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## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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## ABSTRACT

### Introduction

Zika virus (ZIKV) infection in pregnancy has been associated with microcephaly and severe neurological damage to the fetus. Our aim is to document the risks of adverse pregnancy and birth outcomes and the prevalence of laboratory markers of congenital infection in deliveries to women experiencing ZIKV infection during pregnancy, using data from European Commission-funded prospective cohort studies in 20 centres in 11 countries across Latin America and the Caribbean.

### Methods and analysis

We will carry out a centre-by-centre analysis of the risks of adverse pregnancy and birth outcomes, comparing women with confirmed and suspected ZIKV infection in pregnancy to those with no evidence of infection in pregnancy. We will document the proportion of deliveries in which laboratory markers of congenital infection were present. Finally, we will investigate the associations of trimester of maternal infection in pregnancy, presence or absence of maternal symptoms of acute ZIKV infection, and previous flavivirus infections with adverse outcomes and with markers of congenital infection. Centre-specific estimates will be pooled using a two-stage approach.

### Ethics and dissemination

Ethical approval was obtained at each centre. Findings will be presented at international conferences and published in peer-reviewed open access journals, and discussed with local public health officials and representatives of the national Ministries of Health, Pan American Health Organization, and World Health Organization involved with ZIKV prevention and control activities.

### Keywords

Zika virus; pregnancy; vertical transmission; Congenital Zika Syndrome; microcephaly; meta-analysis

### Article summary

#### Strengths and limitations of this study

- This will be a pooled analysis of data from 3 international consortia conducting prospective cohort studies of outcomes following ZIKV infection in pregnancy in 20 centres in 11 countries.

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- Standardised definitions of outcomes will provide clarity about the absolute risks of adverse outcomes, which have not been reported consistently in prospective studies so far.
  - These studies include a control group of women with no evidence of ZIKV infection in pregnancy, allowing improved estimation of the proportion of adverse events attributable to ZIKV in pregnancy.
  - Inferences will be limited by: difficulties in distinguishing between women who did and who did not experience a ZIKV infection in pregnancy, due to the high frequency of mild and asymptomatic infections and the low sensitivity and specificity of diagnostic tools; low diagnostic sensitivity of markers of congenital infection; and outcome data that are not missing at random.



## INTRODUCTION

Following the emergence of Zika virus (ZIKV) in Asia and the Pacific in 2013 (1) and the subsequent introduction to Brazil (2), clusters of neonates with severe neurological complications and microcephaly were observed across Latin America. Following recent experiences with the H1N1 influenza pandemic and Ebola outbreak in Western Africa, the need for coordinated international research on ZIKV was quickly recognized. In January 2016, before the World Health Organization (WHO) declared a Public Health Emergency of International Concern (3), the European Commission (EC) issued a funding call to set up a network in Latin America and the Caribbean with the aim of implementing and coordinating urgently required research, while simultaneously contributing to research capacity and preparedness for other emerging infectious diseases. Three consortia were funded: ZikaPLAN (4) (<https://zikaplan.tghn.org/>), ZIKAlliance (<https://zikalliance.tghn.org/>), and ZIKAction (<http://zikaction.org/>). All are multidisciplinary international collaborations with active investigations in epidemiology, virology, immunology, diagnostics, mathematical modelling, social science, and animal studies. Each consortium includes its own prospective cohort study of ZIKV in pregnancy and a shared work package that aims to ensure the harmonization of protocols and data sets in order to facilitate a pooled analysis of cohort data. The primary aim of the pooled analysis is to investigate the incidence of adverse outcomes of ZIKV infection in pregnancy, including “congenital infection, microcephaly, Zika congenital syndrome, and other sequelae of ZIKV infection”.

The aim of this paper is to present a protocol for this pooled analysis. Data has been or is still being collected in multiple sites in 20 regional coordinating centres spread over 11 countries and regions across Latin America and the Caribbean. There are 15 ZIKAlliance centres: Sao Paulo, Rio de Janeiro, Recife and Belo Horizonte (Brazil); Valencia (Venezuela); Bucaramanga (Colombia); Guayaquil (Ecuador); Lima (Peru); Jalisco, Nayarit, Veracruz, Yucatan (Mexico); Santa Cruz de la Sierra (Bolivia); Havana (Cuba); Guadeloupe (French Territory of the Americas); 3 ZikaPLAN centres: Goiânia, Rio de Janeiro, Recife (Brazil); and 2 ZIKAction centres: Kingston (Jamaica) and Port-au-Prince (Haiti).

Several studies of ZIKV in pregnancy have recently been published. In registry-based studies (5–7), fetuses and newborns of women with confirmed infection in pregnancy have been reported to have “potentially Zika-related” adverse outcomes at rates of up to 15%, with higher rates in the first trimester. Registry-based studies are likely to over-estimate the risk of severe clinical manifestations and underestimate the risk of more mild clinical presentations because they recruit both prospectively ascertained ZIKV-infected pregnant women and women whose infection was recognized retrospectively

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3 following the birth of an infant with congenital abnormalities. Prospective studies of congenital infection  
4 have variously reported 25% “severe” and 21% “mild to moderate” outcomes in French Guiana (8), and  
5 27% adverse outcomes in Brazil (9). The specificity of these outcome definitions for ZIKV in pregnancy is  
6 not known as these studies did not include a control group of women with no ZIKV infection in  
7 pregnancy. In another Brazilian study, the risk of adverse outcomes was reported to be 46% in births to  
8 women with NAAT (Nucleic Acid Amplification Test)-confirmed ZIKV infection in pregnancy compared to  
9 11.5% in NAAT-negative women (10). In a large prospective study based in the French Territories of the  
10 Americas, among infants born to women with NAAT-confirmed ZIKV infection, 7.0% presented with  
11 neurologic or ocular birth defects and 3.1% met the study’s criteria for Congenital Zika Syndrome (CZS)  
12 (14), which is characterized by several unique features (11). An important limitation in the comparison  
13 of the results of these different studies is the lack of a standard definition of CZS and of the clinical and  
14 diagnostic procedures used to evaluate these children, leading to possible misclassification of the  
15 outcomes studied.

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26 The vertical transmission rate is the probability of congenital infection in births to women with infection  
27 in pregnancy. The rates reported so far, 26% (9) and 35% (8), are based on laboratory markers of  
28 congenital infection such as NAAT or IgM in the fetus or newborn. However, a prospective cohort  
29 retrospectively reconstructed from a register study estimated the vertical transmission rate to be only  
30 9% (12). Comparison of these rates is difficult as different markers and different biological samples were  
31 used. In addition, although these tests are analytically sensitive and specific they have poor diagnostic  
32 sensitivity as markers of congenital infection. These markers were absent from serum in a high  
33 proportion of CZS cases (13) (14) and in newborns with other potentially ZIKV-related adverse outcomes  
34 born to women with confirmed ZIKV during pregnancy (8,9). Clearance of virus from amniotic fluid and  
35 fetal blood has been reported in cases of CZS, even when ZIKV is found in brain tissue post-mortem  
36 (15,16). It therefore appears that fetal infection may occur, causing profound damage, but clearing  
37 before delivery and leaving no discernable immunological trace in serum. Consequently, in this study we  
38 will document the prevalence of markers of congenital infection using uniform criteria, recognizing that  
39 this is an underestimate of the true vertical transmission rate.

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49 Regarding effect modifiers, a number of studies have reported a higher incidence of congenital  
50 abnormalities following maternal infections in the first trimester (7,10–11,14). Maternal symptoms  
51 during acute ZIKV infection do not appear to be a risk factor for adverse outcomes (18). There is  
52 evidence of antibody dependent enhancement of ZIKV by dengue virus (DENV) antibody in animal  
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3 models (19), but it is unclear whether previous DENV infection or exposure to other flaviviruses has a  
4 protective, risk-enhancing, or null effect, in maternal or congenital infection in humans (20). It also  
5 remains to be established whether a previous ZIKV infection confers protective immunity. Little is  
6 currently known about risk factors for trans-placental transmission of ZIKV.  
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10 The analysis plan described here complements the recently published protocol of the Zika Virus  
11 Individual Participant Data Consortium (21), which will eventually include data from the three EC  
12 consortia as well as data from many other sources. Although the objectives of the protocols are similar,  
13 different methods are proposed, especially in relation to standardizing diagnostic results and outcomes.  
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16 In light of the unexplained heterogeneity in reported rates of adverse outcomes, and the variation in  
17 prevalence of markers of congenital infection, a pooled analysis of data from 20 centers following  
18 similar protocols with harmonized definitions of clinical and laboratory outcomes will provide important  
19 new information on outcomes of ZIKV in pregnancy.  
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## 24 **OBJECTIVES OF THE JOINT ANALYSIS**

- 25 1. To estimate the risk of adverse outcomes in the fetus, newborn, and child following maternal ZIKV  
26 infection in pregnancy, compared to outcomes in controls with no evidence of maternal infection in  
27 pregnancy.  
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- 29 2. To estimate the prevalence of markers of congenital infection in deliveries to women with ZIKV  
30 infection during pregnancy.  
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- 33 3. To assess the associations between trimester of maternal infection, presence or absence of maternal  
34 symptoms, and previous flavivirus infections with adverse outcomes and markers of congenital  
35 infection.  
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## 41 **METHODS**

### 42 **Participants**

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45 Pregnant women were eligible only if their infection status during pregnancy (infected or not infected)  
46 was ascertained prior to the detection of adverse outcomes, or was not influenced by fetal examination  
47 or outcome on delivery. This definition is compatible with retrospective testing of previously collected  
48 maternal samples, after delivery. Although the unit of recruitment is the mother, the unit of analysis is  
49 the fetus, newborn, and infant; multiple births are sufficiently rare to be treated as independent  
50 observations (22).  
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## Study design

Consenting women were screened in pregnancy for markers of ZIKV infection. Those in whom maternal infection in pregnancy (MIP) was suspected were followed with enhanced investigations. In ZIKAction and ZIKAlliance, all deliveries to these women, including fetal losses, stillbirths, and newborns were examined clinically and tested for markers of congenital infection. This testing was not routinely performed in ZikaPLAN. In all three cohorts, newborns were prospectively followed to identify any adverse outcomes that may develop later. A sample of newborns delivered to women with no evidence of infection in pregnancy served as an unexposed control group.

There were some differences between the protocols adopted by the three consortia in terms of how women were recruited into the study, and the choice and scheduling of tests and investigations (Supplementary Table S1). In ZIKAction and ZIKAlliance, women were recruited regardless of symptoms during pregnancy, although report of symptoms was recorded. In ZikaPLAN, only women with rash, a common sign of ZIKV infection, were recruited. Statistical analyses will therefore be stratified by whether the mother reported symptoms in pregnancy. Patients or the public were not involved in the protocol design.

## Target parameters and terminology of vertical transmission studies

The logic of an idealised prospective study is shown in Table 1. The usual target parameters are the *vertical transmission rate*, which is the probability of congenital infection following MIP,  $(A+B) / (A+B+C+D)$ ; and the *rate of adverse outcomes in those with congenital infection*,  $A/(A+B)$ . The definitions of “adverse outcomes”, congenital infection, and MIP will be determined by a Joint Diagnostics Group and a Joint Endpoint Review Group, after the data have been assembled. Estimates of these parameters are standard in studies of vertical transmission of human immune-deficiency virus (HIV) (23,24), toxoplasmosis (25–27) and cytomegalovirus (CMV) (28). In studies of less specific outcomes, the event rate  $C/(C+D)$  in fetuses and newborns of women with MIP but in whom no congenital infection occurred (Control Group 1 in Table 1) forms a comparison group (29,30) representing the adverse event rate that is due to MIP in the absence of congenital infection.

However, because cases of congenital infection cannot be reliably identified by diagnostic tests, we can only estimate the prevalence of laboratory *markers of vertical infection* (Objective 2). Similarly, the “overall” (unconditional) adverse event rate is taken as the primary outcome for Objective 1; this includes all births to women with MIP,  $(A+C)/(A+B+C+D)$  (Table 1). All three consortia included a further control

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3 group of births to unexposed women, those with no infection in pregnancy (Control Group 2 in Table 1).  
4 The adverse event rates in this group,  $E/(E+F)$ , represents a baseline for comparison with the overall event  
5 rates in exposed women (31).  
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8 Although estimates of the vertical transmission rate are compromised, it may still be of interest to  
9 compare adverse outcome rates in the MIP with congenital infection group (i.e.,  $A/(A+B)$ ), the MIP without  
10 congenital infection group (i.e.,  $C/(C+D)$ ), and the No MIP group (i.e.,  $E/(E+F)$ ), as this may provide insight  
11 into whether adverse fetal outcomes are associated with MIP in the absence of demonstrable fetal  
12 infection.  
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17 In addition to the challenges associated with the laboratory definition of congenital infection, it is also  
18 difficult to discriminate between pregnancies with MIP and with No MIP as required in analyses based on  
19 Table 1. A positive NAAT result or seroconversion during pregnancy are sufficient to confirm MIP.  
20 However, even if tested *per protocol*, MIP may be missed due to the narrow window of detection of NAAT  
21 tests, perhaps as low as 14 days (32). Tests of recent infection, including IgM, IgG3 or avidity assays, may  
22 reflect infection during pregnancy, but may also be the result of infection prior to pregnancy (33), and  
23 immunologic cross-reactivity to DENV antibody may need to be ruled out (34). These tests therefore  
24 indicate suspected but not confirmed MIP. An IgG negative response in the woman or newborn at or  
25 shortly after delivery can be taken as suggestive of No MIP, although the dynamics of ZIKV IgG are not  
26 well documented. While a negative IgG is likely to be uncommon in some sites, as some women will  
27 have experienced a ZIKV infection prior to pregnancy, it lowers the probability that a ZIKV infection would  
28 have occurred in pregnancy. Taking all this together, MIP status will be characterised as “Confirmed”,  
29 “Suspected”, “No Evidence of MIP” (i.e., all NAAT and IgM tests negative), and “No MIP” (i.e., all NAAT and  
30 IgM tests negative AND IgG negative at or shortly after delivery).  
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## 42 **Definitions of variables**

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44 To support the pooled analyses, a Joint Diagnostics Group and a Joint Endpoint Review Group will be  
45 convened to agree on standardized case definitions. Supplementary Table S2 provides some provisional  
46 definitions for: MIP (Confirmed, Suspected, No Evidence of MIP, No MIP), Markers of congenital infection  
47 (present or absent), signs and symptoms compatible with Congenital Zika Syndrome (CZS), other  
48 potentially-Zika-related outcomes (OPZRO), and trimester of MIP. To the greatest extent possible,  
49 definitions will be harmonized across consortia and across centres within consortia.  
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3 An essential aspect of the definitions for MIP status used in the statistical analyses below is that they must  
4 be based exclusively on the *prospective* diagnostic testing. For example, although CZS and/or laboratory  
5 evidence of congenital infection in the newborn provides compelling evidence of MIP, this would not  
6 affect the MIP status as ascertained prospectively. Thus, we expect to observe some newborns with CZS  
7 and/or with laboratory markers of congenital infection delivered to women with Suspected MIP, or even  
8 those categorized as having No Evidence of MIP.  
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## 19 **STATISTICAL ANALYSIS**

### 20 **Analysis of data from individual centres**

#### 21 **Descriptive tables**

22 We will produce descriptive tables providing a breakdown of the numbers in each centre with Confirmed  
23 MIP, Suspected MIP, No Evidence of MIP, and No MIP, and the type of evidence on which this is based  
24 (e.g., NAAT, seroconversion, tests of recent infection) (Supplementary Table S3). The similar  
25 Supplementary Table S4 will document numbers with signs or symptoms compatible with CZS and with  
26 markers of congenital infection, and the type of evidence on which this is based (e.g., NAAT, IgM).  
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#### 39 **Objective 1: Adverse Outcomes**

40 Prospectively ascertained MIP status will be tabulated against overall (i.e., unconditional on congenital  
41 infection status) adverse outcomes (Table 2). Various risks can be estimated within each MIP category,  
42 including: the risk of signs and symptoms compatible with CZS, the risk of OPZRO, the risk of both  
43 combined, of individual signs and symptoms, or of signs and symptoms grouped in clinically (e.g.,  
44 ophthalmologic defects) or embryologically meaningful ways. Outcomes may be binary (e.g.,  
45 microcephaly) or continuous (e.g., head circumference), or multi-category (CZS-related outcomes, OPZRO,  
46 asymptomatic). As well as congenital anomalies, rates of standard outcomes, in the absence of congenital  
47 anomalies, will be documented, including: fetal loss, stillbirth, low birthweight, intra-uterine growth  
48 retardation (IUGR), and premature delivery.  
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3 The rate of adverse outcomes in the No MIP group represents the study-specific background rate of  
4 adverse outcomes (i.e., in the absence of MIP, Control Group 2), to be compared to rates in women with  
5 Confirmed MIP. Absolute risks will be estimated as well as risk ratios and risk differences.  
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9 Parallel sets of estimates will be calculated in the Suspected MIP and No Evidence of MIP groups, as  
10 particular adverse events indicate lack of diagnostic specificity and sensitivity in the testing protocol  
11 during pregnancy and are therefore informative regarding the effectiveness of the maternal testing  
12 protocol in each centre; for example, microcephaly in the No Evidence of MIP group would indicate a lack  
13 of sensitivity. These sets of estimates may be pooled, respectively, with adverse outcome risks in  
14 Confirmed MIP and No MIP groups in sensitivity analyses.  
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## 22 Objective 2: Markers of vertical transmission

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24 Estimates of the prevalence of markers of congenital infection will be produced in all centres with  
25 available data for each MIP group except the No MIP group, as the definition of No MIP is not compatible  
26 with laboratory markers of congenital infection. For this purpose, the numerator will be the number with  
27 markers of congenital infection, and the denominator will be the sum of the numbers with and without  
28 markers of congenital infection. Separate estimates will be obtained for: laboratory markers, clinical  
29 markers, such as microcephaly, and combined laboratory and clinical markers (Table 3). Rates in the No  
30 Evidence of MIP group are of interest as they carry information about the diagnostic accuracy of the  
31 maternal testing protocol.  
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## 41 Objective 3: Effect of covariates

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43 Separate estimates of the prevalence of adverse pregnancy outcomes, and the probability of laboratory  
44 markers of congenital infection, will be produced for each trimester of maternal infection, and by  
45 presence or absence of maternal symptoms.  
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49 Analyses of adverse event frequencies and of markers of congenital infection can be extended to include  
50 multiple covariates, using logistic regression. These might include potential *effect modifiers*, such as  
51 previous arbovirus infection or co-infection, or *confounding factors* such as socio-economic indicators  
52 likely to be associated with both arbovirus exposure and adverse outcomes. However, at the time of  
53 writing it is not known whether sufficient data will be available for regression analyses.  
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## Secondary objectives

ZIKV infection in pregnancy could lead to adverse pregnancy and birth outcomes either following a congenital infection (cell A in Table 1) or in the absence of congenital infection (equally in cells A and C). Although absence of markers of congenital infection does not rule out congenital infection, we might still expect to observe more adverse outcomes in fetuses and newborns with laboratory markers of congenital infection if those outcomes are caused by congenital infection. By contrast, adverse outcomes that are the result of MIP in the absence of congenital infection (Cell E in Table 1) should occur equally with or without laboratory markers of congenital infection. Based on literature on other infections in pregnancy, including dengue virus (35), adverse outcomes associated with MIP in the absence of congenital infection potentially include: fetal loss, stillbirth, prematurity, IUGR, and low birthweight for gestational age (36). The analysis would be based on a tabulation of presence or absence of neonatal symptoms, or sets of symptoms (Supplementary Table S5), and would be stratified by trimester of maternal infection, as this is likely to be associated with the presence of markers of congenital infection and with adverse outcomes.

## Missing Covariates

All analyses will be conducted on a “complete case” basis, in the first instance. Methods for handling missing covariates, such as imputation (37), will be considered after the extent and patterns of missing data have been explored.

## Combining data across centres

The above analyses will generate a series of centre-specific estimates of proportions, relative risks comparing MIP and No MIP groups, risk-differences, and means of continuous variables, stratified by trimester and maternal clinical presentation. If logistic regression is used to examine effect-modifiers and confounders, further estimates of interaction terms or adjusted estimates can also be produced.

All these estimates can be combined across centres using fixed or random effects models in a “two-stage” meta-analysis. Bayesian Markov chain Monte Carlo methods will be used as this will facilitate the use of exact binomial and multinomial likelihoods, which have a better performance with low and zero cells



counts. Vague priors will be employed. Centre-specific random effect estimates will be sampled from beta distributions for binomial outcomes data, Dirichlet distributions for multinomial data, and normal distributions for continuous data.

In combining estimates from different centres, we will take account of the fact that in ZikaPLAN only women with rash were recruited, so that women with no ZIKV infection may have experienced other exanthematic infections (10), including arbovirus infections such as dengue and chikungunya, which may themselves be associated with adverse outcomes (38,39).

Depending on the results of two-stage analyses, and the completeness of covariate data, an individual patient data one-stage meta-analyses will be considered for each objective, as a secondary or sensitivity analysis, with centre as an additional fixed “intercept” term.

## Sensitivity analyses

### Definitions of MIP status, ZIKV-related outcomes, and laboratory markers of congenital infection

We will report differences in adverse event rates between Confirmed MIP and Suspected MIP, and between No Evidence of MIP and No MIP. If the differences are small, we will produce results pooling these categories as a sensitivity analysis.

Further, the No Evidence of MIP category can be subdivided into women who were tested *per protocol* and those who may have been tested less completely. The impact of compliance with protocols will be explored, as it is expected to impact on the proportion of women with MIP who are classified as “No evidence of MIP” and, hence, on the probability of observing adverse outcomes in this group. Similarly, we will conduct sensitivity analyses around the definitions of Confirmed and Suspected MIP on advice from the Joint Diagnostics Group.

Alternative sets of estimates will be generated using alternative criteria for CZS-related outcomes and OPZRO, that are more, or less, specific for ZIKV in pregnancy. Similarly, we will explore the impact of varying the laboratory criteria for congenital infection on the advice of the Joint Diagnostics Group.

## Independent ascertainment of outcomes

A critical requirement of all these analyses is that the ascertainment of markers of congenital infection status and clinical outcomes in the fetuses and newborns, and developing infant are all independent of each other, and also independent of MIP status. For example, ideally the same laboratory testing for congenital infection is carried out regardless of whether the pregnancy outcome is a fetal loss, termination of pregnancy, stillbirth, a case of CZS, or an apparently healthy asymptomatic infant.

These assumptions are difficult, and in certain respects (e.g., first trimester fetal loss and terminations) not possible, to fully implement in practice. To address these inevitable limitations in the analysis, which are expected to impact more on objective 2 than on objective 1, we will carry out exploratory analyses aimed at detecting potential deviations from protocol. For example, the distribution of trimester of MIP should not be associated with prospectively ascertained MIP status. Guided by the results, we will carry out sensitivity analyses that make a range of assumptions about the distribution of missing data, especially data on markers of congenital infection. A series of scenarios will be examined to assess robustness of results to inherent and/or unplanned deviations from the ideal protocols required for unbiased estimation of the target parameters.

## DISCUSSION

Risks of CZS and other adverse birth outcomes of ZIKV infection in pregnancy can only be assessed through studies that recruit women whose infection status is prospectively ascertained, or, if retrospective, ascertainment is independent of outcomes. However, reported risks of adverse outcomes even from prospective studies have been highly variable (8–10,17), as have vertical transmission rates based on laboratory markers of congenital ZIKV infection (8,9,12). An important role for joint analyses of multiple studies is to explore whether this heterogeneity in outcomes can be explained by individual or study-level covariates. To do this, it is essential that incidental sources of variation, such as those arising from differences in outcome reporting or diagnostic testing, are controlled or eliminated as much as possible. One of the most difficult sources of variation between consortia, and between sites within consortia, lies in diagnosis of maternal infection. Our approach is to have an Expert Diagnostics Group produce a harmonised classification of Confirmed MIP, Suspected MIP, No Evidence of MIP, and No MIP, and to compute a range of estimates of the relative effect of maternal infection on outcomes, grouping these in different ways. An analysis based on the Confirmed MIP and No MIP groups alone would be expected to

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3 generate the largest estimates of relative effect, because both poor sensitivity and poor specificity will  
4 tend to bias effect estimates towards the null. An alternative proposal (21) is to treat test sensitivity and  
5 specificity as study-level covariates in a meta-regression. Other statistical methods may have been  
6 developed by the time the data becomes available for these analyses. Whatever form of analysis is  
7 adopted, a standardised pooled analysis from three large consortia comprising 20 centres will provide  
8 valuable information about these parameters, which will assist in framing a public health response and  
9 advice to women who might be exposed in future. It may also throw light on pathologic mechanisms  
10 leading to adverse outcomes, which could help in the development of therapeutic or prophylactic  
11 interventions.  
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### 51 **Author Contributions**

52 The paper was drafted by AEA with the assistance of EBB and NA, following discussions with a steering  
53 group that included CT, TJ, KR and RAAX. The draft was revised based on comments from CT, DB, ASA,  
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3 TJ, KR, MP, DBM, RAAX, PM, MCM, KR, ATH. Comments were then solicited from members of the EC  
4 Zika Consortia Vertical Transmission Study Group collaborators, and a revision was drafted by AEA, EBB  
5 and NA. CG, XdL and AW-S are equal last authors and the consortia PIs. The final draft was agreed by all  
6 authors.  
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10  
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19

20 **Competing interests**  
21

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27 MK has a patent on zika diagnostics pending.  
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33 **Data statement section** Not applicable  
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## TABLES

**Table 1.** Design of an idealized prospective vertical transmission study. The vertical transmission rate is estimated by  $(A+B)/(A+B+C+D)$ . The rate of adverse outcomes conditional on congenital infection is  $A/(A+B)$ . This can be compared with the rate of adverse outcomes in newborns with no congenital infection,  $C/(C+D)$ , who form a control group (Control group 1) to account for potential confounders associated with maternal infection.

An overall, non-conditional estimate of the adverse event rate is  $(A+C)/(A+B+C+D)$ .

Follow-up of births to women with no MIP creates a second control group (Control Group 2), in which the rate of adverse outcomes,  $E/(E+F)$ , can be compared to the rate in births with no congenital infection to women with MIP. Estimated effects of MIP based on Control Group 2 are vulnerable to confounding by factors associated with MIP. Some cells are set to zero as there can be no congenital infection without MIP.

			Maternal Infection Status	
			MIP	No MIP
Congenital Infection Status	congenital infection	Adverse outcomes	A	0
		No Adverse outcomes	B	0
	No congenital infection	Adverse outcomes	C	E
		No Adverse outcomes	D	F

**Table 2.** Scheme for a generic analysis of risks of adverse outcomes by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Presence or absence of fetal and neonatal signs or symptoms (e.g., microcephaly, brain calcifications, arthrogryposis) and other Potentially Zika-related outcomes (e.g., fetal loss) in the different MIP groups will be compared.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Symptom 1				
Symptom 2				
Symptom 3				
:				
No Symptoms				
TOTAL				

**Table 3.** Scheme for generic analysis of markers of congenital infection by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Markers of congenital infection may include laboratory markers, clinical markers, such as microcephaly, or both combined.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Markers of congenital infection present				
Markers of congenital infection absent				
Not tested				
TOTAL				

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For peer review only

## SUPPLEMENTARY MATERIALS

**Supplementary Table S1.** Schedule of tests and examinations in pregnancy, at delivery, and during paediatric follow-up, in each consortium.

Consortium	Mother	Fetus	Newborn and neonate	Pediatric follow-up
ZIKAlliance	ZIKV serology IgG & IgM, NAAT blood and urine, at booking and every 4w.	Laboratory, pathology examination of fetal loss and stillbirth.	NAAT on placenta.  Clinical examination of newborn.  Newborn testing: NAAT, IgM cord blood, urine; TORCH, HIV	Clinical examination  NAAT blood urine; IgM, IgG blood at 4w, 4m, 12m, 24m.  Transcranial U/S at 4w. Hearing, eye, neurodevelopmental assessments
ZIKAction	IgM, IgG serology booking, 20, 28, Delivery, and at pediatric follow-up  NAAT testing, and additional U/S investigations if evidence of infection	Laboratory, pathology examination of fetal loss and stillbirth.	Clinical examination of newborn  Newborn testing: IgM, IgG (serum; CSF only if clinically indicated), urine, saliva.  NAAT, U/S and ophthalmology investigations if evidence of infection	Paediatric testing:  IgG, IgM (serum, urine, saliva) and clinical examination at: 4w, 4m, 9m, 12m, 18m and 24m. NAAT if evidence on infection  Neurodevelopmental assessments
ZikaPLAN	NAAT and serologic testing (PRNT, IgM, IgG3) when symptoms reported and at up to three follow-up visits.  TORCH, DENV, and CHIKV testing	Not routinely tested.	Clinical examination in early infancy.  NAAT testing in the neonatal period in some centres	Clinical examination of the child by specialists at 3, 6, 12, 18, 24, 36, and 48m  Neurodevelopmental assessments

CSF, Cerebro-spinal fluid; DENV, Dengue virus; CHIKV, Chikungunya virus; HIV, Human Immunodeficiency virus; Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test; TORCH, Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes; U/S, Ultrasound; ZIKV, Zika virus

**Supplementary Table S2.** Illustrative and approximate definitions of key explanatory and outcome variables. The final definitions will be determined by the Joint Diagnostics Group and Joint End-point Review Group.

<i>Maternal Symptoms</i>	For example: rash, fever, headache, joint pain
<i>Maternal Infection in Pregnancy (MIP) status, established only on the basis of maternal testing</i>	Confirmed: NAAT, Seroconversion Suspected: Tests of recent infection, including: IgM, IgG3, avidity No Evidence of MIP: Maternal testing protocol was followed, but none of the above were positive. No MIP: As above but with IgG negative at or near time of delivery
<i>Markers of Congenital Infection (CI)</i>	Present: NAAT or IgM any time in first 7 days; Absent: all other findings
<i>Most likely trimester of onset of maternal infection</i>	The highest available from the following hierarchy: <ul style="list-style-type: none"> <li>• Date of first NAAT positive test minus average duration of viremia</li> <li>• Seroconversion: Midpoint between last serological negative and last positive</li> <li>• Tests of recent infection: Date of first positive</li> </ul>
<i>Signs and symptoms compatible with Congenital Zika Syndrome (CZS)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available.
<i>Other Potentially Zika-related Outcomes (OPZRO)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available.

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test.

**Supplementary Table S3.** Prospectively ascertained Maternal Infection in Pregnancy (MIP) status and evidence on which it is based. NB: Data from the same woman can appear in more than one of the last four columns

Centre	Total women	Prospectively ascertained MIP status				Evidence for Confirmed or Suspected MIP			
		Confirmed MIP	Suspected MIP	No Evidence of MIP	No MIP	NAAT	Sero-conversion	IgM/IgG3	PRNT
1									
2									
...									
etc									

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S4.** Summary of evidence on laboratory and clinical markers of congenital infection (CI) and evidence on which it is based. NB: Data from the same fetus/newborn can appear in more than one column

Centre	Total number fetus / newborn	Markers of congenital infection			Evidence on congenital infection				
		Any marker of congenital infection	Laboratory markers of congenital infection	No markers of congenital infection	NAAT	IgM	Signs and symptoms compatible with CZS	Microcephaly	Other potentially Zika-related Symptoms
1									
2									
...									
etc									

CZS, Symptoms compatible with Congenital Zika Syndrome; IgM/G, Immunoglobulin M/G; NAAT, Nucleic Acid Amplification Test; OPZRO, Other potentially Zika-related symptoms; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S5.** Distributions of signs and symptoms by laboratory markers of congenital infection and prospectively ascertained maternal infection in pregnancy (MIP) status. The breakdown shown is an example. Other examples might be "Termination of pregnancy, Pregnancy loss, Stillbirth, Livebirth"; or "Sensorineural hearing loss (SNHL), No SNHL "

<b>CENTRE</b>			
<b>Sign or symptom in fetus, newborn, or infant</b>	<b>MIP with markers of congenital infection</b>	<b>MIP with no markers of congenital infection</b>	<b>No MIP</b>
Signs and symptoms compatible with Congenital Zika Syndrome (CZS)			
Other potentially Zika-related outcomes (OPZRO)			
Asymptomatic			
<b>TOTAL = 100%</b>			

# BMJ Open

## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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## ABSTRACT

### Introduction

Zika virus (ZIKV) infection in pregnancy has been associated with microcephaly and severe neurological damage to the fetus. Our aim is to document the risks of adverse pregnancy and birth outcomes and the prevalence of laboratory markers of congenital infection in deliveries to women experiencing ZIKV infection during pregnancy, using data from European Commission-funded prospective cohort studies in 20 centres in 11 countries across Latin America and the Caribbean.

### Methods and analysis

We will carry out a centre-by-centre analysis of the risks of adverse pregnancy and birth outcomes, comparing women with confirmed and suspected ZIKV infection in pregnancy to those with no evidence of infection in pregnancy. We will document the proportion of deliveries in which laboratory markers of congenital infection were present. Finally, we will investigate the associations of trimester of maternal infection in pregnancy, presence or absence of maternal symptoms of acute ZIKV infection, and previous flavivirus infections with adverse outcomes and with markers of congenital infection. Centre-specific estimates will be pooled using a two-stage approach.

### Ethics and dissemination

Ethical approval was obtained at each centre. Findings will be presented at international conferences and published in peer-reviewed open access journals, and discussed with local public health officials and representatives of the national Ministries of Health, Pan American Health Organization, and World Health Organization involved with ZIKV prevention and control activities.

### Keywords

Zika virus; pregnancy; vertical transmission; Congenital Zika Syndrome; microcephaly; meta-analysis

## Strengths and limitations of this study

- This will be a pooled analysis of data from 3 international consortia conducting prospective cohort studies of outcomes following ZIKV infection in pregnancy in 20 centres in 11 countries.
- Standardised definitions of outcomes will provide clarity about the absolute risks of adverse outcomes, which have not been reported consistently in prospective studies so far.
- These studies include a control group of women with no evidence of ZIKV infection in pregnancy, allowing improved estimation of the proportion of adverse events attributable to ZIKV in pregnancy.
- Inferences will be limited by: difficulties in distinguishing between women who did and who did not experience a ZIKV infection in pregnancy, due to the high frequency of mild and asymptomatic infections and the low sensitivity and specificity of diagnostic tools; low diagnostic sensitivity of markers of congenital infection; and outcome data that are not missing at random.

## INTRODUCTION

Following the emergence of Zika virus (ZIKV) in Asia and the Pacific in 2013 (1) and the subsequent introduction to Brazil (2), clusters of neonates with severe neurological complications and microcephaly were observed across Latin America. Following recent experiences with the H1N1 influenza pandemic and Ebola outbreak in Western Africa, the need for coordinated international research on ZIKV was quickly recognized. In January 2016, before the World Health Organization (WHO) declared a Public Health Emergency of International Concern (3), the European Commission (EC) issued a funding call to set up a network in Latin America and the Caribbean with the aim of implementing and coordinating urgently required research, while simultaneously contributing to research capacity and preparedness for other emerging infectious diseases. Three consortia were funded: ZikaPLAN (4) (<https://zikaplan.tghn.org/>), ZIKAlliance (<https://zikalliance.tghn.org/>) (5,6), and ZIKAction (<http://zikaction.org/>). All are multidisciplinary international collaborations with active investigations in epidemiology, virology, immunology, diagnostics, mathematical modelling, social science, and animal studies. Each consortium includes its own prospective cohort study of ZIKV in pregnancy and a shared work package that aims to ensure the harmonization of protocols and data sets in order to facilitate a pooled analysis of cohort data. The primary aim of the pooled analysis is to investigate the incidence of adverse outcomes of ZIKV infection in pregnancy, including “congenital infection, microcephaly, Zika congenital syndrome, and other sequelae of ZIKV infection”.

The aim of this paper is to present a protocol for this pooled analysis. Data has been or is still being collected in multiple sites in 20 regional coordinating centres spread over 11 countries and regions across Latin America and the Caribbean. There are 15 ZIKAlliance centres: Sao Paulo, Rio de Janeiro, Recife and Belo Horizonte (Brazil); Valencia (Venezuela); Bucaramanga (Colombia); Guayaquil (Ecuador); Lima (Peru); Jalisco, Nayarit, Veracruz, Yucatan (Mexico); Santa Cruz de la Sierra (Bolivia); Havana (Cuba); Guadeloupe (French Territory of the Americas); 3 ZikaPLAN centres: Goiânia, Rio de Janeiro, Recife (Brazil); and 2 ZIKAction centres: Kingston (Jamaica) and Port-au-Prince (Haiti). Recruitment to ZIKAlliance began May 2017, September 2017 for ZIKAction, and December 2015 for ZikaPLAN. Over 700 women with confirmed infection had been recruited by April 2020; recruitment continues in some centres.

Several studies of ZIKV in pregnancy have recently been published. In registry-based studies (7-9), fetuses and newborns of women with confirmed infection in pregnancy have been reported to have “potentially Zika-related” adverse outcomes at rates of up to 15%, with higher rates in the first trimester. Registry-based studies are likely to over-estimate the risk of severe clinical manifestations and underestimate the

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3 risk of more mild clinical presentations because they recruit both prospectively ascertained ZIKV-infected  
4 pregnant women and women whose infection was recognized retrospectively following the birth of an  
5 infant with congenital abnormalities. Prospective studies of congenital infection have variously reported  
6 25% “severe” and 21% “mild to moderate” outcomes in French Guiana (10), and 27% adverse outcomes  
7 in Brazil (11). The specificity of these outcome definitions for ZIKV in pregnancy is not known as these  
8 studies did not include a control group of women with no ZIKV infection in pregnancy. In another Brazilian  
9 study, the risk of adverse outcomes was reported to be 46% in births to women with NAAT (Nucleic Acid  
10 Amplification Test)-confirmed ZIKV infection in pregnancy compared to 11.5% in NAAT-negative women  
11 (12). In a large prospective study based in the French Territories of the Americas, among infants born to  
12 women with NAAT-confirmed ZIKV infection, 7.0% presented with neurologic or ocular birth defects and  
13 3.1% met the study’s criteria for Congenital Zika Syndrome (CZS) (13), which is characterized by several  
14 unique features (14). An important limitation in the comparison of the results of these different studies is  
15 the lack of a standard definition of CZS and of the clinical and diagnostic procedures used to evaluate  
16 these children, leading to possible misclassification of the outcomes studied.  
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19 The vertical transmission rate is the probability of congenital infection in births to women with infection  
20 in pregnancy. The rates reported so far, 26% (11) and 35% (10), are based on laboratory markers of  
21 congenital infection such as NAAT or IgM in the fetus or newborn. However, a prospective cohort  
22 retrospectively reconstructed from a register study estimated the vertical transmission rate to be only 9%  
23 (15). Comparison of these rates is difficult as different markers and different biological samples were used.  
24 In addition, although these tests are analytically sensitive and specific they have poor diagnostic sensitivity  
25 as markers of congenital infection. These markers were absent from serum in a high proportion of CZS  
26 cases (16) (16,17) and in newborns with other potentially ZIKV-related adverse outcomes born to women  
27 with confirmed ZIKV during pregnancy (10,11). Clearance of virus from amniotic fluid and fetal blood has  
28 been reported in cases of CZS, even when ZIKV is found in brain tissue post-mortem (18,19). It therefore  
29 appears that fetal infection may occur, causing profound damage, but clearing before delivery and leaving  
30 no discernable immunological trace in serum. Consequently, in this study we will document the  
31 prevalence of markers of congenital infection using uniform criteria, recognizing that this is an  
32 underestimate of the true vertical transmission rate.  
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35 Regarding effect modifiers, a number of studies have reported a higher incidence of congenital  
36 abnormalities following maternal infections in the first trimester (7,10–11,14). Maternal symptoms during  
37 acute ZIKV infection do not appear to be a risk factor for adverse outcomes (20). There is evidence of  
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3 antibody dependent enhancement of ZIKV by dengue virus (DENV) antibody in animal models (21), but it  
4 is unclear whether previous DENV infection or exposure to other flaviviruses has a protective, risk-  
5 enhancing, or null effect, in maternal or congenital infection in humans (22). It also remains to be  
6 established whether a previous ZIKV infection confers protective immunity. Little is currently known about  
7 risk factors for trans-placental transmission of ZIKV.  
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12 The analysis plan described here complements the recently published protocol of the Zika Virus Individual  
13 Participant Data (IPD) Consortium (23), which will eventually include data from the three EC consortia as  
14 well as data from many other sources. Although the objectives of the protocols are similar, different  
15 methods are proposed in relation to design of included studies, definition of congenital infection, and  
16 approach to imperfect diagnosis of maternal infection.  
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21 In light of the unexplained heterogeneity in reported rates of adverse outcomes, and the variation in  
22 prevalence of markers of congenital infection, a pooled analysis of data from 20 centers following similar  
23 protocols with harmonized definitions of clinical and laboratory outcomes will provide important new  
24 information on outcomes of ZIKV in pregnancy.  
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## 30 **OBJECTIVES OF THE JOINT ANALYSIS**

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33 1. To estimate the risk of adverse outcomes in the fetus, newborn, and child following maternal ZIKV  
34 infection in pregnancy, compared to outcomes in controls with no evidence of maternal infection in  
35 pregnancy.  
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- 38 2. To estimate the prevalence of markers of congenital infection in deliveries to women with ZIKV  
39 infection during pregnancy.  
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- 42 3. To assess the associations between trimester of maternal infection, presence or absence of maternal  
43 symptoms, and previous flavivirus infections with adverse outcomes and markers of congenital  
44 infection.  
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## 50 **METHODS**

### 51 **Participants**

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3 Pregnant women were eligible only if their infection status during pregnancy (infected or not infected)  
4 was ascertained prior to the detection of adverse outcomes, or was not influenced by fetal examination  
5 or outcome on delivery. This definition is compatible with retrospective testing of previously collected  
6 maternal samples, after delivery. Although the unit of recruitment is the mother, the unit of analysis is  
7 the fetus, newborn, and infant; multiple births are sufficiently rare to be treated as independent  
8 observations (24).  
9

## 14 Study design

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16 Consenting women were screened in pregnancy for markers of ZIKV infection. Those in whom maternal  
17 infection in pregnancy (MIP) was suspected were followed with enhanced investigations. In ZIKAction and  
18 ZIKAlliance, all deliveries to these women, including fetal losses, stillbirths, and newborns were examined  
19 clinically and tested for markers of congenital infection. This testing was not routinely performed in  
20 ZikaPLAN. In all three cohorts, newborns were prospectively followed to identify any adverse outcomes  
21 that may develop later. In all three consortia, a sample of newborns delivered to women with no evidence  
22 of infection in pregnancy served as an unexposed control group.  
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28 There were some differences between the protocols adopted by the three consortia in terms of how  
29 women were recruited into the study, and the choice and scheduling of tests and investigations  
30 (Supplementary Table S1). In ZIKAction and ZIKAlliance, women were recruited regardless of symptoms  
31 during pregnancy, although report of symptoms was recorded. In ZikaPLAN, only women with rash, a  
32 common sign of ZIKV infection, were recruited. Statistical analyses will therefore be stratified by whether  
33 the mother reported symptoms in pregnancy. .  
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39 When the studies were designed, there was little information on the risk of adverse outcomes of ZIKV in  
40 pregnancy, on vertical transmission rates, nor on what infection rates among pregnant women might be  
41 expected. Formal sample size calculations were not undertaken.  
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## 47 Target parameters and terminology of vertical transmission studies

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49 Six categories of joint congenital infection status and maternal infection status (A-F) are defined in Table 1,  
50 which illustrates the logic of an idealised prospective study. The usual target parameters are the *vertical*  
51 *transmission rate*, which is the probability of congenital infection following MIP,  $(A+B) / (A+B+C+D)$ ; and  
52 the *rate of adverse outcomes in those with congenital infection*,  $A/(A+B)$ . The definitions of “adverse  
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3 outcomes”, congenital infection, and MIP will be determined by a Joint Diagnostics Group and a Joint  
4 Endpoint Review Group, after the data have been assembled. Estimates of these parameters are standard  
5 in the classic studies of vertical transmission of human immune-deficiency virus (HIV) (25,26),  
6 toxoplasmosis (27–29) and cytomegalovirus (CMV) (30). In studies of less specific outcomes, the event  
7 rate  $C/(C+D)$  in fetuses and newborns of women with MIP but in whom no congenital infection occurred  
8 (Paediatric Control Group 1 in Table 1) forms a comparison group (31,32) representing the adverse event  
9 rate that is due to MIP in the absence of congenital infection. The present analysis plan is modelled closely  
10 on these earlier studies, but includes adaptations to take account of the difficulties in diagnosing maternal  
11 and congenital ZIKV infection.  
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19 For example, because cases of congenital infection cannot be reliably identified by diagnostic tests, we  
20 can only estimate the prevalence of laboratory *markers of vertical infection* (Objective 2). Similarly, the  
21 “overall” (unconditional) adverse event rate is taken as the primary outcome for Objective 1; this includes  
22 all births to women with MIP,  $(A+C)/(A+B+C+D)$  (Table 1). All three consortia included a further control  
23 group of births to unexposed women, those with no infection in pregnancy (Maternal Control Group in  
24 Table 1). The adverse event rates in this group,  $E/(E+F)$ , represents a baseline for comparison with the  
25 overall event rates in exposed women (33).  
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31 Although estimates of the vertical transmission rate are compromised, it may still be of interest to  
32 compare adverse outcome rates in the MIP with congenital infection group (i.e.,  $A/(A+B)$ ), the MIP without  
33 congenital infection group (i.e.,  $C/(C+D)$ ), and the No MIP group (i.e.,  $E/(E+F)$ ), as this may provide insight  
34 into whether adverse fetal outcomes are associated with MIP in the absence of demonstrable fetal  
35 infection.  
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40 In addition to the challenges associated with the laboratory definition of congenital infection, it is also  
41 difficult to discriminate between pregnancies with MIP and with No MIP as required in analyses based on  
42 Table 1. A positive NAAT result or seroconversion during pregnancy are sufficient to confirm MIP.  
43 However, even if tested *per protocol*, MIP may be missed due to the narrow window of detection of NAAT  
44 tests, perhaps as low as 14 days (34). Tests of recent infection, including IgM, IgG3 or avidity assays, may  
45 reflect infection during pregnancy, but may also be the result of infection prior to pregnancy (35), and  
46 immunologic cross-reactivity to DENV antibody may need to be ruled out (36). These tests therefore  
47 indicate suspected but not confirmed MIP. An IgG negative response in the woman or newborn at or tests,  
48 perhaps as low as 14 days (34). Tests of recent infection, including IgM, IgG3 or avidity assays, may reflect  
49 infection during pregnancy, but may also be the result of infection prior to pregnancy (35), and  
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3 immunologic cross-reactivity to DENV antibody may need to be ruled out (36). These tests therefore  
4 indicate suspected but not confirmed MIP. An IgG negative response in the woman or newborn at or  
5 shortly after delivery can be taken as suggestive of No MIP, although the dynamics of ZIKV IgG are not  
6 well documented. While a negative IgG is likely to be uncommon in some sites, as some women will  
7 have experienced a ZIKV infection prior to pregnancy, it lowers the probability that a ZIKV infection would  
8 have occurred in pregnancy. Taking all this together, MIP status will be characterised as "Confirmed",  
9 "Suspected, "No Evidence of MIP" (i.e., all NAAT and IgM tests negative), and "No MIP" (i.e., all NAAT and  
10 IgM tests negative AND IgG negative at or shortly after delivery). To maintain the principle of prospective  
11 ascertainment, confirmation of congenital infection will have no impact on the mother's assigned  
12 infection status.  
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## 21 **Definitions of variables**

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23 To support the pooled analyses, a Joint Diagnostics Group consisting of immunologists and virologists and  
24 a Joint Endpoint Review Group consisting of paediatricians with experience of congenital ZIKV will be  
25 convened to agree on standardized case definitions. The names and qualifications of members of both  
26 groups will be published at the time of the joint data analysis, together with rationale and process for  
27 their recruitment. Supplementary Table S2 provides some provisional definitions for: MIP (Confirmed,  
28 Suspected, No Evidence of MIP, No MIP), laboratory markers of congenital infection (present or absent),  
29 signs and symptoms compatible with Congenital Zika Syndrome (CZS), other potentially-Zika-related  
30 outcomes (OPZRO), and trimester of MIP. The Diagnostics Group will also have responsibility for  
31 definitions of covariates, such as previous flavivirus infection. Definitions of both diagnostic categories  
32 and clinical endpoints will be based on the best information available at the time of analysis, and will to  
33 the greatest extent possible, be harmonized across consortia and across centres within consortia. As the  
34 expert groups will be assembled from representatives of each consortium, they will already have  
35 examined the data prior to a joint analysis, so that blinding will not be possible; however, they will devise  
36 diagnostic and clinical criteria that can be applied objectively across the three consortia.  
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47 An essential aspect of the definitions for MIP status used in the statistical analyses below is that they must  
48 be based exclusively on the *prospective* diagnostic testing. For example, although CZS and/or laboratory  
49 evidence of congenital infection in the newborn provides compelling evidence of MIP, this would not  
50 affect the MIP status as ascertained prospectively. Thus, we expect to observe some newborns with CZS  
51 and/or with laboratory markers of congenital infection delivered to women with Suspected MIP, or even  
52 those categorized as having No Evidence of MIP.  
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## STATISTICAL ANALYSIS

### Analysis of data from individual centres

#### Descriptive tables

We will produce descriptive tables providing a breakdown of the numbers in each centre with Confirmed MIP, Suspected MIP, No Evidence of MIP, and No MIP, and the type of evidence on which this is based (e.g., NAAT, seroconversion, tests of recent infection) (Supplementary Table S3). The similar Supplementary Table S4 will document numbers with signs or symptoms compatible with CZS and with markers of congenital infection, and the type of evidence on which this is based, for example NAAT, IgM, or clinical markers (CZS).

#### Objective 1: Adverse Outcomes

Prospectively ascertained MIP status will be tabulated against overall (i.e., unconditional on congenital infection status) adverse outcomes (Table 2). Various risks can be estimated within each MIP category, including: the risk of signs and symptoms compatible with CZS, the risk of OPZRO, the risk of both combined, of individual signs and symptoms, or of signs and symptoms grouped in clinically (e.g., ophthalmologic defects) or embryologically meaningful ways. Outcomes may be binary (e.g., microcephaly) or continuous (e.g., head circumference), or multi-category (CZS-related outcomes, OPZRO, asymptomatic). As well as congenital anomalies, rates of standard outcomes, in the absence of congenital anomalies, will be documented, including: fetal loss, stillbirth, low birthweight, intra-uterine growth retardation (IUGR), and premature delivery.

The rate of adverse outcomes in the No MIP group represents the study-specific background rate of adverse outcomes (i.e., in the absence of MIP, Control Group 2), to be compared to rates in women with Confirmed MIP. Absolute risks will be estimated as well as risk ratios and risk differences.

Parallel sets of estimates will be calculated in the Suspected MIP and No Evidence of MIP groups, as particular adverse events indicate lack of diagnostic specificity and sensitivity in the testing protocol

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3 during pregnancy and are therefore informative regarding the effectiveness of the maternal testing  
4 protocol in each centre; for example, microcephaly in the No Evidence of MIP group would indicate a lack  
5 of sensitivity. These sets of estimates may be pooled, respectively, with adverse outcome risks in  
6 Confirmed MIP and No MIP groups in sensitivity analyses.  
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## 10 11 12 Objective 2: Laboratory and clinical markers of vertical transmission 13

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15 Estimates of the prevalence of markers of congenital infection will be produced in all centres with  
16 available data for each MIP group except the No MIP group, as the definition of No MIP is not compatible  
17 with laboratory markers of congenital infection. For this purpose, the numerator will be the number with  
18 markers of congenital infection, and the denominator will be the sum of the numbers with and without  
19 markers of congenital infection. Separate estimates will be obtained for: laboratory markers, clinical  
20 markers (namely CZS) , and combined laboratory and clinical markers (Table 3). Rates in the No Evidence  
21 of MIP group are of interest as they carry information about the diagnostic accuracy of the maternal  
22 testing protocol.  
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## 32 Objective 3: Effect of covariates 33

34 Separate estimates of the prevalence of adverse pregnancy outcomes, and the probability of laboratory  
35 and clinical markers of congenital infection, will be produced for each trimester of maternal infection, and  
36 by presence or absence of maternal symptoms.  
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39 Analyses of adverse event frequencies and of markers of congenital infection can be extended to include  
40 multiple covariates, using logistic regression. These might include potential *effect modifiers*, such as  
41 previous arbovirus infection or co-infection, or *confounding factors* such as socio-economic indicators  
42 likely to be associated with both arbovirus exposure and adverse outcomes. However, at the time of  
43 writing it is not known whether sufficient data will be available for regression analyses.  
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## 52 Secondary objectives 53

54 ZIKV infection in pregnancy could lead to adverse pregnancy and birth outcomes either following a  
55 congenital infection (cell A in Table 1) or in the absence of congenital infection (equally in cells A and C).  
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3 Although absence of markers of congenital infection does not rule out congenital infection, we might still  
4 expect to observe more adverse outcomes in fetuses and newborns with laboratory markers of congenital  
5 infection if those outcomes are caused by congenital infection. By contrast, adverse outcomes that are  
6 the result of MIP in the absence of congenital infection (Cell E in Table 1) should occur equally with or  
7 without laboratory markers of congenital infection. Based on literature on other infections in pregnancy,  
8 including dengue virus (37), adverse outcomes associated with MIP in the absence of congenital infection  
9 potentially include: fetal loss, stillbirth, prematurity, IUGR, and low birthweight for gestational age (38).  
10 The analysis would be based on a tabulation of presence or absence of neonatal symptoms, or sets of  
11 symptoms (Supplementary Table S5), and would be stratified by trimester of maternal infection, as this is  
12 likely to be associated with the presence of markers of congenital infection and with adverse outcomes.  
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### 23 Missing Covariates

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25 All analyses will be conducted on a “complete case” basis, in the first instance. Methods for handling  
26 missing covariates, such as imputation (39), will be considered after the extent and patterns of missing  
27 data have been explored.  
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### 33 Combining data across centres

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36 The above analyses will generate a series of centre-specific estimates of proportions, relative risks  
37 comparing MIP and No MIP groups, risk-differences, and means of continuous variables, stratified by  
38 trimester and maternal clinical presentation. If logistic regression is used to examine effect-modifiers and  
39 confounders, further estimates of interaction terms or adjusted estimates can also be produced.  
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43 All these estimates can be combined across centres using fixed or random effects models in a “two-stage”  
44 meta-analysis. Bayesian Markov chain Monte Carlo methods will be used as this will facilitate the use of  
45 exact binomial and multinomial likelihoods, which have a better performance with low and zero cells  
46 counts. Vague priors will be employed. Centre-specific random effect estimates will be sampled from beta  
47 distributions for binomial outcomes data, Dirichlet distributions for multinomial data, and normal  
48 distributions for continuous data. We will report ranges, between-centre standard deviations, mean  
49 effects and predictive effects with 95% credible intervals for each estimate.  
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3 In combining estimates from different centres, we will take account of the fact that in ZikaPLAN only  
4 women with rash were recruited, so that women with no ZIKV infection may have experienced other  
5 exanthematic infections (12), including arbovirus infections such as dengue and chikungunya, which may  
6 themselves be associated with adverse outcomes (40,41).  
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11 Depending on the results of two-stage analyses, and the completeness of covariate data, an individual  
12 patient data one-stage meta-analyses will be considered for each objective, as a secondary or sensitivity  
13 analysis, with centre as an additional fixed “intercept” term.  
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## 17 18 19 **Sensitivity analyses**

### 20 21 22 Definitions of MIP status, ZIKV-related outcomes, and laboratory markers of 23 congenital infection 24 25

26 We will report differences in adverse event rates between Confirmed MIP and Suspected MIP, and  
27 between No Evidence of MIP and No MIP. If the differences are small, we will produce results pooling  
28 these categories as a sensitivity analysis.  
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32 Further, the No Evidence of MIP category can be subdivided into women who were tested *per protocol*  
33 and those who may have been tested less completely. The impact of compliance with protocols will be  
34 explored, as it is expected to impact on the proportion of women with MIP who are classified as “No  
35 evidence of MIP” and, hence, on the probability of observing adverse outcomes in this group. Similarly,  
36 we will conduct sensitivity analyses around the definitions of Confirmed and Suspected MIP on advice  
37 from the Joint Diagnostics Group.  
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42 Alternative sets of estimates will be generated using alternative criteria for CZS-related outcomes and  
43 OPZRO, that are more, or less, specific for ZIKV in pregnancy. Similarly, we will explore the impact of  
44 varying the laboratory criteria for congenital infection on the advice of the Joint Diagnostics Group.  
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### 50 51 **Independent ascertainment of outcomes**

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53 A critical requirement of all these analyses is that the ascertainment of markers of congenital infection  
54 status and clinical outcomes in the fetuses and newborns, and developing infant are all independent of  
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3 each other, and also independent of MIP status. For example, ideally the same laboratory testing for  
4 congenital infection is carried out regardless of whether the pregnancy outcome is a fetal loss, termination  
5 of pregnancy, stillbirth, a case of CZS, or an apparently healthy asymptomatic infant.  
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8 These assumptions are difficult, and in certain respects (e.g., first trimester fetal loss and terminations)  
9 not possible, to fully implement in practice. To address these inevitable limitations in the analysis, which  
10 are expected to impact more on objective 2 than on objective 1, we will carry out exploratory analyses  
11 aimed at detecting potential deviations from protocol. For example, the distribution of trimester of MIP  
12 should not be associated with prospectively ascertained MIP status. Guided by the results, we will carry  
13 out sensitivity analyses that make a range of assumptions about the distribution of missing data, especially  
14 data on markers of congenital infection. A series of scenarios will be examined to assess robustness of  
15 results to inherent and/or unplanned deviations from the ideal protocols required for unbiased estimation  
16 of the target parameters.  
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## 27 **DISCUSSION**

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29 Risks of CZS and other adverse birth outcomes of ZIKV infection in pregnancy can only be assessed through  
30 studies that recruit women whose infection status is prospectively ascertained, or, if retrospective,  
31 ascertainment is independent of outcomes. However, reported risks of adverse outcomes even from  
32 prospective studies have been highly variable (10–13), as have vertical transmission rates based on  
33 laboratory markers of congenital ZIKV infection (10,11,15). An important role for joint analyses of multiple  
34 studies is to explore whether this heterogeneity in outcomes can be explained by individual or study-level  
35 covariates. To do this, it is essential that incidental sources of variation, such as those arising from  
36 differences in outcome reporting or diagnostic testing, are controlled or eliminated as much as possible.  
37 One of the most difficult sources of variation between consortia, and between sites within consortia, lies  
38 in diagnosis of maternal infection. Our approach is to have an Expert Diagnostics Group produce a  
39 harmonised classification of Confirmed MIP, Suspected MIP, No Evidence of MIP, and No MIP, and to  
40 compute a range of estimates of the relative effect of maternal infection on outcomes, grouping these in  
41 different ways. An analysis based on the Confirmed MIP and No MIP groups alone would be expected to  
42 generate the largest estimates of relative effect, because both poor sensitivity and poor specificity will  
43 tend to bias effect estimates towards the null.  
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54 An alternative proposal (23) in relation to maternal infection status is to treat test sensitivity and  
55 specificity as study-level covariates in a meta-regression, but the risk of false positive diagnosis depends  
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3 more on the incidence of ZIKV and cross-reacting antibodies to other arbovirus infections such as dengue  
4 than on test specificity. The Zika Virus IPD Consortium protocol differs in two other ways. First it proposes  
5 to include surveillance studies, which may result in over-estimating the risk of adverse outcomes due to  
6 retrospective ascertainment of infected women following adverse newborn outcomes (7,42). Second,  
7 congenital infection is to be defined by clinical and radiological criteria alone.  
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11 Other statistical methods may have been developed by the time the data becomes available for these  
12 analyses. Whatever form of analysis is adopted, a standardised pooled analysis from three large consortia  
13 comprising 20 centres will provide valuable information about these parameters, which will assist in  
14 framing a public health response and advice to women who might be exposed in future. It may also throw  
15 light on pathologic mechanisms leading to adverse outcomes, which could help in the development of  
16 therapeutic or prophylactic interventions.  
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### 23 **Patient and Public Involvement**

24 No patients were involved in the development of this protocol  
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**Contributors** A steering group (CT, TJ, KDR, RAAX, AEA, EBB, NA) was formed to prepare a Joint Statistical Analysis Plan pursuant to the terms of the three EC Zika Consortia funding, at the direction of the consortia PIs (CG, XdL and AW-S). After initial discussions the steering group delegated the task to AEA, EBB and NA. The SAP was conceptualised and drafted by AEA with EBB and NA. After several iterations, the draft was revised based on comments from CT, DB, ASA, TJ, KDR, MP, DBM, RAAX, MCM, AT, CG, XdL and AW-S. Comments were then solicited from TVBA, VIAS, SEBC, VHBA, PB, CDCC, WVVS, JEGH, BH, MK, CMTM, MMT, ETAM, URM, MEM, JGM, BR, PMSV, CSS and MDT and a revision was drafted by AEA, EBB and NA. The final draft was agreed by all authors.

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**Competing interests** None

## TABLES

**Table 1.** Design of an idealized prospective vertical transmission study. The vertical transmission rate is estimated by  $(A+B)/(A+B+C+D)$ . The rate of adverse outcomes conditional on congenital infection is  $A/(A+B)$ . This can be compared with the rate of adverse outcomes in newborns with no congenital infection,  $C/(C+D)$ , who form a control group (Pediatric Control Group) to account for potential confounders associated with maternal infection.

An overall, non-conditional estimate of the adverse event rate is  $(A+C)/(A+B+C+D)$ .

Follow-up of births to women with no MIP creates a second control group (Maternal Control Group), in which the rate of adverse outcomes,  $E/(E+F)$ , can be compared to the rate in births with no congenital infection to women with MIP. Estimated effects of MIP based on the Maternal Control Group are vulnerable to confounding by factors associated with MIP. Some cells are set to zero as there can be no congenital infection without MIP.

			Maternal Infection Status	
			MIP	No MIP
Congenital Infection Status	congenital infection	Adverse outcomes	A	0
		No Adverse outcomes	B	0
	No congenital infection	Adverse outcomes	C	E
		No Adverse outcomes	D	F

**Table 2.** Scheme for a generic analysis of risks of adverse outcomes by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Presence or absence of fetal and neonatal signs or symptoms (e.g., microcephaly, brain calcifications, arthrogyposis) and other Potentially Zika-related outcomes (e.g., fetal loss) in the different MIP groups will be compared.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Symptom 1				
Symptom 2				
Symptom 3				
:				
No Symptoms				
TOTAL				

**Table 3.** Scheme for generic analysis of markers of congenital infection by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Markers of congenital infection may include laboratory markers, clinical markers, such as microcephaly, or both combined.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Both laboratory and clinical markers of congenital infection				
Only laboratory markers of congenital infection				
Only clinical markers of congenital infection				

<b>No markers of congenital infection</b>				
<b>Not tested</b>				
<b>TOTAL</b>				

For peer review only

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**SUPPLEMENTARY MATERIALS**

**Supplementary Table S1.** Schedule of tests and examinations in pregnancy, at delivery, and during paediatric follow-up, in each consortium.

Consortium	Mother	Fetus	Newborn and neonate	Paediatric follow-up
ZIKAlliance	ZIKV serology IgG & IgM, NAAT, blood and urine, at booking, every 4w, <u>at birth, and at paediatric follow-up.</u>  <u>NAAT placenta and amniotic fluid at birth; NAAT breast milk and saliva at birth, if evidence of infection.</u>  <u>TORCH at enrolment / birth, HIV at birth according to local / national guidelines.</u>	Laboratory, pathology examination of fetal loss and stillbirth.	Clinical examination of newborn.  Newborn testing: NAAT, IgM, <u>blood, cord blood; TORCH according to local / national guidelines.</u>	Clinical examination, <u>anthropometry,</u> NAAT blood <u>and</u> urine; IgM, IgG blood at 4w, 4m, 12m, 24m.  Transcranial U/S at 4w. Hearing, eye, neurodevelopmental assessments.
ZIKAction	IgM, IgG serology booking, 20, 28, Delivery, and at <u>paediatric follow-up</u>  NAAT testing, and additional U/S investigations if evidence of infection	Laboratory, pathology examination of fetal loss and stillbirth.	Clinical examination of newborn  Newborn testing: IgM, IgG (serum; CSF only if clinically indicated), urine, saliva. <u>TORCH testing if clinically indicated</u>  NAAT, U/S and ophthalmology investigations if evidence of infection	Paediatric testing: IgG, IgM (serum, urine, saliva) and clinical examination at: 4w, 4m, 9m, 12m, 18m and 24m. NAAT if evidence on infection  Neurodevelopmental assessments

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ZikaPLAN	NAAT and serologic testing (PRNT, IgM, IgG3) when symptoms reported and at up to three follow-up visits.  TORCH, DENV, and CHIKV testing	Not routinely tested.	Clinical examination in early infancy.  NAAT testing in the neonatal period in some centres. <a href="#">TORCH testing if clinically indicated</a>	Clinical examination of the child by specialists at 3, 6, 12, 18, 24, 36, and 48m  Neurodevelopmental assessments
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CSF, Cerebro-spinal fluid; DENV, Dengue virus; CHIKV, Chikungunya virus; HIV, Human Immunodeficiency virus; Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test; TORCH, Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes; U/S, Ultrasound; ZIKV, Zika virus

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**Supplementary Table S2.** Illustrative and approximate definitions of key explanatory and outcome variables. The final definitions will be determined by the Joint Diagnostics Group and Joint End-point Review Group.

<i>Maternal Symptoms</i>	For example: rash, fever, headache, joint pain
<i>Maternal Infection in Pregnancy (MIP) status, established only on the basis of maternal testing</i>	Confirmed: NAAT, Seroconversion Suspected: <a href="#">Serological</a> tests of recent infection, including: IgM, IgG3, avidity No Evidence of MIP: Maternal testing protocol was followed, but none of the above were positive. No MIP: As above but with IgG negative at or near time of delivery
<i>Laboratory markers of Congenital Infection (CI)</i>	Present: NAAT or IgM any time in first 7 days; Absent: all other findings
<i>Most likely trimester of onset of maternal infection</i>	The highest available from the following hierarchy: <ul style="list-style-type: none"> <li>• Date of first NAAT positive test minus average duration of viremia</li> <li>• Seroconversion: Midpoint between last serological negative and last positive</li> <li>• <a href="#">Serological</a> tests of recent infection: Date of first positive</li> </ul>
<i>Signs and symptoms compatible with Congenital Zika Syndrome (CZS)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available. <a href="#">The intention will be to produce a definition that is virtually 100% specific for congenital ZIKV infection in mothers exposed to a ZIKV outbreak, especially if other TORCH infections can be ruled out.</a>
<i>Other Potentially Zika-related Outcomes (OPZRO)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available.

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test.

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**Supplementary Table S3.** Prospectively ascertained Maternal Infection in Pregnancy (MIP) status and evidence on which it is based. NB: Data from the same woman can appear in more than one of the last four columns

Centre	Total women	Prospectively ascertained MIP status				Evidence for Confirmed or Suspected MIP			
		Confirmed MIP	Suspected MIP	No Evidence of MIP	No MIP	NAAT	Sero-conversion	IgM/IgG3	PRNT
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Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S4.** Summary of evidence on laboratory and clinical markers of congenital infection (CI) and evidence on which it is based. NB: Data from the same fetus/newborn can appear in more than one column

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Centre	Total number fetus/newborn	Markers of congenital infection						Other evidence of congenital infection			
		Laboratory or clinical markers of congenital infection	Laboratory markers of congenital infection only	Signs and symptoms compatible with CZS	NAAT	IgM	No markers of congenital infection	Microcephaly	Neurological abnormalities	Ocular abnormalities	Other, to be defined
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CZS, Symptoms compatible with Congenital Zika Syndrome; IgM/G, Immunoglobulin M/G; NAAT, Nucleic Acid Amplification Test; OPZRO, Other potentially Zika-related symptoms; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S5.** Distributions of signs and symptoms by laboratory markers of congenital infection and prospectively ascertained maternal infection in pregnancy (MIP) status. The breakdown shown is an example. Other examples might be "Termination of pregnancy, Pregnancy loss, Stillbirth, Livebirth"; or "Sensorineural hearing loss (SNHL), No SNHL "

CENTRE			
Sign or symptom in fetus, newborn, or infant	MIP with <u>laboratory</u> markers of congenital infection	MIP with no <u>laboratory</u> markers of congenital infection	No MIP
Signs and symptoms compatible with Congenital Zika Syndrome (CZS)			
Other potentially Zika-related outcomes (OPZRO)			
Asymptomatic			
<b>TOTAL = 100%</b>			

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## SUPPLEMENTARY MATERIALS

**Supplementary Table S1.** Schedule of tests and examinations in pregnancy, at delivery, and during paediatric follow-up, in each consortium.

Consortium	Mother	Fetus	Newborn and neonate	Pediatric follow-up
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ZIKAction	<p>IgM, IgG serology booking, 20, 28, Delivery, and at paediatric follow-up</p> <p>NAAT testing, and additional U/S investigations if evidence of infection</p>	Laboratory, pathology examination of fetal loss and stillbirth.	<p>Clinical examination of newborn</p> <p>Newborn testing: IgM, IgG (serum; CSF only if clinically indicated), urine, saliva. TORCH testing if clinically indicated</p> <p>NAAT, U/S and ophthalmology investigations if evidence of infection</p>	<p>Paediatric testing: IgG, IgM (serum, urine, saliva) and clinical examination at: 4w, 4m, 9m, 12m, 18m and 24m. NAAT if evidence on infection</p> <p>Neurodevelopmental assessments</p>

ZikaPLAN	NAAT and serologic testing (PRNT, IgM, IgG3) when symptoms reported and at up to three follow-up visits.  TORCH, DENV, and CHIKV testing	Not routinely tested.	Clinical examination in early infancy.  NAAT testing in the neonatal period in some centres. TORCH testing if clinically indicated	Clinical examination of the child by specialists at 3, 6, 12, 18, 24, 36, and 48m  Neurodevelopmental assessments
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CSF, Cerebro-spinal fluid; DENV, Dengue virus; CHIKV, Chikungunya virus; HIV, Human Immunodeficiency virus; Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test; TORCH, Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes; U/S, Ultrasound; ZIKV, Zika virus

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<i>Laboratory markers of Congenital Infection (CI)</i>	Present: NAAT or IgM any time in first 7 days; Absent: all other findings
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<i>Signs and symptoms compatible with Congenital Zika Syndrome (CZS)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available. <i>The intention will be to produce a definition that is virtually 100% specific for congenital ZIKV infection in mothers exposed to a ZIKV outbreak, especially if other TORCH infections can be ruled out.</i>
<i>Other Potentially Zika-related Outcomes (OPZRO)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available.

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test.



**Supplementary Table S3.** Prospectively ascertained Maternal Infection in Pregnancy (MIP) status and evidence on which it is based. NB: Data from the same woman can appear in more than one of the last four columns

Centre	Total women	Prospectively ascertained MIP status				Evidence for Confirmed or Suspected MIP			
		Confirmed MIP	Suspected MIP	No Evidence of MIP	No MIP	NAAT	Sero-conversion	IgM/IgG3	PRNT
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		Laboratory or clinical markers of congenital infection	Laboratory markers of congenital infection only	Signs and symptoms compatible with CZS	NAAT	IgM	No markers of congenital infection	Microcephaly	Neurological abnormalities	Ocular abnormalities
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CZS, Symptoms compatible with Congenital Zika Syndrome; IgM/G, Immunoglobulin M/G; NAAT, Nucleic Acid Amplification Test; OPZRO, Other potentially Zika-related symptoms; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S5.** Distributions of signs and symptoms by laboratory markers of congenital infection and prospectively ascertained maternal infection in pregnancy (MIP) status. The breakdown shown is an example. Other examples might be "Termination of pregnancy, Pregnancy loss, Stillbirth, Livebirth"; or "Sensorineural hearing loss (SNHL), No SNHL "

<b>CENTRE</b>			
<b>Sign or symptom in fetus, newborn, or infant</b>	<b>MIP with laboratory markers of congenital infection</b>	<b>MIP with no laboratory markers of congenital infection</b>	<b>No MIP</b>
Signs and symptoms compatible with Congenital Zika Syndrome (CZS)			
Other potentially Zika-related outcomes (OPZRO)			
Asymptomatic			
<b>TOTAL = 100%</b>			

# BMJ Open

## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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Secondary Subject Heading:	Epidemiology, Infectious diseases
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## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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## ABSTRACT

### Introduction

Zika virus (ZIKV) infection in pregnancy has been associated with microcephaly and severe neurological damage to the fetus. Our aim is to document the risks of adverse pregnancy and birth outcomes and the prevalence of laboratory markers of congenital infection in deliveries to women experiencing ZIKV infection during pregnancy, using data from European Commission-funded prospective cohort studies in 20 centres in 11 countries across Latin America and the Caribbean.

### Methods and analysis

We will carry out a centre-by-centre analysis of the risks of adverse pregnancy and birth outcomes, comparing women with confirmed and suspected ZIKV infection in pregnancy to those with no evidence of infection in pregnancy. We will document the proportion of deliveries in which laboratory markers of congenital infection were present. Finally, we will investigate the associations of trimester of maternal infection in pregnancy, presence or absence of maternal symptoms of acute ZIKV infection, and previous flavivirus infections with adverse outcomes and with markers of congenital infection. Centre-specific estimates will be pooled using a two-stage approach.

### Ethics and dissemination

Ethical approval was obtained at each centre. Findings will be presented at international conferences and published in peer-reviewed open access journals, and discussed with local public health officials and representatives of the national Ministries of Health, Pan American Health Organization, and World Health Organization involved with ZIKV prevention and control activities.

### Keywords

Zika virus; pregnancy; vertical transmission; Congenital Zika Syndrome; microcephaly; meta-analysis



## Strengths and limitations of this study

- This will be a pooled analysis of data from 3 international consortia conducting prospective cohort studies of outcomes following ZIKV infection in pregnancy in 20 centres in 11 countries.
- Standardised definitions of outcomes will provide clarity about the absolute risks of adverse outcomes, which have not been reported consistently in prospective studies so far.
- These studies include a control group of women with no evidence of ZIKV infection in pregnancy, allowing improved estimation of the proportion of adverse events attributable to ZIKV in pregnancy.
- Inferences will be limited by: difficulties in distinguishing between women who did and who did not experience a ZIKV infection in pregnancy, due to the high frequency of mild and asymptomatic infections and the low sensitivity and specificity of diagnostic tools; low diagnostic sensitivity of markers of congenital infection; and outcome data that are not missing at random.

## INTRODUCTION

Following the emergence of Zika virus (ZIKV) in Asia and the Pacific in 2013 (1) and the subsequent introduction to Brazil (2), clusters of neonates with severe neurological complications and microcephaly were observed across Latin America. Following recent experiences with the H1N1 influenza pandemic and Ebola outbreak in Western Africa, the need for coordinated international research on ZIKV was quickly recognized. In January 2016, before the World Health Organization (WHO) declared a Public Health Emergency of International Concern (3), the European Commission (EC) issued a funding call to set up a network in Latin America and the Caribbean with the aim of implementing and coordinating urgently required research, while simultaneously contributing to research capacity and preparedness for other emerging infectious diseases. Three consortia were funded: ZIKAlliance (<https://zikalliance.tghn.org/>) (4,5), ZikaPLAN (6) (<https://zikaplan.tghn.org/>), and ZIKAction (<http://zikaction.org/>). All are multidisciplinary international collaborations with active investigations in epidemiology, virology, immunology, diagnostics, mathematical modelling, social science, and animal studies. Each consortium includes its own prospective cohort study of ZIKV in pregnancy and a shared work package that aims to ensure the harmonization of protocols and data sets in order to facilitate a pooled analysis of cohort data. The primary aim of the pooled analysis is to investigate the incidence of adverse outcomes of ZIKV infection in pregnancy, including “congenital infection, microcephaly, Zika congenital syndrome, and other sequelae of ZIKV infection”.

The aim of this paper is to present a protocol for this pooled analysis. Data has been or is still being collected in multiple sites in 20 regional coordinating centres spread over 11 countries and regions across Latin America and the Caribbean. There are 15 ZIKAlliance centres: Sao Paulo, Rio de Janeiro, Recife and Belo Horizonte (Brazil); Valencia (Venezuela); Bucaramanga (Colombia); Guayaquil (Ecuador); Lima (Peru); Jalisco, Nayarit, Veracruz, Yucatan (Mexico); Santa Cruz de la Sierra (Bolivia); Havana (Cuba); Guadeloupe (French Territory of the Americas); 3 ZikaPLAN centres: Goiânia, Rio de Janeiro, Recife (Brazil); and 2 ZIKAction centres: Kingston (Jamaica) and Port-au-Prince (Haiti). Recruitment to ZIKAlliance began May 2017, December 2015 for ZikaPLAN, and September 2017 for ZIKAction. Over 700 women with confirmed infection had been recruited by April 2020; recruitment continues in some centres.

Several studies of ZIKV in pregnancy have recently been published. In registry-based studies (7-9), fetuses and newborns of women with confirmed infection in pregnancy have been reported to have “potentially Zika-related” adverse outcomes at rates of up to 15%, with higher risk of Zika-associated adverse outcomes in the first trimester. Registry-based studies are likely to over-estimate the risk of severe clinical

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3 manifestations and underestimate the risk of more mild clinical presentations because they recruit both  
4 prospectively ascertained ZIKV-infected pregnant women and women whose infection was recognized  
5 retrospectively following the birth of an infant with congenital abnormalities. Prospective studies of  
6 congenital infection have variously reported 25% “severe” and 21% “mild to moderate” outcomes in  
7 French Guiana (10), and 27% adverse outcomes in Brazil (11). The specificity of these outcome definitions  
8 for ZIKV in pregnancy is not known as these studies did not include a control group of women with no  
9 ZIKV infection in pregnancy. In another Brazilian study, the risk of adverse outcomes was reported to be  
10 46% in births to women with NAAT (Nucleic Acid Amplification Test)-confirmed ZIKV infection in  
11 pregnancy compared to 11.5% in NAAT-negative women (12). In a large prospective study based in the  
12 French Territories of the Americas, among infants born to women with NAAT-confirmed ZIKV infection,  
13 7.0% presented with neurologic or ocular birth defects and 3.1% met the study’s criteria for Congenital  
14 Zika Syndrome (CZS) (13), which is characterized by several unique features (14). An important limitation  
15 in the comparison of the results of these different studies is the lack of a standard definition of CZS and  
16 of the clinical and diagnostic procedures used to evaluate these children, leading to possible  
17 misclassification of the outcomes studied.  
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29 The vertical transmission rate is the probability of congenital infection in births to women with infection  
30 in pregnancy. The rates reported so far, 26% (11) and 35% (10), are based on laboratory markers of  
31 congenital infection such as NAAT or IgM in the fetus or newborn. However, a prospective cohort  
32 retrospectively reconstructed from a register study estimated the vertical transmission rate to be only 9%  
33 (15). Comparison of these rates is difficult as different markers and different biological samples were used.  
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52 In addition, although these tests are analytically sensitive and specific they have poor diagnostic sensitivity  
53 as markers of congenital infection. These markers were absent from serum in a high proportion of CZS  
54 cases (16) (16,17) and in newborns with other potentially ZIKV-related adverse outcomes born to women  
55 with confirmed ZIKV during pregnancy (10,11). Clearance of virus from amniotic fluid and fetal blood has  
56 been reported in cases of CZS, even when ZIKV is found in brain tissue post-mortem (18,19). It therefore  
57 appears that fetal infection may occur, causing profound damage, but clearing before delivery and leaving  
58 no discernable immunological trace in serum. Consequently, in this study we will document the  
59 prevalence of markers of congenital infection using uniform criteria, recognizing that this is an  
60 underestimate of the true vertical transmission rate.

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3 acute ZIKV infection do not appear to be a risk factor for adverse outcomes (20). There is evidence of  
4 antibody dependent enhancement of ZIKV by dengue virus (DENV) antibody in animal models (21), but it  
5 is unclear whether previous DENV infection or exposure to other flaviviruses has a protective, risk-  
6 enhancing, or null effect, in maternal or congenital infection in humans (22). It also remains to be  
7 established whether a previous ZIKV infection confers protective immunity. Little is currently known about  
8 risk factors for trans-placental transmission of ZIKV.  
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13 The analysis plan described here complements the recently published protocol of the Zika Virus Individual  
14 Participant Data (IPD) Consortium (23), which will eventually include data from the three EC consortia as  
15 well as data from many other sources. Although the objectives of the protocols are similar, different  
16 methods are proposed in relation to design of included studies, definition of congenital infection, and  
17 approach to imperfect diagnosis of maternal infection.  
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23 In light of the unexplained heterogeneity in reported rates of adverse outcomes, and the variation in  
24 prevalence of markers of congenital infection, a pooled analysis of data from 20 centers following similar  
25 protocols with harmonized definitions of clinical and laboratory outcomes will provide important new  
26 information on outcomes of ZIKV in pregnancy.  
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## 32 **OBJECTIVES OF THE JOINT ANALYSIS**

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35 1. To estimate the risk of adverse outcomes in the fetus, newborn, and child following maternal ZIKV  
36 infection in pregnancy, compared to outcomes in controls with no evidence of maternal infection in  
37 pregnancy.  
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40 2. To estimate the prevalence of markers of congenital infection among fetuses and liveborn infants  
41 following maternal ZIKV infection during pregnancy.  
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44 3. To assess the associations between trimester of maternal infection, presence or absence of maternal  
45 symptoms, and previous flavivirus infections with adverse outcomes and markers of congenital  
46 infection.  
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## 52 **METHODS**

### 53 **Participants**

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3 Pregnant women were eligible only if their infection status during pregnancy (infected or not infected)  
4 was ascertained prior to the detection of adverse outcomes, or was not influenced by fetal examination  
5 or outcome on delivery. This definition is compatible with retrospective testing of previously collected  
6 maternal samples, after delivery. Although the unit of recruitment is the mother, the unit of analysis is  
7 the fetus, newborn, and infant; multiple births are sufficiently rare to be treated as independent  
8 observations (24).  
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## 14 Study design

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16 Consenting women were screened in pregnancy for markers of ZIKV infection. Those in whom maternal  
17 infection in pregnancy (MIP) was suspected were followed with enhanced investigations. In ZIKAction and  
18 ZIKAlliance, all deliveries to these women, including fetal losses, stillbirths, and newborns were examined  
19 clinically and tested for markers of congenital infection. This testing was not routinely performed in  
20 ZikaPLAN. In all three cohorts, newborns were prospectively followed to identify any adverse outcomes  
21 that may develop later. In all three consortia, a sample of newborns delivered to women with no evidence  
22 of infection in pregnancy served as an unexposed control group.  
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28 There were some differences between the protocols adopted by the three consortia in terms of how  
29 women were recruited into the study, and the choice and scheduling of tests and investigations  
30 (Supplementary Table S1). In ZIKAction and ZIKAlliance, women were recruited regardless of symptoms  
31 during pregnancy, although report of symptoms was recorded. In ZikaPLAN, only women with rash, a  
32 common sign of ZIKV infection, were recruited. Statistical analyses will therefore be stratified by whether  
33 the mother reported symptoms in pregnancy. .  
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39 When the studies were designed, there was little information on the risk of adverse outcomes of ZIKV in  
40 pregnancy, on vertical transmission rates, nor on what infection rates among pregnant women might be  
41 expected. Formal sample size calculations were not undertaken.  
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## 47 Target parameters and terminology of vertical transmission studies

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49 Six categories of joint congenital infection status and maternal infection status (A-F) are defined in Table 1,  
50 which illustrates the logic of an idealised prospective study. The usual target parameters are the *vertical*  
51 *transmission rate*, which is the probability of congenital infection following MIP,  $(A+B) / (A+B+C+D)$ ; and  
52 the *rate of adverse outcomes in those with congenital infection*,  $A/(A+B)$ . The definitions of “adverse  
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3 outcomes”, congenital infection, and MIP will be determined by a Joint Diagnostics Group and a Joint  
4 Endpoint Review Group, after the data have been assembled. Estimates of these parameters are standard  
5 in the classic studies of vertical transmission of human immune-deficiency virus (HIV) (25,26),  
6 toxoplasmosis (27–29) and cytomegalovirus (CMV) (30). In studies of less specific outcomes, the event  
7 rate  $C/(C+D)$  in fetuses and newborns of women with MIP but in whom no congenital infection occurred  
8 (Paediatric Control Group 1 in Table 1) forms a comparison group (31,32) representing the adverse event  
9 rate that is due to MIP in the absence of congenital infection. The present analysis plan is modelled closely  
10 on these earlier studies, but includes adaptations to take account of the difficulties in diagnosing maternal  
11 and congenital ZIKV infection.  
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19 For example, because cases of congenital infection cannot be reliably identified by diagnostic tests, we  
20 can only estimate the prevalence of laboratory *markers of vertical infection* (Objective 2). Similarly, the  
21 “overall” (unconditional) adverse event rate is taken as the primary outcome for Objective 1; this includes  
22 all births to women with MIP,  $(A+C)/(A+B+C+D)$  (Table 1). All three consortia included a further control  
23 group of births to unexposed women, those with no infection in pregnancy (Maternal Control Group in  
24 Table 1). The adverse event rates in this group,  $E/(E+F)$ , represents a baseline for comparison with the  
25 overall event rates in exposed women (33).  
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31 Although estimates of the vertical transmission rate are compromised, it may still be of interest to  
32 compare adverse outcome rates in the MIP with congenital infection group (i.e.,  $A/(A+B)$ ), the MIP without  
33 congenital infection group (i.e.,  $C/(C+D)$ ), and the No MIP group (i.e.,  $E/(E+F)$ ), as this may provide insight  
34 into whether adverse fetal outcomes are associated with MIP in the absence of demonstrable fetal  
35 infection.  
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40 In addition to the challenges associated with the laboratory definition of congenital infection, it is also  
41 difficult to discriminate between pregnancies with MIP and with No MIP as required in analyses based on  
42 Table 1. A positive NAAT result or seroconversion during pregnancy are sufficient to confirm MIP.  
43 However, even if tested *per protocol*, MIP may be missed due to the narrow window of detection of NAAT  
44 tests, perhaps as low as 14 days (34). Tests of recent infection, including IgM, IgG3 or avidity assays, may  
45 reflect infection during pregnancy, but may also be the result of infection prior to pregnancy (35), and  
46 immunologic cross-reactivity to DENV antibody may need to be ruled out (36). These tests therefore  
47 indicate suspected but not confirmed MIP. An IgG negative response in the woman or newborn at or tests,  
48 perhaps as low as 14 days (34). Tests of recent infection, including IgM, IgG3 or avidity assays, may reflect  
49 infection during pregnancy, but may also be the result of infection prior to pregnancy (35), and  
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3 immunologic cross-reactivity to DENV antibody may need to be ruled out (36). These tests therefore  
4 indicate suspected but not confirmed MIP. An IgG negative response in the woman or newborn at or  
5 shortly after delivery can be taken as suggestive of No MIP, although the dynamics of ZIKV IgG are not  
6 well documented. While a negative IgG is likely to be uncommon in some sites, as some women will  
7 have experienced a ZIKV infection prior to pregnancy, it lowers the probability that a ZIKV infection would  
8 have occurred in pregnancy. Taking all this together, MIP status will be characterised as "Confirmed",  
9 "Suspected, "No Evidence of MIP" (i.e., all NAAT and IgM tests negative), and "No MIP" (i.e., all NAAT and  
10 IgM tests negative AND IgG negative at or shortly after delivery). To maintain the principle of prospective  
11 ascertainment, confirmation of congenital infection will have no impact on the mother's assigned  
12 infection status.  
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## 21 **Definitions of variables**

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23 To support the pooled analyses, a Joint Diagnostics Group consisting of immunologists and virologists and  
24 a Joint Endpoint Review Group consisting of paediatricians with experience of congenital ZIKV will be  
25 convened to agree on standardized case definitions. The names and qualifications of members of both  
26 groups will be published at the time of the joint data analysis, together with rationale and process for  
27 their recruitment. Supplementary Table S2 provides some provisional definitions for: MIP (Confirmed,  
28 Suspected, No Evidence of MIP, No MIP), laboratory markers of congenital infection (present or absent),  
29 signs and symptoms compatible with Congenital Zika Syndrome (CZS), other potentially-Zika-related  
30 outcomes (OPZRO), and trimester of MIP. The Diagnostics Group will also have responsibility for  
31 definitions of covariates, such as previous flavivirus infection. Definitions of both diagnostic categories  
32 and clinical endpoints will be based on the best information available at the time of analysis, and will to  
33 the greatest extent possible, be harmonized across consortia and across centres within consortia. As the  
34 expert groups will be assembled from representatives of each consortium, they will already have  
35 examined the data prior to a joint analysis, so that blinding will not be possible; however, they will devise  
36 diagnostic and clinical criteria that can be applied objectively across the three consortia.  
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47 An essential aspect of the definitions for MIP status used in the statistical analyses below is that they must  
48 be based exclusively on the *prospective* diagnostic testing. For example, although CZS and/or laboratory  
49 evidence of congenital infection in the newborn provides compelling evidence of MIP, this would not  
50 affect the MIP status as ascertained prospectively. Thus, we expect to observe some newborns with CZS  
51 and/or with laboratory markers of congenital infection delivered to women with Suspected MIP, or even  
52 those categorized as having No Evidence of MIP.  
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## STATISTICAL ANALYSIS

### Analysis of data from individual centres

#### Descriptive tables

We will produce descriptive tables providing a breakdown of the numbers in each centre with Confirmed MIP, Suspected MIP, No Evidence of MIP, and No MIP, and the type of evidence on which this is based (e.g., NAAT, seroconversion, tests of recent infection) (Supplementary Table S3). The similar Supplementary Table S4 will document numbers with signs or symptoms compatible with CZS and with markers of congenital infection, and the type of evidence on which this is based, for example NAAT, IgM, or clinical markers (CZS).

#### Objective 1: Adverse Outcomes

Prospectively ascertained MIP status will be tabulated against overall (i.e., unconditional on congenital infection status) adverse outcomes (Table 2). Various risks can be estimated within each MIP category, including: the risk of signs and symptoms compatible with CZS, the risk of OPZRO, the risk of both combined, of individual signs and symptoms, or of signs and symptoms grouped in clinically (e.g., ophthalmologic defects) or embryologically meaningful ways. Outcomes may be binary (e.g., microcephaly) or continuous (e.g., head circumference), or multi-category (CZS-related outcomes, OPZRO, asymptomatic). As well as congenital anomalies, rates of standard outcomes, in the absence of congenital anomalies, will be documented, including: fetal loss, stillbirth, low birthweight, intra-uterine growth retardation (IUGR), and premature delivery.

The rate of adverse outcomes in the No MIP group represents the study-specific background rate of adverse outcomes (i.e., in the absence of MIP, Control Group 2), to be compared to rates in women with Confirmed MIP. Absolute risks will be estimated as well as risk ratios and risk differences.

Parallel sets of estimates will be calculated in the Suspected MIP and No Evidence of MIP groups, as particular adverse events indicate lack of diagnostic specificity and sensitivity in the testing protocol



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3 during pregnancy and are therefore informative regarding the effectiveness of the maternal testing  
4 protocol in each centre; for example, microcephaly in the No Evidence of MIP group would indicate a lack  
5 of sensitivity. These sets of estimates may be pooled, respectively, with adverse outcome risks in  
6 Confirmed MIP and No MIP groups in sensitivity analyses.  
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## 10 11 12 13 Objective 2: Laboratory and clinical markers of vertical transmission

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15 Estimates of the prevalence of markers of congenital infection will be produced in all centres with  
16 available data for each MIP group except the No MIP group, as the definition of No MIP is not compatible  
17 with laboratory markers of congenital infection. For this purpose, the numerator will be the number with  
18 markers of congenital infection, and the denominator will be the sum of the numbers with and without  
19 markers of congenital infection. Separate estimates will be obtained for: laboratory markers, clinical  
20 markers (namely CZS) , and combined laboratory and clinical markers (Table 3). Rates in the No Evidence  
21 of MIP group are of interest as they carry information about the diagnostic accuracy of the maternal  
22 testing protocol.  
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## 32 Objective 3: Effect of covariates

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34 Separate estimates of the prevalence of adverse pregnancy outcomes, and the probability of laboratory  
35 and clinical markers of congenital infection, will be produced for each trimester of maternal infection, and  
36 by presence or absence of maternal symptoms.  
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40 Analyses of adverse event frequencies and of markers of congenital infection can be extended to include  
41 multiple covariates, using logistic regression. These might include potential *effect modifiers*, such as  
42 previous arbovirus infection or co-infection, or *confounding factors* such as socio-economic indicators  
43 likely to be associated with both arbovirus exposure and adverse outcomes. However, at the time of  
44 writing it is not known whether sufficient data will be available for regression analyses.  
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## 52 Secondary objectives

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54 ZIKV infection in pregnancy could lead to adverse pregnancy and birth outcomes either following a  
55 congenital infection (cell A in Table 1) or in the absence of congenital infection (equally in cells A and C).  
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3 Although absence of markers of congenital infection does not rule out congenital infection, we might still  
4 expect to observe more adverse outcomes in fetuses and newborns with laboratory markers of congenital  
5 infection if those outcomes are caused by congenital infection. By contrast, adverse outcomes that are  
6 the result of MIP in the absence of congenital infection (Cell E in Table 1) should occur equally with or  
7 without laboratory markers of congenital infection. Based on literature on other infections in pregnancy,  
8 including dengue virus (37), adverse outcomes associated with MIP in the absence of congenital infection  
9 potentially include: fetal loss, stillbirth, prematurity, IUGR, and low birthweight for gestational age (38).  
10 The analysis would be based on a tabulation of presence or absence of neonatal symptoms, or sets of  
11 symptoms (Supplementary Table S5), and would be stratified by trimester of maternal infection, as this is  
12 likely to be associated with the presence of markers of congenital infection and with adverse outcomes.  
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### 23 **Missing Covariates**

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25 All analyses will be conducted on a “complete case” basis, in the first instance. Methods for handling  
26 missing covariates, such as imputation (39), will be considered after the extent and patterns of missing  
27 data have been explored.  
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### 33 **Combining data across centres**

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36 The above analyses will generate a series of centre-specific estimates of proportions, relative risks  
37 comparing MIP and No MIP groups, risk-differences, and means of continuous variables, stratified by  
38 trimester and maternal clinical presentation. If logistic regression is used to examine effect-modifiers and  
39 confounders, further estimates of interaction terms or adjusted estimates can also be produced.  
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43 All these estimates can be combined across centres using fixed or random effects models in a “two-stage”  
44 meta-analysis. Bayesian Markov chain Monte Carlo methods will be used as this will facilitate the use of  
45 exact binomial and multinomial likelihoods, which have a better performance with low and zero cells  
46 counts. Vague priors will be employed. Centre-specific random effect estimates will be sampled from beta  
47 distributions for binomial outcomes data, Dirichlet distributions for multinomial data, and normal  
48 distributions for continuous data. We will report ranges, between-centre standard deviations, mean  
49 effects and predictive effects with 95% credible intervals for each estimate.  
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3 In combining estimates from different centres, we will take account of the fact that in ZikaPLAN only  
4 women with rash were recruited, so that women with no ZIKV infection may have experienced other  
5 exanthematic infections (12), including arbovirus infections such as dengue and chikungunya, which may  
6 themselves be associated with adverse outcomes (40,41).  
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11 Depending on the results of two-stage analyses, and the completeness of covariate data, an individual  
12 patient data one-stage meta-analyses will be considered for each objective, as a secondary or sensitivity  
13 analysis, with centre as an additional fixed “intercept” term.  
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## 17 18 19 **Sensitivity analyses**

### 20 21 22 Definitions of MIP status, ZIKV-related outcomes, and laboratory markers of 23 congenital infection 24 25

26 We will report differences in adverse event rates between Confirmed MIP and Suspected MIP, and  
27 between No Evidence of MIP and No MIP. If the differences are small, we will produce results pooling  
28 these categories as a sensitivity analysis.  
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32 Further, the No Evidence of MIP category can be subdivided into women who were tested *per protocol*  
33 and those who may have been tested less completely. The impact of compliance with protocols will be  
34 explored, as it is expected to impact on the proportion of women with MIP who are classified as “No  
35 evidence of MIP” and, hence, on the probability of observing adverse outcomes in this group. Similarly,  
36 we will conduct sensitivity analyses around the definitions of Confirmed and Suspected MIP on advice  
37 from the Joint Diagnostics Group.  
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42 Alternative sets of estimates will be generated using alternative criteria for CZS-related outcomes and  
43 OPZRO, that are more, or less, specific for ZIKV in pregnancy. Similarly, we will explore the impact of  
44 varying the laboratory criteria for congenital infection on the advice of the Joint Diagnostics Group.  
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## 50 51 **Independent ascertainment of outcomes**

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53 A critical requirement of all these analyses is that the ascertainment of markers of congenital infection  
54 status and clinical outcomes in the fetuses and newborns, and developing infant are all independent of  
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3 each other, and also independent of MIP status. For example, ideally the same laboratory testing for  
4 congenital infection is carried out regardless of whether the pregnancy outcome is a fetal loss, termination  
5 of pregnancy, stillbirth, a case of CZS, or an apparently healthy asymptomatic infant.  
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8 These assumptions are difficult, and in certain respects (e.g., first trimester fetal loss and terminations)  
9 not possible, to fully implement in practice. To address these inevitable limitations in the analysis, which  
10 are expected to impact more on objective 2 than on objective 1, we will carry out exploratory analyses  
11 aimed at detecting potential deviations from protocol. For example, the distribution of trimester of MIP  
12 should not be associated with prospectively ascertained MIP status. Guided by the results, we will carry  
13 out sensitivity analyses that make a range of assumptions about the distribution of missing data, especially  
14 data on markers of congenital infection. A series of scenarios will be examined to assess robustness of  
15 results to inherent and/or unplanned deviations from the ideal protocols required for unbiased estimation  
16 of the target parameters.  
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## 27 **DISCUSSION**

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29 Risks of CZS and other adverse birth outcomes of ZIKV infection in pregnancy can only be assessed through  
30 studies that recruit women whose infection status is prospectively ascertained, or, if retrospective,  
31 ascertainment is independent of outcomes. However, reported risks of adverse outcomes even from  
32 prospective studies have been highly variable (10–13), as have vertical transmission rates based on  
33 laboratory markers of congenital ZIKV infection (10,11,15). An important role for joint analyses of multiple  
34 studies is to explore whether this heterogeneity in outcomes can be explained by individual or study-level  
35 covariates. To do this, it is essential that incidental sources of variation, such as those arising from  
36 differences in outcome reporting or diagnostic testing, are controlled or eliminated as much as possible.  
37 One of the most difficult sources of variation between consortia, and between sites within consortia, lies  
38 in diagnosis of maternal infection. Our approach is to have an Expert Diagnostics Group produce a  
39 harmonised classification of Confirmed MIP, Suspected MIP, No Evidence of MIP, and No MIP, and to  
40 compute a range of estimates of the relative effect of maternal infection on outcomes, grouping these in  
41 different ways. An analysis based on the Confirmed MIP and No MIP groups alone would be expected to  
42 generate the largest estimates of relative effect, because both poor sensitivity and poor specificity will  
43 tend to bias effect estimates towards the null.  
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54 An alternative proposal (23) in relation to maternal infection status is to treat test sensitivity and  
55 specificity as study-level covariates in a meta-regression, but the risk of false positive diagnosis depends  
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3 more on the incidence of ZIKV and cross-reacting antibodies to other arbovirus infections such as dengue  
4 than on test specificity. The Zika Virus IPD Consortium protocol differs in two other ways. First it proposes  
5 to include surveillance studies, which may result in over-estimating the risk of adverse outcomes due to  
6 retrospective ascertainment of infected women following adverse newborn outcomes (7,42). Second,  
7 congenital infection is to be defined by clinical and radiological criteria alone.  
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11 Other statistical methods may have been developed by the time the data becomes available for these  
12 analyses. Whatever form of analysis is adopted, a standardised pooled analysis from three large consortia  
13 comprising 20 centres will provide valuable information about these parameters, which will assist in  
14 framing a public health response and advice to women who might be exposed in future. It may also throw  
15 light on pathologic mechanisms leading to adverse outcomes, which could help in the development of  
16 therapeutic or prophylactic interventions.  
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### 23 **Patient and Public Involvement**

24 No patients were involved in the development of this protocol  
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30

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**Contributors** A steering group (CT, TJ, KDR, RAAX, AEA, EBB, NA) was formed to prepare a Joint Statistical Analysis Plan pursuant to the terms of the three EC Zika Consortia funding, at the direction of the consortia PIs (CG, XdL and AW-S). After initial discussions the steering group delegated the task to AEA, EBB and NA. The SAP was conceptualised and drafted by AEA with EBB and NA. After several iterations, the draft was revised based on comments from CT, DB, ASA, TJ, KDR, MP, DBM, RAAX, MCM, AT, CG, XdL and AW-S. Comments were then solicited from TVBA, VIAS, SEBC, VHBA, PB, CDCC, WVS, JEGH, BH, MK, CMTM, MMT, ETAM, URM, MEM, JGM, BR, PMSV, CSS and MDT and a revision was drafted by AEA, EBB and NA. The final draft was agreed by all authors.

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For peer review only

## TABLES

**Table 1.** Design of an idealized prospective vertical transmission study. The vertical transmission rate is estimated by  $(A+B)/(A+B+C+D)$ . The rate of adverse outcomes conditional on congenital infection is  $A/(A+B)$ . This can be compared with the rate of adverse outcomes in newborns with no congenital infection,  $C/(C+D)$ , who form a control group (Pediatric Control Group) to account for potential confounders associated with maternal infection.

An overall, non-conditional estimate of the adverse event rate is  $(A+C)/(A+B+C+D)$ .

Follow-up of births to women with no MIP creates a second control group (Maternal Control Group), in which the rate of adverse outcomes,  $E/(E+F)$ , can be compared to the rate in births with no congenital infection to women with MIP. Estimated effects of MIP based on the Maternal Control Group are vulnerable to confounding by factors associated with MIP. Some cells are set to zero as there can be no congenital infection without MIP.

			Maternal Infection Status	
			MIP	No MIP
Congenital Infection Status	congenital infection	Adverse outcomes	A	0
		No Adverse outcomes	B	0
	No congenital infection	Adverse outcomes	C	E
		No Adverse outcomes	D	F



**Table 2.** Scheme for a generic analysis of risks of adverse outcomes by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Presence or absence of fetal and neonatal signs or symptoms (e.g., microcephaly, brain calcifications, arthrogyposis) and other Potentially Zika-related outcomes (e.g., fetal loss) in the different MIP groups will be compared.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Symptom 1				
Symptom 2				
Symptom 3				
:				
No Symptoms				
TOTAL				

**Table 3.** Scheme for generic analysis of markers of congenital infection by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Markers of congenital infection may include laboratory markers, clinical markers, such as microcephaly, or both combined.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Both laboratory and clinical markers of congenital infection				
Only laboratory markers of congenital infection				
Only clinical markers of congenital infection				

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<b>No markers of congenital infection</b>				
<b>Not tested</b>				
<b>TOTAL</b>				

For peer review only

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## SUPPLEMENTARY MATERIALS

**Supplementary Table S1.** Schedule of tests and examinations in pregnancy, at delivery, and during paediatric follow-up, in each consortium.

Consortium	Mother	Fetus	Newborn and neonate	Pediatric follow-up
ZIKAlliance	<p>ZIKV serology IgG &amp; IgM, NAAT blood and urine, at booking, every 4w, at birth, and at paediatric follow-up.</p> <p>NAAT placenta and amniotic fluid at birth; NAAT breast milk and saliva at birth, if evidence of infection.</p> <p>TORCH at enrolment / birth, HIV at birth according to local / national guidelines.</p>	Laboratory, pathology examination of fetal loss and stillbirth.	<p>Clinical examination of newborn.</p> <p>Newborn testing: NAAT, IgM blood, cord blood; TORCH according to local / national guidelines.</p>	<p>Clinical examination, anthropometry, NAAT blood and urine; IgM, IgG blood at 4w, 4m, 12m, 24m.</p> <p>Transcranial U/S at 4w. Hearing, eye, neurodevelopmental assessments.</p>
ZIKAction	<p>IgM, IgG serology booking, 20, 28, Delivery, and at paediatric follow-up</p> <p>NAAT testing, and additional U/S investigations if evidence of infection</p>	Laboratory, pathology examination of fetal loss and stillbirth.	<p>Clinical examination of newborn</p> <p>Newborn testing: IgM, IgG (serum; CSF only if clinically indicated), urine, saliva. TORCH testing if clinically indicated</p> <p>NAAT, U/S and ophthalmology investigations if evidence of infection</p>	<p>Paediatric testing: IgG, IgM (serum, urine, saliva) and clinical examination at: 4w, 4m, 9m, 12m, 18m and 24m. NAAT if evidence on infection</p> <p>Neurodevelopmental assessments</p>

ZikaPLAN	NAAT and serologic testing (PRNT, IgM, IgG3) when symptoms reported and at up to three follow-up visits.  TORCH, DENV, and CHIKV testing	Not routinely tested.	Clinical examination in early infancy.  NAAT testing in the neonatal period in some centres. TORCH testing if clinically indicated	Clinical examination of the child by specialists at 3, 6, 12, 18, 24, 36, and 48m  Neurodevelopmental assessments
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CSF, Cerebro-spinal fluid; DENV, Dengue virus; CHIKV, Chikungunya virus; HIV, Human Immunodeficiency virus; Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test; TORCH, Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes; U/S, Ultrasound; ZIKV, Zika virus

**Supplementary Table S2.** Illustrative and approximate definitions of key explanatory and outcome variables. The final definitions will be determined by the Joint Diagnostics Group and Joint End-point Review Group.

<i>Maternal Symptoms</i>	For example: rash, fever, headache, joint pain
<i>Maternal Infection in Pregnancy (MIP) status, established only on the basis of maternal testing</i>	Confirmed: NAAT, Seroconversion Suspected: Serological tests of recent infection, including: IgM, IgG3, avidity No Evidence of MIP: Maternal testing protocol was followed, but none of the above were positive. No MIP: As above but with IgG negative at or near time of delivery
<i>Laboratory markers of Congenital Infection (CI)</i>	Present: NAAT or IgM any time in first 7 days; Absent: all other findings
<i>Most likely trimester of onset of maternal infection</i>	The highest available from the following hierarchy: <ul style="list-style-type: none"> <li>• Date of first NAAT positive test minus average duration of viremia</li> <li>• Seroconversion: Midpoint between last serological negative and last positive</li> <li>• Serological tests of recent infection: Date of first positive</li> </ul>
<i>Signs and symptoms compatible with Congenital Zika Syndrome (CZS)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available. <i>The intention will be to produce a definition that is virtually 100% specific for congenital ZIKV infection in mothers exposed to a ZIKV outbreak, especially if other TORCH infections can be ruled out.</i>
<i>Other Potentially Zika-related Outcomes (OPZRO)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available.

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test.



**Supplementary Table S3.** Prospectively ascertained Maternal Infection in Pregnancy (MIP) status and evidence on which it is based. NB: Data from the same woman can appear in more than one of the last four columns

Centre	Total women	Prospectively ascertained MIP status				Evidence for Confirmed or Suspected MIP			
		Confirmed MIP	Suspected MIP	No Evidence of MIP	No MIP	NAAT	Sero-conversion	IgM/IgG3	PRNT
1									
2									
...									
etc									

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S4.** Summary of evidence on laboratory and clinical markers of congenital infection (CI) and evidence on which it is based. NB: Data from the same fetus/newborn can appear in more than one column

Centre	Total number fetus/newborn	Markers of congenital infection					Other evidence of congenital infection			
		Laboratory or clinical markers of congenital infection	Laboratory markers of congenital infection only	Signs and symptoms compatible with CZS	NAAT	IgM	No markers of congenital infection	Microcephaly	Neurological abnormalities	Ocular abnormalities
1										
2										
...										
etc										

CZS, Symptoms compatible with Congenital Zika Syndrome; IgM/G, Immunoglobulin M/G; NAAT, Nucleic Acid Amplification Test; OPZRO, Other potentially Zika-related symptoms; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S5.** Distributions of signs and symptoms by laboratory markers of congenital infection and prospectively ascertained maternal infection in pregnancy (MIP) status. The breakdown shown is an example. Other examples might be "Termination of pregnancy, Pregnancy loss, Stillbirth, Livebirth"; or "Sensorineural hearing loss (SNHL), No SNHL "

<b>CENTRE</b>			
<b>Sign or symptom in fetus, newborn, or infant</b>	<b>MIP with laboratory markers of congenital infection</b>	<b>MIP with no laboratory markers of congenital infection</b>	<b>No MIP</b>
Signs and symptoms compatible with Congenital Zika Syndrome (CZS)			
Other potentially Zika-related outcomes (OPZRO)			
Asymptomatic			
<b>TOTAL = 100%</b>			

# BMJ Open

## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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## Zika virus infection in pregnancy: A protocol for the joint analysis of the prospective cohort studies of the ZIKAlliance, ZikaPLAN and ZIKAction consortia

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## ABSTRACT

### Introduction

Zika virus (ZIKV) infection in pregnancy has been associated with microcephaly and severe neurological damage to the fetus. Our aim is to document the risks of adverse pregnancy and birth outcomes and the prevalence of laboratory markers of congenital infection in deliveries to women experiencing ZIKV infection during pregnancy, using data from European Commission-funded prospective cohort studies in 20 centres in 11 countries across Latin America and the Caribbean.

### Methods and analysis

We will carry out a centre-by-centre analysis of the risks of adverse pregnancy and birth outcomes, comparing women with confirmed and suspected ZIKV infection in pregnancy to those with no evidence of infection in pregnancy. We will document the proportion of deliveries in which laboratory markers of congenital infection were present. Finally, we will investigate the associations of trimester of maternal infection in pregnancy, presence or absence of maternal symptoms of acute ZIKV infection, and previous flavivirus infections with adverse outcomes and with markers of congenital infection. Centre-specific estimates will be pooled using a two-stage approach.

### Ethics and dissemination

Ethical approval was obtained at each centre. Findings will be presented at international conferences and published in peer-reviewed open access journals, and discussed with local public health officials and representatives of the national Ministries of Health, Pan American Health Organization, and World Health Organization involved with ZIKV prevention and control activities.

### Keywords

Zika virus; pregnancy; vertical transmission; Congenital Zika Syndrome; microcephaly; meta-analysis



## Strengths and limitations of this study

- This will be a pooled analysis of data from 3 international consortia conducting prospective cohort studies of outcomes following ZIKV infection in pregnancy in 20 centres in 11 countries.
- Standardised definitions of outcomes will provide clarity about the absolute risks of adverse outcomes, which have not been reported consistently in prospective studies so far.
- These studies include a control group of women with no evidence of ZIKV infection in pregnancy, allowing improved estimation of the proportion of adverse events attributable to ZIKV in pregnancy.
- Inferences will be limited by: difficulties in distinguishing between women who did and who did not experience a ZIKV infection in pregnancy, due to the high frequency of mild and asymptomatic infections and the low sensitivity and specificity of diagnostic tools; low diagnostic sensitivity of markers of congenital infection; and outcome data that are not missing at random.

## INTRODUCTION

Following the emergence of Zika virus (ZIKV) in Asia and the Pacific in 2013 (1) and the subsequent introduction to Brazil (2), clusters of neonates with severe neurological complications and microcephaly were observed across Latin America. Following recent experiences with the H1N1 influenza pandemic and Ebola outbreak in Western Africa, the need for coordinated international research on ZIKV was quickly recognized. In January 2016, before the World Health Organization (WHO) declared a Public Health Emergency of International Concern (3), the European Commission (EC) issued a funding call to set up a network in Latin America and the Caribbean with the aim of implementing and coordinating urgently required research, while simultaneously contributing to research capacity and preparedness for other emerging infectious diseases. Three consortia were funded: ZIKAlliance (<https://zikalliance.tghn.org/>) (4,5), ZikaPLAN (6) (<https://zikaplan.tghn.org/>), and ZIKAction (<http://zikaction.org/>). All are multidisciplinary international collaborations with active investigations in epidemiology, virology, immunology, diagnostics, mathematical modelling, social science, and animal studies. Each consortium includes its own prospective cohort study of ZIKV in pregnancy and a shared work package that aims to ensure the harmonization of protocols and data sets in order to facilitate a pooled analysis of cohort data. The primary aim of the pooled analysis is to investigate the incidence of adverse outcomes of ZIKV infection in pregnancy, including “congenital infection, microcephaly, Zika congenital syndrome, and other sequelae of ZIKV infection”.

The aim of this paper is to present a protocol for this pooled analysis. Data has been or is still being collected in multiple sites in 20 regional coordinating centres spread over 11 countries and regions across Latin America and the Caribbean. There are 15 ZIKAlliance centres: Sao Paulo, Rio de Janeiro, Recife and Belo Horizonte (Brazil); Valencia (Venezuela); Bucaramanga (Colombia); Guayaquil (Ecuador); Lima (Peru); Jalisco, Nayarit, Veracruz, Yucatan (Mexico); Santa Cruz de la Sierra (Bolivia); Havana (Cuba); Guadeloupe (French Territory of the Americas); 3 ZikaPLAN centres: Goiânia, Rio de Janeiro, Recife (Brazil); and 2 ZIKAction centres: Kingston (Jamaica) and Port-au-Prince (Haiti). Recruitment to ZIKAlliance began May 2017, December 2015 for ZikaPLAN, and September 2017 for ZIKAction. Over 700 women with confirmed infection had been recruited by April 2020.

Several studies of ZIKV in pregnancy have recently been published. In registry-based studies (7-9), fetuses and newborns of women with confirmed infection in pregnancy have been reported to have “potentially Zika-related” adverse outcomes at rates of up to 15%, with higher risk of Zika-associated adverse outcomes in the first trimester. Registry-based studies are likely to over-estimate the risk of severe clinical

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3 manifestations and underestimate the risk of more mild clinical presentations because they recruit both  
4 prospectively ascertained ZIKV-infected pregnant women and women whose infection was recognized  
5 retrospectively following the birth of an infant with congenital abnormalities. Prospective studies of  
6 congenital infection have variously reported 25% “severe” and 21% “mild to moderate” outcomes in  
7 French Guiana (10), and 27% adverse outcomes in Brazil (11). The specificity of these outcome definitions  
8 for ZIKV in pregnancy is not known as these studies did not include a control group of women with no  
9 ZIKV infection in pregnancy. In another Brazilian study, the risk of adverse outcomes was reported to be  
10 46% in births to women with NAAT (Nucleic Acid Amplification Test)-confirmed ZIKV infection in  
11 pregnancy compared to 11.5% in NAAT-negative women (12). In a large prospective study based in the  
12 French Territories of the Americas, among infants born to women with NAAT-confirmed ZIKV infection,  
13 7.0% presented with neurologic or ocular birth defects and 3.1% met the study’s criteria for Congenital  
14 Zika Syndrome (CZS) (13), which is characterized by several unique features (14). An important limitation  
15 in the comparison of the results of these different studies is the lack of a standard definition of CZS and  
16 of the clinical and diagnostic procedures used to evaluate these children, leading to possible  
17 misclassification of the outcomes studied.

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20 The vertical transmission rate is the probability of congenital infection in births to women with infection  
21 in pregnancy. The rates reported so far, 26% (11) and 35% (10), are based on laboratory markers of  
22 congenital infection such as NAAT or IgM in the fetus or newborn. However, a prospective cohort  
23 retrospectively reconstructed from a register study estimated the vertical transmission rate to be only 9%  
24 (15). Comparison of these rates is difficult as different markers and different biological samples were used.  
25 In addition, although these tests (NAAT and IgM) are analytically sensitive and specific they have poor  
26 diagnostic sensitivity as markers of congenital infection. These markers were absent from serum in a high  
27 proportion of CZS cases (16) (16,17) and in newborns with other potentially ZIKV-related adverse  
28 outcomes born to women with confirmed ZIKV during pregnancy (10,11). Clearance of virus from amniotic  
29 fluid and fetal blood has been reported in cases of CZS, even when ZIKV is found in brain tissue post-  
30 mortem (18,19). It therefore appears that fetal infection may occur, causing profound damage, but  
31 clearing before delivery and leaving no discernable immunological trace in serum. Consequently, in this  
32 study we will document the prevalence of markers of congenital infection using uniform criteria,  
33 recognizing that this is an underestimate of the true vertical transmission rate.

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36 Regarding effect modifiers, a number of studies have reported a higher incidence of congenital  
37 abnormalities following maternal infections in the first trimester (7,10–11,14). Maternal symptoms during  
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3 acute ZIKV infection do not appear to be a risk factor for adverse outcomes (20). There is evidence of  
4 antibody dependent enhancement of ZIKV by dengue virus (DENV) antibody in animal models (21), but it  
5 is unclear whether previous DENV infection or exposure to other flaviviruses has a protective, risk-  
6 enhancing, or null effect, in maternal or congenital infection in humans (22). It also remains to be  
7 established whether a previous ZIKV infection confers protective immunity. Little is currently known about  
8 risk factors for trans-placental transmission of ZIKV.  
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13 The analysis plan described here complements the recently published protocol of the Zika Virus Individual  
14 Participant Data (IPD) Consortium (23), which will eventually include data from the three EC consortia as  
15 well as data from many other sources. Although the objectives of the protocols are similar, different  
16 methods are proposed in relation to design of included studies, definition of congenital infection, and  
17 approach to imperfect diagnosis of maternal infection.  
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22 In light of the unexplained heterogeneity in reported rates of adverse outcomes, and the variation in  
23 prevalence of markers of congenital infection, a pooled analysis of data from 20 centers following similar  
24 protocols with harmonized definitions of clinical and laboratory outcomes will provide important new  
25 information on outcomes of ZIKV in pregnancy.  
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## 32 **OBJECTIVES OF THE JOINT ANALYSIS**

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35 1. To estimate the risk of adverse outcomes in the fetus, newborn, and child following maternal ZIKV  
36 infection in pregnancy, compared to outcomes in controls with no evidence of maternal infection in  
37 pregnancy.  
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40 2. To estimate the prevalence of markers of congenital infection among fetuses and liveborn infants  
41 following maternal ZIKV infection during pregnancy.  
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- 43  
44 3. To assess the associations between trimester of maternal infection, presence or absence of maternal  
45 symptoms, and previous flavivirus infections with adverse outcomes and markers of congenital  
46 infection.  
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## 52 **METHODS**

### 53 **Participants**

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3 Pregnant women were eligible only if their infection status during pregnancy (infected or not infected)  
4 was ascertained prior to the detection of adverse outcomes, or was not influenced by fetal examination  
5 or outcome on delivery. This definition is compatible with retrospective testing of previously collected  
6 maternal samples, after delivery. Although the unit of recruitment is the mother, the unit of analysis is  
7 the fetus, newborn, and infant; multiple births are sufficiently rare to be treated as independent  
8 observations (24).  
9

## 14 Study design

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16 Consenting women were screened in pregnancy for markers of ZIKV infection. Those in whom maternal  
17 infection in pregnancy (MIP) was suspected were followed with enhanced investigations. In ZIKAction and  
18 ZIKAlliance, all deliveries to these women, including fetal losses, stillbirths, and newborns were examined  
19 clinically and tested for markers of congenital infection. This testing was not routinely performed in  
20 ZikaPLAN. In all three cohorts, newborns were prospectively followed to identify any adverse outcomes  
21 that may develop later. In all three consortia, a sample of newborns delivered to women with no evidence  
22 of infection in pregnancy served as an unexposed control group.  
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28 There were some differences between the protocols adopted by the three consortia in terms of how  
29 women were recruited into the study, and the choice and scheduling of tests and investigations  
30 (Supplementary Table S1). In ZIKAction and ZIKAlliance, women were recruited regardless of symptoms  
31 during pregnancy, although report of symptoms was recorded. In ZikaPLAN, only women with rash, a  
32 common sign of ZIKV infection, were recruited. Statistical analyses will therefore be stratified by whether  
33 the mother reported symptoms in pregnancy. .  
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39 When the studies were designed, there was little information on the risk of adverse outcomes of ZIKV in  
40 pregnancy, on vertical transmission rates, nor on what infection rates among pregnant women might be  
41 expected. Formal sample size calculations were not undertaken.  
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45 **Patient and public involvement statement:** There was no patient or public involvement in  
46 this study  
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## 50 Target parameters and terminology of vertical transmission studies

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52 Six categories of joint congenital infection status and maternal infection status (A-F) are defined in Table 1,  
53 which illustrates the logic of an idealised prospective study. The usual target parameters are the *vertical*  
54 *transmission rate*, which is the probability of congenital infection following MIP,  $(A+B) / (A+B+C+D)$ ; and  
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3 the *rate of adverse outcomes in those with congenital infection*,  $A/(A+B)$ . The definitions of “adverse  
4 outcomes”, congenital infection, and MIP will be determined by a Joint Diagnostics Group and a Joint  
5 Endpoint Review Group, after the data have been assembled. Estimates of these parameters are standard  
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7 in the classic studies of vertical transmission of human immune-deficiency virus (HIV) (25,26),  
8 toxoplasmosis (27–29) and cytomegalovirus (CMV) (30). In studies of less specific outcomes, the event  
9 rate  $C/(C+D)$  in fetuses and newborns of women with MIP but in whom no congenital infection occurred  
10 (Paediatric Control Group 1 in Table 1) forms a comparison group (31,32) representing the adverse event  
11 rate that is due to MIP in the absence of congenital infection. The present analysis plan is modelled closely  
12 on these earlier studies, but includes adaptations to take account of the difficulties in diagnosing maternal  
13 and congenital ZIKV infection.  
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17 For example, because cases of congenital infection cannot be reliably identified by diagnostic tests, we  
18 can only estimate the prevalence of laboratory *markers of vertical infection* (i.e. NAAT or IgM) (Objective  
19 2). Similarly, the “overall” (unconditional) adverse event rate is taken as the primary outcome for  
20 Objective 1; this includes all births to women with MIP,  $(A+C)/(A+B+C+D)$  (Table 1). All three consortia  
21 included a further control group of births to unexposed women, those with no infection in pregnancy  
22 (Maternal Control Group in Table 1). The adverse event rates in this group,  $E/(E+F)$ , represents a baseline  
23 for comparison with the overall event rates in exposed women (33).  
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27 Although estimates of the vertical transmission rate are compromised, it may still be of interest to  
28 compare adverse outcome rates in the MIP with congenital infection group (i.e.,  $A/(A+B)$ ), the MIP without  
29 congenital infection group (i.e.,  $C/(C+D)$ ), and the No MIP group (i.e.,  $E/(E+F)$ ), as this may provide insight  
30 into whether adverse fetal outcomes are associated with MIP in the absence of demonstrable fetal  
31 infection.  
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35 In addition to the challenges associated with the laboratory definition of congenital infection, it is also  
36 difficult to discriminate between pregnancies with MIP and with No MIP as required in analyses based on  
37 Table 1. A positive NAAT result or seroconversion during pregnancy are sufficient to confirm MIP.  
38 However, even if tested *per protocol*, MIP may be missed due to the narrow window of detection of NAAT  
39 tests, perhaps as low as 14 days (34). Tests of recent infection, including IgM, IgG3 or avidity assays, may  
40 reflect infection during pregnancy, but may also be the result of infection prior to pregnancy (35), and  
41 immunologic cross-reactivity to DENV antibody may need to be ruled out (36). These tests therefore  
42 indicate suspected but not confirmed MIP. An IgG negative response in the woman or newborn at or tests,  
43 perhaps as low as 14 days (34). An IgG negative response in the woman or newborn at or shortly after  
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3 delivery can be taken as suggestive of No MIP, although the dynamics of ZIKV IgG are not well  
4 documented. While a negative IgG is likely to be uncommon in some sites, as some women will  
5 have experienced a ZIKV infection prior to pregnancy, it lowers the probability that a ZIKV infection would  
6 have occurred in pregnancy. Taking all this together, MIP status will be characterised as “Confirmed”,  
7 “Suspected, “No Evidence of MIP” (i.e., all NAAT and IgM tests negative), and “No MIP” (i.e., all NAAT and  
8 IgM tests negative AND IgG negative at or shortly after delivery). To maintain the principle of prospective  
9 ascertainment, confirmation of congenital infection will have no impact on the mother’s assigned  
10 infection status.  
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## 18 **Definitions of variables**

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20 To support the pooled analyses, a Joint Diagnostics Group consisting of immunologists and virologists and  
21 a Joint Endpoint Review Group consisting of paediatricians with experience of congenital ZIKV will be  
22 convened to agree on standardized case definitions. The names and qualifications of members of both  
23 groups will be published at the time of the joint data analysis, together with rationale and process for  
24 their recruitment. Supplementary Table S2 provides some provisional definitions for: MIP (Confirmed,  
25 Suspected, No Evidence of MIP, No MIP), laboratory markers of congenital infection (present or absent),  
26 signs and symptoms compatible with Congenital Zika Syndrome (CZS), other potentially-Zika-related  
27 outcomes (OPZRO), and trimester of MIP. The Diagnostics Group will also have responsibility for  
28 definitions of covariates, such as previous flavivirus infection. Definitions of both diagnostic categories  
29 and clinical endpoints will be based on the best information available at the time of analysis, and will to  
30 the greatest extent possible, be harmonized across consortia and across centres within consortia. As the  
31 expert groups will be assembled from representatives of each consortium, they will already have  
32 examined the data prior to a joint analysis, so that blinding will not be possible; however, they will devise  
33 diagnostic and clinical criteria that can be applied objectively across the three consortia.  
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44 An essential aspect of the definitions for MIP status used in the statistical analyses below is that they must  
45 be based exclusively on the *prospective* diagnostic testing. For example, although CZS and/or laboratory  
46 evidence of congenital infection in the newborn provides compelling evidence of MIP, this would not  
47 affect the MIP status as ascertained prospectively. Thus, we expect to observe some newborns with CZS  
48 and/or with laboratory markers of congenital infection delivered to women with Suspected MIP, or even  
49 those categorized as having No Evidence of MIP.  
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## STATISTICAL ANALYSIS

### Analysis of data from individual centres

#### Descriptive tables

We will produce descriptive tables providing a breakdown of the numbers in each centre with Confirmed MIP, Suspected MIP, No Evidence of MIP, and No MIP, and the type of evidence on which this is based (e.g., NAAT, seroconversion, tests of recent infection) (Supplementary Table S3). The similar Supplementary Table S4 will document numbers with signs or symptoms compatible with CZS and with markers of congenital infection, and the type of evidence on which this is based, for example NAAT, IgM, or clinical markers (CZS).

#### Objective 1: Adverse Outcomes

Prospectively ascertained MIP status will be tabulated against overall (i.e., unconditional on congenital infection status) adverse outcomes (Table 2). Various risks can be estimated within each MIP category, including: the risk of signs and symptoms compatible with CZS, the risk of OPZRO, the risk of both combined, of individual signs and symptoms, or of signs and symptoms grouped in clinically (e.g., ophthalmologic defects) or embryologically meaningful ways. Outcomes may be binary (e.g., microcephaly) or continuous (e.g., head circumference), or multi-category (CZS-related outcomes, OPZRO, asymptomatic). As well as congenital anomalies, rates of standard outcomes, in the absence of congenital anomalies, will be documented, including: fetal loss, stillbirth, low birthweight, intra-uterine growth retardation (IUGR), and premature delivery.

The rate of adverse outcomes in the No MIP group represents the study-specific background rate of adverse outcomes (i.e., in the absence of MIP, Control Group 2), to be compared to rates in women with Confirmed MIP. Absolute risks will be estimated as well as risk ratios and risk differences.

Parallel sets of estimates will be calculated in the Suspected MIP and No Evidence of MIP groups, as particular adverse events indicate lack of diagnostic specificity and sensitivity in the testing protocol during pregnancy and are therefore informative regarding the effectiveness of the maternal testing



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3 protocol in each centre; for example, microcephaly in the No Evidence of MIP group would indicate a lack  
4 of sensitivity. These sets of estimates may be pooled, respectively, with adverse outcome risks in  
5 Confirmed MIP and No MIP groups in sensitivity analyses.  
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## 10 Objective 2: Laboratory and clinical markers of vertical transmission

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13 Estimates of the prevalence of markers of congenital infection will be produced in all centres with  
14 available data for each MIP group except the No MIP group, as the definition of No MIP is not compatible  
15 with laboratory markers of congenital infection. For this purpose, the numerator will be the number with  
16 markers of congenital infection, and the denominator will be the sum of the numbers with and without  
17 markers of congenital infection. Separate estimates will be obtained for: laboratory markers, clinical  
18 markers (namely CZS) , and combined laboratory and clinical markers (Table 3). Rates in the No Evidence  
19 of MIP group are of interest as they carry information about the diagnostic accuracy of the maternal  
20 testing protocol.  
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## 30 Objective 3: Effect of covariates

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33 Separate estimates of the prevalence of adverse pregnancy outcomes, and the probability of laboratory  
34 and clinical markers of congenital infection, will be produced for each trimester of maternal infection, and  
35 by presence or absence of maternal symptoms.  
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39 Analyses of adverse event frequencies and of markers of congenital infection can be extended to include  
40 multiple covariates, using logistic regression. These might include potential *effect modifiers*, such as  
41 previous arbovirus infection or co-infection, or *confounding factors* such as socio-economic indicators  
42 likely to be associated with both arbovirus exposure and adverse outcomes. However, at the time of  
43 writing it is not known whether sufficient data will be available for regression analyses.  
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## 50 Secondary objectives

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53 ZIKV infection in pregnancy could lead to adverse pregnancy and birth outcomes either following a  
54 congenital infection (cell A in Table 1) or in the absence of congenital infection (equally in cells A and C).  
55 Although absence of markers of congenital infection does not rule out congenital infection, we might still  
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3 expect to observe more adverse outcomes in fetuses and newborns with laboratory markers of congenital  
4 infection if those outcomes are caused by congenital infection. By contrast, adverse outcomes that are  
5 the result of MIP in the absence of congenital infection (Cell E in Table 1) should occur equally with or  
6 without laboratory markers of congenital infection. Based on literature on other infections in pregnancy,  
7 including dengue virus (37), adverse outcomes associated with MIP in the absence of congenital infection  
8 potentially include: fetal loss, stillbirth, prematurity, IUGR, and low birthweight for gestational age (38).  
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10 The analysis would be based on a tabulation of presence or absence of neonatal symptoms, or sets of  
11 symptoms (Supplementary Table S5), and would be stratified by trimester of maternal infection, as this is  
12 likely to be associated with the presence of markers of congenital infection and with adverse outcomes.  
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## 21 Missing Covariates

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23 All analyses will be conducted on a “complete case” basis, in the first instance. Methods for handling  
24 missing covariates, such as imputation (39), will be considered after the extent and patterns of missing  
25 data have been explored.  
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## 32 Combining data across centres

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34 The above analyses will generate a series of centre-specific estimates of proportions, relative risks  
35 comparing MIP and No MIP groups, risk-differences, and means of continuous variables, stratified by  
36 trimester and maternal clinical presentation. If logistic regression is used to examine effect-modifiers and  
37 confounders, further estimates of interaction terms or adjusted estimates can also be produced.  
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41 All these estimates can be combined across centres using fixed or random effects models in a “two-stage”  
42 meta-analysis. Bayesian Markov chain Monte Carlo methods will be used as this will facilitate the use of  
43 exact binomial and multinomial likelihoods, which have a better performance with low and zero cells  
44 counts. Vague priors will be employed. Centre-specific random effect estimates will be sampled from beta  
45 distributions for binomial outcomes data, Dirichlet distributions for multinomial data, and normal  
46 distributions for continuous data. We will report ranges, between-centre standard deviations, mean  
47 effects and predictive effects with 95% credible intervals for each estimate.  
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3 In combining estimates from different centres, we will take account of the fact that in ZikaPLAN only  
4 women with rash were recruited, so that women with no ZIKV infection may have experienced other  
5 exanthematic infections (12), including arbovirus infections such as dengue and chikungunya, which may  
6 themselves be associated with adverse outcomes (40,41).  
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11 Depending on the results of two-stage analyses, and the completeness of covariate data, an individual  
12 patient data one-stage meta-analyses will be considered for each objective, as a secondary or sensitivity  
13 analysis, with centre as an additional fixed “intercept” term.  
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## 17 18 19 **Sensitivity analyses**

### 20 21 22 **Definitions of MIP status, ZIKV-related outcomes, and laboratory markers of** 23 **congenital infection** 24 25

26 We will report differences in adverse event rates between Confirmed MIP and Suspected MIP, and  
27 between No Evidence of MIP and No MIP. If the differences are small, we will produce results pooling  
28 these categories as a sensitivity analysis.  
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32 Further, the No Evidence of MIP category can be subdivided into women who were tested *per protocol*  
33 and those who may have been tested less completely. The impact of compliance with protocols will be  
34 explored, as it is expected to impact on the proportion of women with MIP who are classified as “No  
35 evidence of MIP” and, hence, on the probability of observing adverse outcomes in this group. Similarly,  
36 we will conduct sensitivity analyses around the definitions of Confirmed and Suspected MIP on advice  
37 from the Joint Diagnostics Group.  
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42 Alternative sets of estimates will be generated using alternative criteria for CZS-related outcomes and  
43 OPZRO, that are more, or less, specific for ZIKV in pregnancy. Similarly, we will explore the impact of  
44 varying the laboratory criteria for congenital infection on the advice of the Joint Diagnostics Group.  
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## 50 51 **Independent ascertainment of outcomes**

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53 A critical requirement of all these analyses is that the ascertainment of markers of congenital infection  
54 status and clinical outcomes in the fetuses and newborns, and developing infant are all independent of  
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3 each other, and also independent of MIP status. For example, ideally the same laboratory testing for  
4 congenital infection is carried out regardless of whether the pregnancy outcome is a fetal loss, termination  
5 of pregnancy, stillbirth, a case of CZS, or an apparently healthy asymptomatic infant.  
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8 These assumptions are difficult, and in certain respects (e.g., first trimester fetal loss and terminations)  
9 not possible, to fully implement in practice. To address these inevitable limitations in the analysis, which  
10 are expected to impact more on objective 2 than on objective 1, we will carry out exploratory analyses  
11 aimed at detecting potential deviations from protocol. For example, the distribution of trimester of MIP  
12 should not be associated with prospectively ascertained MIP status. Guided by the results, we will carry  
13 out sensitivity analyses that make a range of assumptions about the distribution of missing data, especially  
14 data on markers of congenital infection. A series of scenarios will be examined to assess robustness of  
15 results to inherent and/or unplanned deviations from the ideal protocols required for unbiased estimation  
16 of the target parameters.  
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## 27 **DISCUSSION**

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29 Risks of CZS and other adverse birth outcomes of ZIKV infection in pregnancy can only be assessed through  
30 studies that recruit women whose infection status is prospectively ascertained, or, if retrospective,  
31 ascertainment is independent of outcomes. However, reported risks of adverse outcomes even from  
32 prospective studies have been highly variable (10–13), as have vertical transmission rates based on  
33 laboratory markers of congenital ZIKV infection (10,11,15). An important role for joint analyses of multiple  
34 studies is to explore whether this heterogeneity in outcomes can be explained by individual or study-level  
35 covariates. To do this, it is essential that incidental sources of variation, such as those arising from  
36 differences in outcome reporting or diagnostic testing, are controlled or eliminated as much as possible.  
37 One of the most difficult sources of variation between consortia, and between sites within consortia, lies  
38 in diagnosis of maternal infection. Our approach is to have an Expert Diagnostics Group produce a  
39 harmonised classification of Confirmed MIP, Suspected MIP, No Evidence of MIP, and No MIP, and to  
40 compute a range of estimates of the relative effect of maternal infection on outcomes, grouping these in  
41 different ways. An analysis based on the Confirmed MIP and No MIP groups alone would be expected to  
42 generate the largest estimates of relative effect, because both poor sensitivity and poor specificity will  
43 tend to bias effect estimates towards the null.  
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54 An alternative proposal (23) in relation to maternal infection status is to treat test sensitivity and  
55 specificity as study-level covariates in a meta-regression, but the risk of false positive diagnosis depends  
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3 more on the incidence of ZIKV and cross-reacting antibodies to other arbovirus infections such as dengue  
4 than on test specificity. The Zika Virus IPD Consortium protocol differs in two other ways. First it proposes  
5 to include surveillance studies, which may result in over-estimating the risk of adverse outcomes due to  
6 retrospective ascertainment of infected women following adverse newborn outcomes (7,42). Second,  
7 congenital infection is to be defined by clinical and radiological criteria alone.  
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11 Other statistical methods may have been developed by the time the data becomes available for these  
12 analyses. Whatever form of analysis is adopted, a standardised pooled analysis from three large consortia  
13 comprising 20 centres will provide valuable information about these parameters, which will assist in  
14 framing a public health response and advice to women who might be exposed in future. It may also throw  
15 light on pathologic mechanisms leading to adverse outcomes, which could help in the development of  
16 therapeutic or prophylactic interventions.  
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7 Luis Angel VILLAR (Universidad Industrial de Santander, Bucaramanga, Colombia)  
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14 Statistical Analysis Plan pursuant to the terms of the three EC Zika Consortia funding, at the direction of  
15 the consortia PIs (CG, XdL and AW-S). After initial discussions the steering group delegated the task to  
16 AEA, EBB and NA. The SAP was conceptualised and drafted by AEA with EBB and NA. After several  
17 iterations, the draft was revised based on comments from CT, DB, ASA, TJ, KDR, MP, DBM, RAAX, MCM,  
18 AT, CG, XdL and AW-S. Comments were then solicited from TVBA, VIAS, SEBC, VHBA, PB, CDCC, WVS,  
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38

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#### 50 **Ethics and dissemination**

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52 The following ethical committees / institutional review boards have approved the participating studies:  
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3 Bolivia: El Tribunal de Ética del Colegio Médico de Santa Cruz -Tribunal Departamental de Ética y  
4 Deontología Médica (TEDM) (018/2017; 019/2017).  
5  
6 Brazil: Comissão Nacional de Ética em Pesquisa (CONEP) (2.493.696/CAAE 67410117.3.1001.5262);  
7  
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9  
10 67410117.3.3001.5132); Comitê de Ética em Pesquisa da UFMG (COEPE-UFMG), Belo Horizonte  
11 (2.877.160/CAAE 67410117.3.2002.5149); Comitê de Ética em Pesquisa do Instituto Nacional de  
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14 63567917.6.0000.0065; 2.262.420/CAAE 74853517.0.0000.0065; 1.938.335/CAAE  
15 54699416.0.0000.0065); Comitê de Ética em Pesquisa (CEP) do Instituto Aggeu Magalhães, Recife  
16 (2.777.436/CAAE 80243817.5.0000.5190; 2.737.404/CAAE 67410117.3.2001.5190); CEP Universidade  
17 Federal de Goiás, Goiânia (64534017.7.0000.5083); CEP Instituto Fernandes Figueira, Rio de Janeiro  
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19  
20 Colombia: Comité de Ética en Investigación Científica de la Universidad Industrial de Santander (CEINCI-  
21 UIS) (4110)  
22  
23 Cuba: Comité de Ética de la Investigación del Instituto de Medicina Tropical “Pedro Kourí” (25–18; 59–  
24 16; 40–16)  
25  
26 Ecuador: Comité de Ética del Hospital Clínica Kennedy, Guayaquil (HCK-CEISH-17-0026)  
27  
28 Haiti: Ministère de la Santé Publique et de la Population, Comité National de Bioéthique (1718-7)  
29  
30 Jamaica: University of the West Indies (Mona Campus) Research Ethics Committee (104-16/17)  
31  
32 Mexico: Comité de Ética en Investigación y Comité de Investigación de la Comisión Nacional de  
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39  
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41  
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3 Findings will be presented at international conferences and published in peer-reviewed open access  
4 journals, and discussed with local public health officials and representatives of the national Ministries of  
5 Health, Pan American Health Organization, and World Health Organization involved with ZIKV prevention  
6 and control activities.  
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## TABLES

**Table 1.** Design of an idealized prospective vertical transmission study. The vertical transmission rate is estimated by  $(A+B)/(A+B+C+D)$ . The rate of adverse outcomes conditional on congenital infection is  $A/(A+B)$ . This can be compared with the rate of adverse outcomes in newborns with no congenital infection,  $C/(C+D)$ , who form a control group (Pediatric Control Group) to account for potential confounders associated with maternal infection.

An overall, non-conditional estimate of the adverse event rate is  $(A+C)/(A+B+C+D)$ .

Follow-up of births to women with no MIP creates a second control group (Maternal Control Group), in which the rate of adverse outcomes,  $E/(E+F)$ , can be compared to the rate in births with no congenital infection to women with MIP. Estimated effects of MIP based on the Maternal Control Group are vulnerable to confounding by factors associated with MIP. Some cells are set to zero as there can be no congenital infection without MIP.

			Maternal Infection Status	
			MIP	No MIP
Congenital Infection Status	congenital infection	Adverse outcomes	A	0
		No Adverse outcomes	B	0
	No congenital infection	Adverse outcomes	C	E
		No Adverse outcomes	D	F

**Table 2.** Scheme for a generic analysis of risks of adverse outcomes by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Presence or absence of fetal and neonatal signs or symptoms (e.g., microcephaly, brain calcifications, arthrogyposis) and other Potentially Zika-related outcomes (e.g., fetal loss) in the different MIP groups will be compared.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Symptom 1				
Symptom 2				
Symptom 3				
:				
No Symptoms				
TOTAL				

**Table 3.** Scheme for generic analysis of markers of congenital infection by prospectively ascertained Maternal Infection in Pregnancy (MIP) status. Markers of congenital infection may include laboratory markers, clinical markers, such as microcephaly, or both combined.

CENTRE	Maternal Infection in Pregnancy status			
	Confirmed	Suspected	No Evidence of MIP	No MIP
Both laboratory and clinical markers of congenital infection				
Only laboratory markers of congenital infection				
Only clinical markers of congenital infection				

<b>No markers of congenital infection</b>				
<b>Not tested</b>				
<b>TOTAL</b>				

For peer review only

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## SUPPLEMENTARY MATERIALS

**Supplementary Table S1.** Schedule of tests and examinations in pregnancy, at delivery, and during paediatric follow-up, in each consortium.

Consortium	Mother	Fetus	Newborn and neonate	Pediatric follow-up
ZIKAlliance	<p>ZIKV serology IgG &amp; IgM, NAAT blood and urine, at booking, every 4w, at birth, and at paediatric follow-up.</p> <p>NAAT placenta and amniotic fluid at birth; NAAT breast milk and saliva at birth, if evidence of infection.</p> <p>TORCH at enrolment / birth, HIV at birth according to local / national guidelines.</p>	Laboratory, pathology examination of fetal loss and stillbirth.	<p>Clinical examination of newborn.</p> <p>Newborn testing: NAAT, IgM blood, cord blood; TORCH according to local / national guidelines.</p>	<p>Clinical examination, anthropometry, NAAT blood and urine; IgM, IgG blood at 4w, 4m, 12m, 24m.</p> <p>Transcranial U/S at 4w. Hearing, eye, neurodevelopmental assessments.</p>
ZIKAction	<p>IgM, IgG serology booking, 20, 28, Delivery, and at paediatric follow-up</p> <p>NAAT testing, and additional U/S investigations if evidence of infection.</p> <p>PRNT for sub-set of samples.</p>	Laboratory, pathology examination of fetal loss and stillbirth.	<p>Clinical examination of newborn</p> <p>Newborn testing: IgM, IgG (serum; CSF only if clinically indicated), urine, saliva. TORCH testing if clinically indicated</p> <p>NAAT, U/S and ophthalmology investigations if evidence of infection</p>	<p>Paediatric testing: IgG, IgM (serum, urine, saliva) and clinical examination at: 4w, 4m, 9m, 12m, 18m and 24m. NAAT if evidence on infection</p> <p>Neurodevelopmental assessments</p>

ZikaPLAN	NAAT and serologic testing (PRNT, IgM, IgG3) when symptoms reported and at up to three follow-up visits.  TORCH, DENV, and CHIKV testing	Not routinely tested.	Clinical examination in early infancy.  NAAT testing in the neonatal period in some centres. TORCH testing if clinically indicated	Clinical examination of the child by specialists at 3, 6, 12, 18, 24, 36, and 48m  Neurodevelopmental assessments
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CSF, Cerebro-spinal fluid; DENV, Dengue virus; CHIKV, Chikungunya virus; HIV, Human Immunodeficiency virus; Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test; TORCH, Toxoplasmosis, Other, Rubella, Cytomegalovirus, Herpes; U/S, Ultrasound; ZIKV, Zika virus



**Supplementary Table S2.** Illustrative and approximate definitions of key explanatory and outcome variables. The final definitions will be determined by the Joint Diagnostics Group and Joint End-point Review Group.

<i>Maternal Symptoms</i>	For example: rash, fever, headache, joint pain
<i>Maternal Infection in Pregnancy (MIP) status, established only on the basis of maternal testing</i>	Confirmed: NAAT, Seroconversion Suspected: Serological tests of recent infection, including: IgM, IgG3, avidity No Evidence of MIP: Maternal testing protocol was followed, but none of the above were positive. No MIP: As above but with IgG negative at or near time of delivery
<i>Laboratory markers of Congenital Infection (CI)</i>	Present: NAAT or IgM any time in first 7 days; Absent: all other findings
<i>Most likely trimester of onset of maternal infection</i>	The highest available from the following hierarchy: <ul style="list-style-type: none"> <li>• Date of first NAAT positive test minus average duration of viremia</li> <li>• Seroconversion: Midpoint between last serological negative and last positive</li> <li>• Serological tests of recent infection: Date of first positive</li> </ul>
<i>Signs and symptoms compatible with Congenital Zika Syndrome (CZS)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available. <i>The intention will be to produce a definition that is virtually 100% specific for congenital ZIKV infection in mothers exposed to a ZIKV outbreak, especially if other TORCH infections can be ruled out.</i>
<i>Other Potentially Zika-related Outcomes (OPZRO)</i>	A definition will be prepared by the Joint End-point Review Group, based on best evidence available.

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test.

**Supplementary Table S3.** Prospectively ascertained Maternal Infection in Pregnancy (MIP) status and evidence on which it is based. NB: Data from the same woman can appear in more than one of the last four columns

Centre	Total women	Prospectively ascertained MIP status				Evidence for Confirmed or Suspected MIP			
		Confirmed MIP	Suspected MIP	No Evidence of MIP	No MIP	NAAT	Sero-conversion	IgM/IgG3	PRNT
1									
2									
...									
etc									

Ig, Immunoglobulin; NAAT, Nucleic Acid Amplification Test; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S4.** Summary of evidence on laboratory and clinical markers of congenital infection (CI) and evidence on which it is based. NB: Data from the same fetus/newborn can appear in more than one column

Centre	Total number fetus/newborn	Markers of congenital infection					Other evidence of congenital infection			
		Laboratory or clinical markers of congenital infection	Laboratory markers of congenital infection only	Signs and symptoms compatible with CZS	NAAT	IgM	No markers of congenital infection	Microcephaly	Neurological abnormalities	Ocular abnormalities
1										
2										
...										
etc										

CZS, Symptoms compatible with Congenital Zika Syndrome; IgM/G, Immunoglobulin M/G; NAAT, Nucleic Acid Amplification Test; OPZRO, Other potentially Zika-related symptoms; PRNT, Plaque Reduction Neutralisation Test.

**Supplementary Table S5.** Distributions of signs and symptoms by laboratory markers of congenital infection and prospectively ascertained maternal infection in pregnancy (MIP) status. The breakdown shown is an example. Other examples might be "Termination of pregnancy, Pregnancy loss, Stillbirth, Livebirth"; or "Sensorineural hearing loss (SNHL), No SNHL "

<b>CENTRE</b>			
<b>Sign or symptom in fetus, newborn, or infant</b>	<b>MIP with laboratory markers of congenital infection</b>	<b>MIP with no laboratory markers of congenital infection</b>	<b>No MIP</b>
Signs and symptoms compatible with Congenital Zika Syndrome (CZS)			
Other potentially Zika-related outcomes (OPZRO)			
Asymptomatic			
<b>TOTAL = 100%</b>			