (red) in the
 836 full-term placentas of the ZIKV-infected patients. The white arrows indicate
 837 co-localisation of the ZIKV antigen and CD163 protein within the villous
 838 stroma of the placenta disc. All images were captured at 40X magnification.

839

840 **Figure 2. Immune characterization of ZIKV-infected pregnant women.**
 841 Blood samples were obtained from three pregnant women during the acute
 842 and convalescent phases of ZIKV infection. The women were infected in
 843 different trimesters of their pregnancy: P1, trimester 1; P2, trimester 2; P3,
 844 trimester 3. Levels of peripheral blood immune cells and immune mediators
 845 were quantified by luminex and immunophenotyping, respectively. Healthy
 846 controls (n = 3) were included. (A) PCA scores of the infected and healthy
 847 women based on a collective analysis of immunophenotyping and cytokine
 848 measurements with Multiple Factor Analysis (MFA). (B) Correlation of

849 immunophenotyping and cytokine measurements with the first two principal
850 component axes (PC1, PC2) of the MFA. (C) Heatmaps comparing the
851 cytokine and immunophenotyping data for each subject. (D) Correlation
852 between infection (% ZIKV NS3+ monocytes) and neutrophil numbers in
853 whole blood samples.

854

855 **Figure 3. Principal component analysis of gene expression in placenta**
856 **samples.** Full-term placentas from ZIKV-infected pregnant patients were
857 obtained and separated into the fetal membrane, and CD45- non-immune and
858 CD45+ immune cells of the placenta disc. Placentas from two healthy women
859 were obtained in parallel as a control. The transcriptomes of the three cell
860 types (Placenta disc CD45+, Placenta disc Maternal CD45- and Fetal
861 membrane cells) were assessed by RNA-sequencing (RNA-seq). PCA was
862 performed by analyzing RNA-seq data either (A) collectively from all three cell
863 types, or (B) individually as each specific cell type.

864

865 **Figure 4. Placental gene expression associated with ZIKV infection.**
866 Heatmaps comparing gene expression in the different placental specimens
867 (Placenta disc CD45+, Placenta disc CD45- and Fetal membrane cells) from
868 ZIKV-infected women infected during the first (P1), second (P2) or third (P3)
869 trimesters of pregnancy. Placental samples from two healthy women (H1, H2)
870 were obtained in parallel as a control. Samples from H2, P1 and P2 were
871 processed in triplicates. The most highly differentially regulated genes and
872 pathways in P1 included EIF2 signaling, oxidative phosphorylation, histones
873 and ribosomal RNAs. Gene expression measurements from RNA-seq in

874 transcripts per million (TPM) scale were used to calculate a Z-score on each
875 row.

876

877 **Figure 5. ZIKV infection during pregnancy first trimester triggers**
878 **disparate cellular response in placenta disc CD45+ cells.** (A) Relative
879 proportions of immune subsets in placenta disc CD45+ cells in ZIKV-infected
880 patients (n=3) and healthy controls (n=2). (B) PCA of gene expression profiles
881 of placenta disc CD45+ cells from ZIKV patients and healthy controls. The
882 PCA was performed on 30,717 genes selected by standard deviation > 0. (C)
883 Correlation plot of immune-cell subset frequencies in placenta disc CD45+
884 cells with the first two principal components (PC1, PC2) of the gene
885 expression profiles. (D) Enrichment plot of the gene set
886 GSE2405_0H_VS_9H_A_PHAGOCYTOPHILUM_STIM_NEUTROPHIL_DN,
887 which was reported by GSEA as most enriched among all immunological
888 gene sets (C7 of MSigDB) in the placenta disc CD45+ fraction of Patient 1 vs.
889 Healthy placenta. The profile shows the running enrichment score (green
890 curve) and the positions of gene-set members (black vertical bars) on the
891 rank-ordered list of differential gene expression comparing the Patient 1 vs.
892 Healthy placenta disc CD45+ fraction.

893

Figure 1. ZIKV NS3 antigen in CD163-positive macrophages impacts stromal changes in full-term placentas. Representative hematoxylin and eosin (H&E)-stained sections showing the placenta disc (chorionic villi) and fetal membranes of three patients infected by ZIKV at different gestational age. (A) Aggregates of vacuolated cells morphologically in keeping with Hofbauer cells are observed in the chorionic villi of all three infected patients. Areas highlighted by the black boxes are magnified in the lower panel. (B) Increased stromal and mononuclear cells are noted in the sub-amniotic connective tissue of the fetal membranes of the infected patients. All images were captured at 40X magnification. (C) Immunofluorescence microscopy was used to visualize ZIKV NS3 antigen (green) and CD163 protein (red) in the full-term placentas of the ZIKV-infected patients. The white arrows indicate co-localisation of the ZIKV antigen and CD163 protein within the villous stroma of the placenta disc. All images were captured at 40X magnification.

Figure 2. Immune characterization of ZIKV-infected pregnant women. Blood samples were obtained from three pregnant women during the acute and convalescent phases of ZIKV infection. The women were infected in different trimesters of their pregnancy: P1, trimester 1; P2, trimester 2; P3, trimester 3. Levels of peripheral blood immune cells and immune mediators were quantified by luminex and immunophenotyping, respectively. Healthy controls ($n = 3$) were included. (A) PCA scores of the infected and healthy women based on a collective analysis of immunophenotyping and cytokine measurements with Multiple Factor Analysis (MFA). (B) Correlation of immunophenotyping and cytokine measurements with the first two principal component axes (PC1, PC2) of the MFA. (C) Heatmaps comparing the cytokine and immunophenotyping data for each subject. (D) Correlation between infection (% ZIKV NS3+ monocytes) and neutrophil numbers in whole blood samples.

Figure 3. Principal component analysis of gene expression in placenta samples. Full-term placentas from ZIKV-infected pregnant patients were obtained and separated into the fetal membrane, and CD45- non-immune and CD45+ immune cells of the placenta disc. Placentas from two healthy women were obtained in parallel as a control. The transcriptomes of the three cell types (Placenta disc CD45+, Placenta disc Maternal CD45- and Fetal membrane cells) were assessed by RNA-sequencing (RNA-seq). PCA was performed by analyzing RNA-seq data either (A) collectively from all three cell types, or (B) individually as each specific cell type.

Figure 3 Lum et al., 2019

Figure 5. ZIKV infection during pregnancy first trimester triggers disparate cellular response in placenta disc CD45+ cells. (A) Relative proportions of immune subsets in placenta disc CD45+ cells in ZIKV-infected patients (n=3) and healthy controls (n=2). (B) PCA of gene expression profiles of placenta disc CD45+ cells from ZIKV patients and healthy controls. The PCA was performed on 30,717 genes selected by standard deviation > 0. (C) Correlation plot of immune-cell subset frequencies in placenta disc CD45+ cells with the first two principal components (PC1, PC2) of the gene expression profiles. (D) Enrichment plot of the gene set GSE2405_0H_VS_9H_A_PHAGOCYTOPHILUM_STIM_NEUTROPHIL_DN, which was reported by GSEA as most enriched among all immunological gene sets (C7 of MSigDB) in the placenta disc CD45+ fraction of Patient 1 vs. Healthy placenta. The profile shows the running enrichment score (green curve) and the positions of gene-set members (black vertical bars) on the rank-ordered list of differential gene expression comparing the Patient 1 vs. Healthy placenta disc CD45+ fraction.

Table 1. Profiles of ZIKV-infected pregnant patients

	Acute phase			Convalescent phase			
	Patient 1	Patient 2	Patient 3	Patient 1	Patient 2	Patient 3	Healthy ^g
Samples collection (PIO) ^a	5	7	6	14	13	9	-
Age (years)	33	34	28	-	-	-	-
Presence of symptom ^b							
<i>Fever</i>	No	No	Yes	-	-	-	-
<i>Headache</i>	Yes	No	Yes	-	-	-	-
<i>Rash</i>	Yes	No	No	-	-	-	-
<i>Pain around eye area</i>	Yes	No	No	-	-	-	-
Presence of ZIKV RNA in urine ^c	Yes	Yes	Yes	-	-	-	-
Percentage ZIKV antigen in blood	15	0.44	2.66	7.61	0.19	10.1	
(Infection level) ^d	(moderate)	(low)	(low)	(low)	(low)	(moderate)	-
Cell counts (numbers/ μ l blood) ^e							

<i>Neutrophils</i>	9378	4915	4868	8934	3668	5936	3784 ± 428
<i>Monocytes</i>	243	257	37	323	239	99	253 ± 96
<i>CD4 T cells</i>	301	954	393	501	491	407	993 ± 105
<i>CD8 T cells</i>	129	341	125	237	236	154	358 ± 83
<i>DNT cells</i>	41	30	27	132	29	25	93 ± 30
<i>NK cells</i>	57	72	126	232	103	115	163 ± 75
<i>NKT cells</i>	81	53	79	264	50	69	87 ± 39
<i>CD56^{hi} cells</i>	16	13	15	28	12	15	11 ± 1
<i>CD14⁺CD56⁺ cells</i>	30	43	9	41	43	29	31 ± 13
<i>B cells</i>	74	309	85	105	114	82	211 ± 100
Antibody titer (fold increase) ^f							
<i>IgM</i>	1.29	1.91	2.72	0.92	1.79	2.68	-
<i>IgG</i>	1.10	3.79	3.14	0.88	3.85	2.86	-

^a Samples were collected during both acute (1-7 days post-illness onset (PIO)) and convalescent (8-14 days PIO) phases. Patient 1, 2 and 3 were infected during the first, second and third trimester, respectively.

^b Presence of any symptoms were recorded during the patients' first visit to the hospital

^c Presence of ZIKV RNA in urine samples was determined by qRT-PCR during the patients' first visit to the hospital.

^d Infection is determined by the percentage of ZIKV Ag positive CD14+ monocytes and the level of infection can be classified as moderate (>10%) or low (<10%)

^e Cellular numbers of the peripheral blood immune subset were calculated with the following formula: [Percentages of specific immune subset × total leucocyte numbers = cellular numbers of specific immune subset]. Percentages of specific immune subset were obtained with immune-phenotyping. Total leucocyte numbers were obtained with a hematology analyzer.

^f Antibody titer is expressed as fold change increase relative to levels obtained in healthy controls

^g Average numbers of peripheral blood immune cells obtained from healthy control are shown as mean ± SD