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Effect of acute ZIKA virus infection on sperm and virus clearance in body fluids: a prospective observational study

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Summary

Background Evidence of human sexual transmission during ZIKA virus (ZIKV) emergence is a matter of concern particularly in procreation but to date kinetics of seminal shedding and the effects of infection on human reproductive function need to be described. To investigate the effects of ZIKA virus (ZIKV) infection on semen and clearance of ZIKV from semen and body fluids, we prospectively studied a cohort of ZIKV-infected men.

Methods This prospective observational study recruited men presenting with acute ZIKV infection in a university hospital on Guadeloupe Island, French Caribbean, where a ZIKV outbreak occurred in 2016. Fifteen male volunteers (mean age \pm SD 35 ± 5 years, range 25–44) with acute ZIKV infection and positive ZIKV RNA detection in blood or urine were enrolled. Blood, urine and semen were collected at days 7, 11, 20, 30, 60, 90 and 120 after symptom onset, and semen characteristics and reproductive hormone levels were assessed. At days 7, 11 and 20, semen was processed to isolate motile spermatozoa. ZIKV RNA was detected by reverse-transcriptase polymerase chain reaction (RT-PCR) on whole blood, serum, urine, seminal plasma, semen cells, and motile spermatozoa fractions. ZIKV was isolated from different sperm fractions on Vero E6 cultures.

Findings Total sperm count (TSC) was decreased from median 119×10^6 spermatozoa [interquartile range Q1–Q3 22–234] at day 7 to 45.2×10^6 [16.5–89.6] and 70 [28.5–81.4] at days 30 and 60, respectively, after ZIKV infection. Inhibin values increased from 93.5 [55–162] pg/mL at day 7 to 150 [78–209] at day 120 when TSC recovered. In motile spermatozoa obtained after density gradient separation ZIKV RNA was found in 3/14,

4/15 and 4/15 patients at days 7, 11 and 20, respectively, and replication-competent virus was found in the tested patient. Seminal shedding kinetics appeared heterogeneous among patients. Whole blood was the fluid most frequently positive for ZIKV RNA (62 of 92 samples) and 3 patients remained positive at day 120. **Interpretation** Semen alterations early after acute ZIKV infection may affect fertility and could be explained by virus effects on the testis and epididymis. Frequency of shedding and high viral load in semen, together with the presence of replicative virus in a motile spermatozoa fraction, can lead to ZIKV transmission during sexual contact and assisted reproduction procedures. Whole blood appears to be the best specimen for ZIKV RNA detection, diagnosis and follow-up.

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Research in context

Evidence before this study

In February, 2016, when this study was proposed, very few data were available on ZIKA virus and human semen and no prospective study was available to determine the kinetics of ZIKA virus in semen and its correlation with presence of the virus in other body fluids. During the major 2016 ZIKA virus epidemic, sexual transmission of the virus, in particular from male to female, and adverse fetal outcomes linked to in utero transmission, such as microcephaly, were confirmed. To date, with an exception of a prospective study, only several case reports or very short series have been published showing that ZIKA virus persisted in semen after clinical remission, with a prolonged risk of sexual transmission

(number: 27; PubMed search terms: ZIKA and semen or sexual transmission, before June 2017 in any language). Animal studies demonstrated ZIKA virus localisation in the testes and also in several organs of the male genital tract. In an immune-modified male mouse model, severe damage due to ZIKA virus infection was observed in the testes and epididymis. However, no data were available on semen characteristics in men during and after infection. The precise localisation of ZIKA virus in semen (seminal plasma, semen cells, and spermatozoa) was not known, despite a case report of ZIKA virus antigen within spermatozoa. In February, 2017, the preliminary results of a prospective study reported clearance of the virus in different body fluids, except whole blood samples, and including semen but semen characteristics and ZIKA virus localisation in semen were not analysed.

Added value of this study

This prospective study provides longitudinal data stressing that replicative ZIKA virus can persist in semen after clinical remission despite its clearance in other fluids such as blood and urine. It investigates semen characteristics during 120 days follow-up and ZIKA virus localisation in different fractions of semen. Depending on semen viral load, different shedding patterns were observed. Moreover, we showed quantitative and qualitative harmful effects on spermatozoa production. Finally, replication-competent virus was found in seminal plasma and also in spermatozoa after spermatozoa isolation by semen preparation methods.

Implications of all the available evidence

Data on frequency and duration of replicative ZIKA virus shedding in semen are of vital importance in planning strategies for prevention of sexual transmission. Moreover, although they were transient in this study, quantitative and qualitative sperm production alterations challenge human reproductive potential. Detection of replication-competent virus in isolated spermatozoa from an infected patient is also a matter of concern for sexual transmission, reproduction, and particularly for assisted medical procreation guidelines. Further studies are necessary to identify the factors responsible for ZIKA virus shedding in semen and for sperm alterations. Lastly, whole blood is shown as the most sensitive sample for molecular diagnosis of ZIKA virus infection. The results of this study increase our knowledge of ZIKA virus infection and its impact on human male reproduction. They will help physicians to counsel infected patients and public health specialists to make policy recommendations.

Introduction

ZIKA virus (ZIKV) is an *Aedes* mosquito-borne flavivirus, first isolated in the rhesus monkey in Uganda in 1947 and in man in 1952.¹ The first large outbreaks occurred in the Yap islands in Micronesia (2007)² and then in French Polynesia (2013–2014).³ A recent outbreak in Brazil extended to several countries of the Americas. Acute ZIKV infection is symptomatic in 20 to 50% of cases. Microcephaly and central nervous system abnormalities in fetuses and newborn have been described after infection during pregnancy. In adults, neurological diseases such as Guillain-Barré syndrome and myelitis have been reported. In February 2016, WHO designated the ZIKV epidemic “a public health emergency of international concern”. This emergency situation ended mid-November 2016. Nevertheless, WHO underlined the urgent need for greater understanding of the pathophysiology of ZIKV disease and transmission.

ZIKV has been isolated from numerous human fluids⁴ and identified in human semen⁵ and vaginal secretions.⁶ In semen, ZIKV RNA can persist up to 188 days after symptom onset.⁷ The localisation of ZIKV in the human genital tract and its consequences are not yet known. However, studies in monkeys⁸ and mice^{9–11} have evidenced ZIKV in different male genital organs and have shown the consequences of genital infection. A ZIKV antigen was detected inside the spermatozoa of an infected man.¹² Sexual transmission has been described.¹³ In Brazil, higher ZIKV incidence in sexually active women than in men suggests that sexual transmission is involved in viral spread.¹⁴

Understanding the localisation of ZIKV within the male genital tract, its dynamics and shedding in semen, is of paramount importance to prevent sexual transmission. The consequences of ZIKV infection on the testis, genital tract and fertility in men are unknown. Mice studies using immune-deficient animals and/or modified ZIKV strains have shown a highly deleterious effect of ZIKV infection on the testis and epididymis.

In humans, the association of a mosquito-borne virus with sexual and transplacental transmissions and with birth defects had not previously been described. This association raises increased concerns about the consequences of the ZIKV outbreaks.

We conducted a prospective study to investigate the relationships between whole blood, serum, urine and semen ZIKV loads over time, to identify ZIKV in semen and within the different semen compartments, and to determine semen characteristics and reproductive hormone levels immediately following infection in men.

Methods

Study design and subjects

This was a prospective observational study to determine the presence and clearance of ZIKV in different body fluids and to research the effect of ZIKV infection on reproductive function. Inclusion criteria: men aged between 18 and 45 years with diagnosed ZIKV infection. Exclusion criteria: men with other acute illnesses, inability to provide a semen sample, ejaculation disorders, semen volume < 1.5 ml, or negative ZIKV RNA in serum

or urine. Patients attended follow-up visits 7, 11, 20, 30, 60, 90 and 120 days after symptom onset (Appendix p4). Whole blood, serum, urine and semen samples were collected at each visit.

Nineteen male patients presenting with clinical symptoms of acute ZIKV virus infection were recruited through physicians after a press information campaign in Guadeloupe Island and underwent a ZIKV RNA test in urine and serum. Of these 19 men, 3 men with a negative ZIKV RNA test and 1 with very low semen volume were excluded. Fifteen men diagnosed with acute symptomatic ZIKV infection and positive for ZIKV RNA in serum or urine were enrolled in Pointe-à-Pitre University Hospital, Guadeloupe Island. This area was officially designated a ZIKV outbreak area from April to November 2016. One patient had been taking levothyroxine for hypothyroidism for several years and one patient had chronic migraine treated by oxetorone. Seven patients were married or cohabiting and 8 were single. Eleven were of African or mixed descent and 4 were Caucasian.

The study was registered at ClinicalTrials.gov (NCT02874456) and approved by the institutional ethics review board (CPP Sud-Ouest et Outre-Mer II). All volunteers gave written informed consent and received compensation (400€) for their participation. At each visit, a questionnaire was completed about any unusual events since the last visit to the laboratory.

Semen Specimens and Analyses

One hundred semen samples were obtained by masturbation after a recommended 3–6 days abstinence period and processed within 1 h of ejaculation for analysis. Seminal plasma and whole semen cells were isolated from a semen aliquot by centrifugation at 600g and frozen at -80°C.

Semen analysis was performed according to WHO guidelines on an aliquot (200 µL) of semen (Appendix, p3).

At days 7, 11 and 20, an aliquot of semen was processed to isolate spermatozoa cell populations (90% fraction and swim-up fraction) according to previously published methods used for HIV-infected men (Appendix, p3).¹⁵ Seminal plasma and spermatozoa underwent ZIKV RNA analyses and viral isolation.

Urine and Blood Samples

Urine, whole blood and sera were collected in the morning and frozen until ZIKV reverse-transcriptase polymerase chain reaction (RT-PCR) assays.

Hormonal Analyses

Serum FSH, LH and testosterone levels were assessed by automated immunoassay (Cobas® 8000e602, Roche Diagnostics, Meylan, France). Serum inhibin B levels were quantified in duplicate using a manual ELISA assay (AnshLabs, Webster, USA) with a quantification limit of 4.6 pg/mL.

Virological Methods: ZIKA Virus Detection

RNA was extracted from whole blood, serum, urine and semen fractions with the MagNA Pure 96 instrument using the DNA and Viral NA Small Volume Kit (Roche Diagnostics, Meylan, France) (input and output volumes 200–100 µl). For semen cell fractions, input volumes were adjusted to 2×10^6 cells. ZIKV RNA was quantified using the RealStar Zika RNA RT-PCR kit 1.0 (Altona Diagnostics GmbH, Hamburg, Germany; limit of detection 2.48 log copies/mL). The manufacturer's internal control was systematically used to check for PCR inhibitors.

Virological Methods: ZIKA Virus Isolation

Samples from one patient (patient 13) positive for ZIKV RNA in seminal plasma and cell fractions, including motile spermatozoa, were tested for infectivity in Vero E6 cell cultures (Appendix, p3).

Serology

Anti-ZIKV IgG and IgM antibodies were detected using Diapro ZIKV IgG or IgM ELISA immunoassay (Launch Diagnostics, Longfield, England) according to the manufacturer's protocols. Results are expressed as signal to cut-off ratios (S/CO). Anti-DENV IgG and anti-CHIKV IgG antibodies were detected using Dengue virus IgG and CHIKV IgG ELISA immunoassays (Diapro, Diagnostic Bioprobes Srl, Milano, Italy). HIV and HTLV-1 screening tests were performed on Abbott Diagnostics Architect i2000SR.

Centralisation of Biological Specimens and Data Collection

All frozen samples were transferred to the GERMETHEQUE biobank (BB-0033-00081) and data from case report forms were centralised at Toulouse University Hospital.

Statistical Analysis

Data are presented as median and interquartile range Q1–Q3 due to the number of patients and as boxplot for graphic representation.

Data were compared between positive and negative ZIKV RNA samples (whole blood, serum and seminal plasma) using the nonparametric Mann-Whitney test for quantitative variables (semen parameters, sperm pellet). To study correlation between quantitative variables, Spearman's rank correlation coefficient was used.

Sperm characteristics were compared between day 7 and following days (days 11, 20, 30, 60, 90, 120) using the Wilcoxon signed rank sum test.

Hormone values were compared between day 7 and days 30, 60 and 120. As there was multiple comparisons, a Bonferroni correction was used and a p-value of 0.83% was considered significant for sperm characteristics and of 1.66% for hormone values.

Statistical analyses were performed using SAS software (9.3, SAS Institute). A p-value of 5% was considered significant in the case of no Bonferroni correction.

Role of the funding source: the sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Fifteen ZIKV-positive men (mean age \pm SD 35 ± 5 years, range 25–44) were included in the study. All patients were symptomatic (Appendix p7). They were followed prospectively for 120 days, except one patient who withdrew after day 30 visit (no reason was given). All were negative for HIV and HTLV-1 antibodies. Four participants provided further samples at day 150, and two at day 180.

ZIKV Serology

All patients developed an immune response against ZIKV. Anti-ZIKV IgM antibodies were detected as early as day 7 in 80% of patients and in all patients at day 20. After day 20, anti-ZIKV IgM appeared to decrease but with an average still above unity (Appendix p5).

DENV and CHIKV Serologies

Two patients have negative anti-DENV IgG sera: one (N°7) had no ZIKV in semen and the other (N°10) only semen ZIKV excretion at D7. 13 patients have anti-DENV IgG positive: 3 of these patients have no semen excretion of ZIKV (N° 1, 12, 14). Only patients 4, 6, 13, and 15 had their D7 serum reactive for anti-CHIV IgG

ZIKV RNA Detection

ZIKV RNA detection in the different body fluid samples during follow-up is presented in Figure 1, and viral load for each body fluid in Table S4 (Appendix, p10).

Serum

ZIKV RNA was detected in at least one serum sample in all patients. Thirty-six of 92 (39%) sera were ZIKV RNA-positive, maximum value 4.38 log copies/mL. Four patients still had positive serum samples at day 30.

Whole Blood

All volunteers but one had at least one ZIKV RNA-positive sample. Sixty-two of 92 (67%) samples were ZIKV RNA-positive (highest viral load: 4.70 log copies/mL). Three volunteers (23%) remained ZIKV RNA-positive at day 120.

Urine

All volunteers had at least one ZIKV RNA-positive urine specimen. Thirty-six of 91 (40%) urine specimens were ZIKV RNA-positive (highest viral load: 5.38 log copies/mL). Two urine samples tested positive at day 30.

Semen

Eleven men (73%) had at least one ZIKV RNA-positive seminal plasma or semen sample at day 7. In 4 patients (27%), ZIKV RNA was never detected in semen or its fractions.

Only one sample exhibited PCR inhibitors. Thirty-five samples (35%) were ZIKV RNA-positive (highest seminal viral load: 10.20 log copies/ml).

Of the 4 patients who provided seminal plasma after 120 days, one remained ZIKV RNA-positive at day 160 (3.40 log copies/ml).

ZIKV RNA was detected in 27 of 100 (27%) native semen cell pellets. The highest viral load in native semen cell pellets was 9.12 log copies/ 2×10^6 cells.

The results of ZIKV RNA detection in seminal plasma showed 3 different patterns of viral seminal shedding (Figure 2): A) non-shedding patients, with consistently negative ZIKV RNA detection in seminal plasma during follow-up (n=4, patients 1, 7, 12 and 14); B) seminal shedders with concomitant sera and/or urine shedding (n=6, patients 2, 3, 4, 8, 9 and 10); C) persistent seminal shedders after virus clearance in sera and

urines, i.e. discordant shedding patients (n=5, patients 5, 6, 11, 13, 15). Intermittence of seminal excretion was observed for 3 patients (patients 5, 11 and 15) from this last group.

Sperm Fractions

We isolated spermatozoa fractions at days 7, 11 and 20 in all semen samples except one (Appendix p9). Eleven 90% fractions (25%) containing only spermatozoa were ZIKV RNA-positive. All were from semen with high ZIKV RNA load in seminal plasma (>5 log copies/ml). All these positive 90% fractions were submitted to a swim-up method to isolate motile spermatozoa. Seven (64%) swim-up fractions later tested positive for ZIKV RNA (maximum 7.20 log copies/ 2×10^6 cells).

No sample was positive in cell fractions obtained after sperm preparation if native semen was negative. Seminal plasma was positive and semen cells of native semen were negative in 7 of 45 samples (15.5%), and seminal plasma was negative and native semen cells were positive in 2 of 45 samples (2%).

ZIKV Isolation

ZIKV was isolated from seminal plasma, 90% fractions (spermatozoa) and swim-up fractions (motile spermatozoa) in one volunteer (patient 13). Viral replication was observed in all these specimens, as assessed by cytopathic effect observation and measurement of increased ZIKV RNA levels on Vero E6 cell culture (Appendix p6).

Semen and Hormonal Characteristics

Semen characteristics are reported in Table 1 and Figure 3. Total sperm count (TSC) and total motile sperm count (TMSC) were significantly decreased (~50% lower) at day 60 compared with day 7. The multiple anomalies index (MAI) was already higher at day 30. At day 120, sperm characteristics were not different from day 7.

Compared with day 7, FSH level was higher at day 30 and decreased after this time with lower values at D60 and D120 than day 7. Lowest Inhibin β value was observed at D7 with a progressive increase over time. (Table 2). While LH values decreased after day 7, testosterone values were not significantly different while a trend toward lower values was observed at day 7.

When ZIKV RNA was positive in native semen (seminal plasma or semen cell pellets), sperm characteristics tended to have significantly lower values compared with ZIKV RNA-negative semen (Appendix p8).

Discussion

This prospective study is the first to: 1) perform longitudinal assessment of different biological samples (whole blood, urine and semen) jointly with the investigation of semen characteristics and reproductive hormones following acute ZIKV infection, and 2) determine the detection and clearance of ZIKV RNA in different semen fractions and the presence of replicative virus (including motile spermatozoa fractions, generally used in assisted reproductive procedures).

Fifteen symptomatic patients provided the first samples at day 7 after clinical onset. All patients showed an immune response to ZIKV since IgM antibodies were systematically detected 7–20 days after clinical onset. All but two are immunized against DENV.

Serum and urine samples were ZIKV RNA-positive for all patients at day 7 after clinical onset. In agreement with Paz-Bailey *et al.*,¹⁶ ZIKV RNA detection was more frequent in serum than in urine during follow-up. Two urine samples and four sera were ZIKV RNA-positive at day 30 but none after. Notably, ZIKV RNA was detected in whole blood in all patients except one. This whole blood-negative patient showed only brief and low viremia/viruria without viral excretion in semen. In our study, whole blood was more frequently ZIKV RNA-positive than urine or sera, and remained so for longer periods (up to 120 days). Similarly, Murray *et al.* detected ZIKV RNA in erythrocytes after infection.¹⁷ We confirm the higher sensitivity of ZIKV RNA detection in whole blood compared with urine or serum compartments.¹⁸ This should probably have an impact on the recommendations of the samples to be used for molecular detection of ZIKV RNA detection, whether for diagnosis of an acute infection or for detection of the virus in a biological product of human origin.

Several case reports have described ZIKV shedding in semen from symptomatic and asymptomatic men, and sexual transmission of the virus has also been reported. Sexual transmission is a major concern, particularly for pregnant women and couples wishing to conceive, because of the adverse effects of the virus during pregnancy. However, very few cohort studies have analysed the persistence and clearance of ZIKV in semen.^{16,19} The majority (73%) of our ZIKV-infected patients excreted the virus in semen seven days after symptom onset. Our prevalence of patients with ZIKV-positive semen appears higher than in other studies.^{16,19} These differences could be explained by the fact that in our study, unlike other studies, ZIKV RNA was detected both in seminal plasma and in semen cells. This leads to increased detection in semen samples, and consequently is a better reflection of the presence of ZIKV in semen. According to ZIKV RNA seminal shedding, we defined three patient patterns: non-shedding patients, with consistently negative ZIKV RNA detection in seminal plasma during follow-up (27%), seminal shedders with concomitant blood and/or urine shedding (40%) and discordant shedder patients with persistent seminal shedding after virus clearance in sera and urines (33%).

The causes of these different patterns need further investigation, as several hypotheses could be suggested: differences in viral seeding and local replication within genital organs and cells, differences in local host innate and adaptive immune defences, and/or differences in virus strain tropism (although all patients came from the same localised area and were recruited over a short period of time). Thus specific male organs/cells could act as mid or long-term reservoirs for ZIKV once infected, as has been reported for a number of systemic viruses.^{20,21} Our results confirm that seminal viral loads can be much higher than blood and urine viral loads.⁵

The precise replication site of ZIKV in semen is currently unknown in man. The fact that vasectomised men^{22,23} can shed ZIKV in semen suggests that male genital organs distal from the testis could contribute to viral production. The persistence of ZIKV-infected cells in the testis, seminal vesicles and prostate of non-human primates with cleared viremia has also recently been reported.⁸ The haemospermia reported during acute ZIKV infection^{24, 25} could suggest local genital tract infection.

Density gradient centrifugation and swim-up methods are effective in obtaining HIV- or HCV-free spermatozoa populations.¹⁵ In some of our patients, high ZIKV RNA loads were still detected in the swim-up fraction, which contains only motile spermatozoa. This could reflect ZIKV adherence to spermatozoa or their infection, although technical contamination of the preparation due to the high viral load in semen cannot totally be ruled out, as reported for HIV virus.²⁶ In support of ZIKV interaction with sperm cells, we previously detected a ZIKV antigen in spermatozoa from an infected patient.¹² The fact that we were able to rescue replication-competent virus from all fractions, including the swim-up fraction of motile spermatozoa, argues against contamination with genomic RNA or defective virus particles.

To date, the effect of acute ZIKV infection on semen and hormonal characteristics in men has not been studied. Studies in mouse models described drastic effects such as orchitis and epididymitis, following systemic infection of immune-compromised animals.^{10,11} In non-human-primates, foci of ZIKV-infected cells were localised in the testes, prostate and seminal vesicles.⁸ We studied sperm characteristics over time following ZIKV infection. As early as 30 days post symptom onset, the multiple anomalies index increased, the median percentage of normal forms was significantly decreased at day 90, and recovery of both parameters was observed at day 120. The lowest median values of TSC and

TMSC were found at day 30, with statistical significance at day 60 after clinical onset. Recovery was seen at day 90-120. Soon after ZIKV symptom onset, FSH levels were higher and inhibin levels lower than at day 120, and this could be related to an initial direct or indirect impact of infection on Sertoli cell function. This could be associated with subtle early Leydig cell dysfunction as there was a trend for low testosterone values and significantly higher LH at day 7. This is concordant with the decreased inhibin and testosterone levels observed in testis homogenates but from ZIKV-infected immune-deficient mice at 14 and 21 days of ZIKV inoculation.^{10,27} Early sperm alterations could result from viral infection of the epididymis with a direct or indirect effect on spermatozoa during epididymal transit (mean duration: 12 days) and/or testis infection affecting late spermiogenesis (spermiogenesis duration: 23 days). The sperm modifications may also be related to fever effect. This has previously been reported, but after high fever.^{28,29} However, while the majority of our patients reported subjective fever, pyrexia during ZIKV infection is usually moderate. Moreover, we found that semen characteristics were more altered in seminal plasma samples with positive viral loads than in those with undetectable loads. This suggests that ZIKV infection by itself could probably be directly responsible for sperm alterations, although further studies with a large number of men are needed to confirm our results.

However further studies are required to confirm these results as one limitation of this study was the absence of a control group, i.e. non-infected men, for the study of semen and hormonal characteristics.

Studies, particularly on ZIKV-infected and asymptomatic men (i.e. without fever) including also a control group, as well as in relevant animal or *ex vivo* human models, are needed in order to decipher the origins of sperm alterations and to investigate the infectivity and functions of genital glands following ZIKV infection.^{5,30}

In conclusion, this study provides a longitudinal assessment of the detection and clearance of ZIKV in different body compartments. We show that ZIKV RNA is detected in whole blood longer than in serum and urine. We describe three different patterns of ZIKV excretion in semen according to their viral excretion in semen, blood and urine. Importantly, we show that ZIKV infection modifies semen characteristics in men and that replication-competent virus can be isolated from motile spermatozoa. Although our knowledge of ZIKV infection and the reproductive tract is incomplete, these findings have implications for public health policy, contributing to increase diagnostic efficiency and limit sexual transmission of ZIKV, as well as guiding counselling of ZIKV-infected patients and couples who wish for a child.

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Conflicts of interests: The authors declare that they have no conflicts of interest.

Contributors: LB, CP, GJ performed the literature search and designed the study. LP, GJ, PL recruited the volunteers. LP, NP and GJ performed clinical and biological follow-up of the volunteers, SG performed the first biological ZIKV diagnosis tests for volunteer inclusion, JMM and CP performed all virological and immune analyses, GM and NDR performed virus isolation, SH performed hormonal investigations, MW carried out all data treatment, statistical analyses and designed the article figures. GJ, JMM, GM, MW, LP, NP, NDR, CP and LB participated in interpretation of the data and writing the discussion. All the authors have approved the manuscript. The study protocol and manuscript writing was coordinated by LB.

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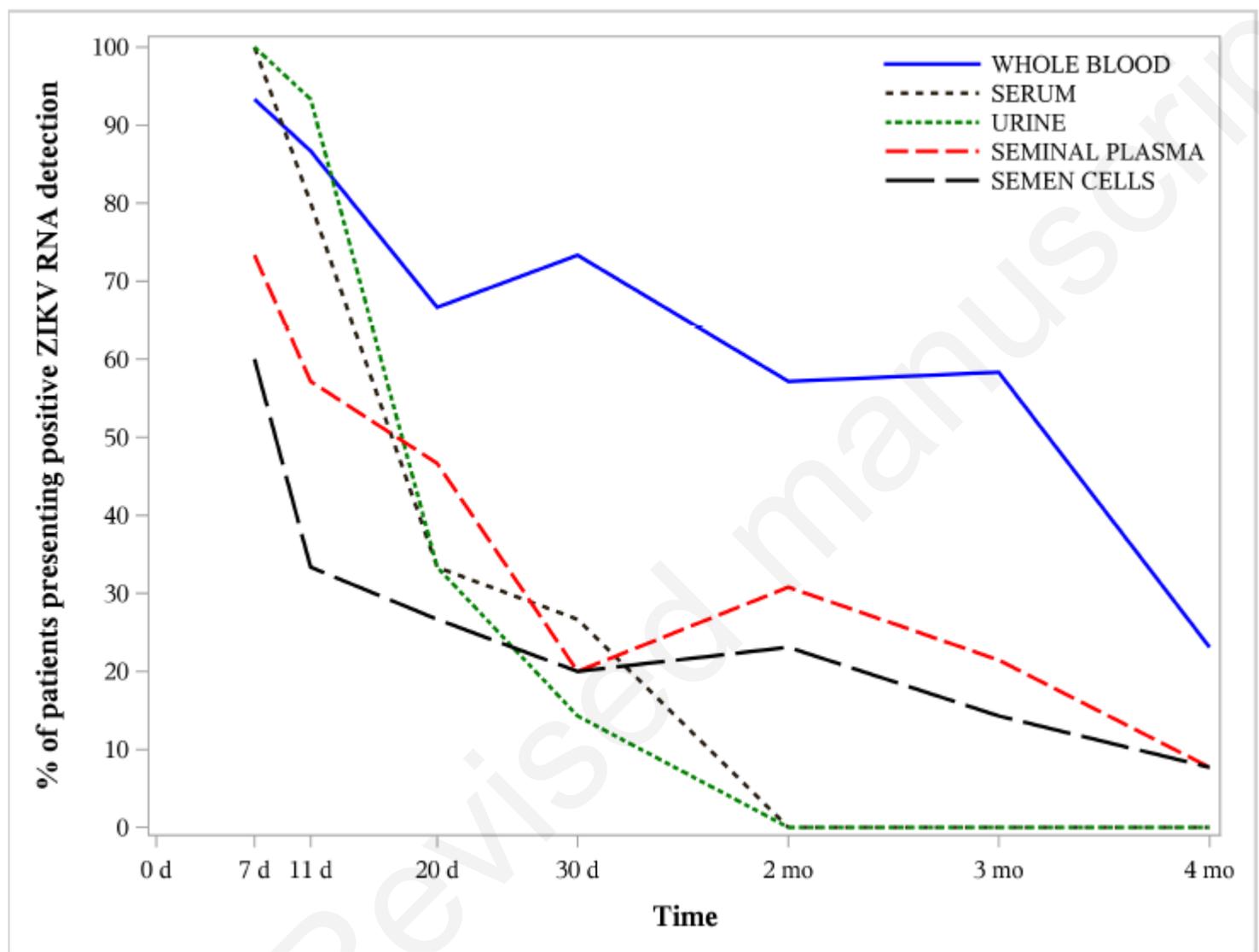


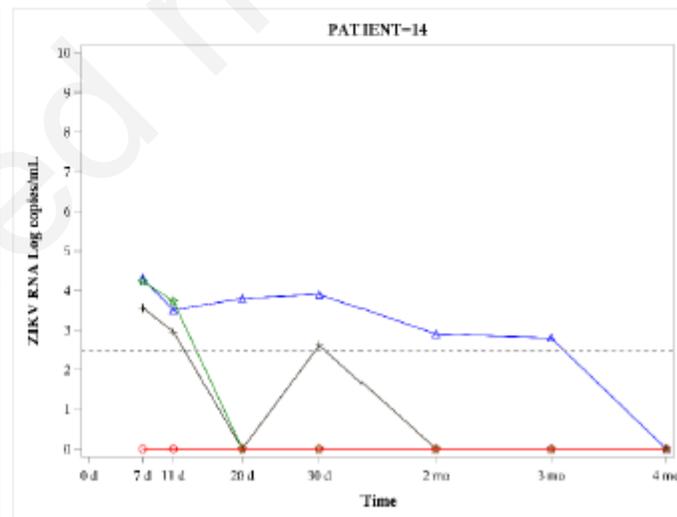
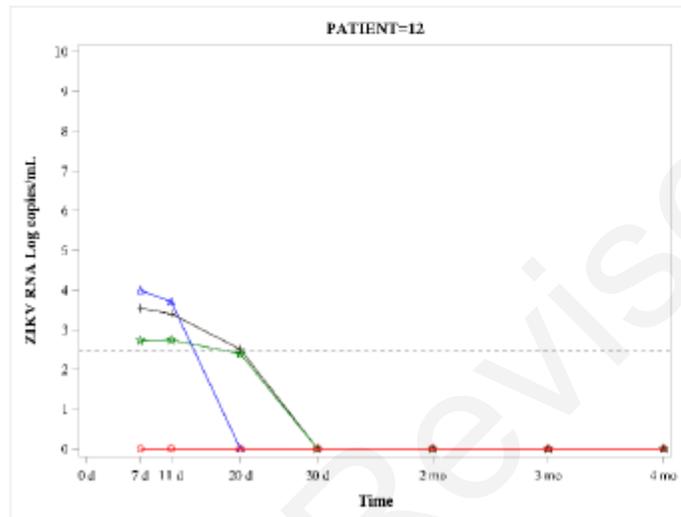
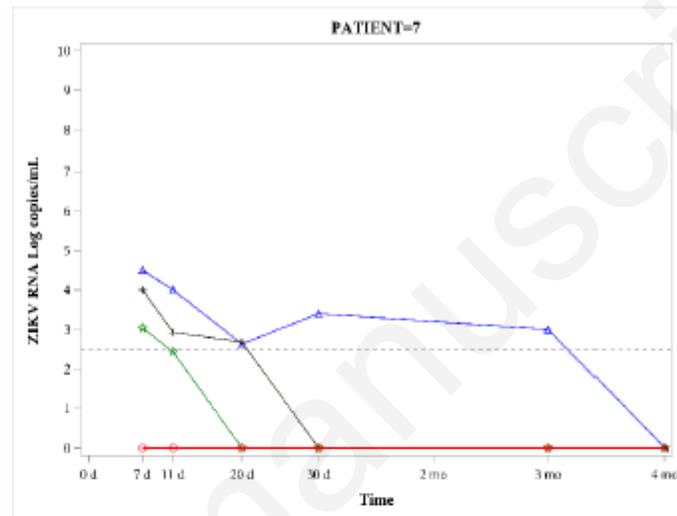
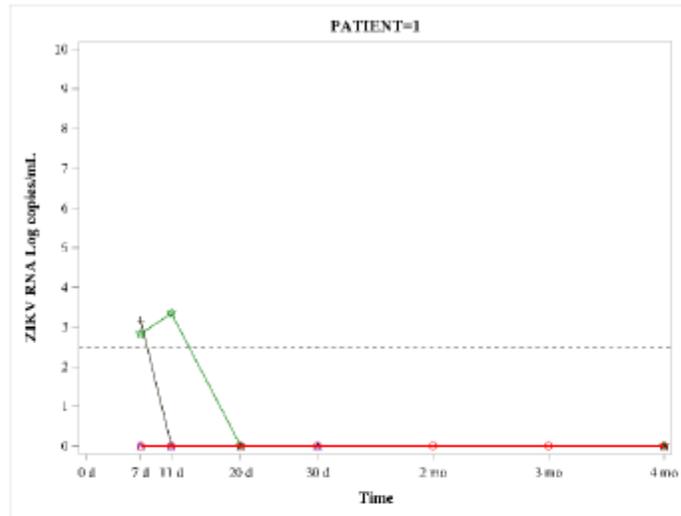
Figure 1. Frequency of ZIKV RNA Detection in the Different Fluids According to Time Points after ZIKV Infection Symptoms Onset in 15

Patients.

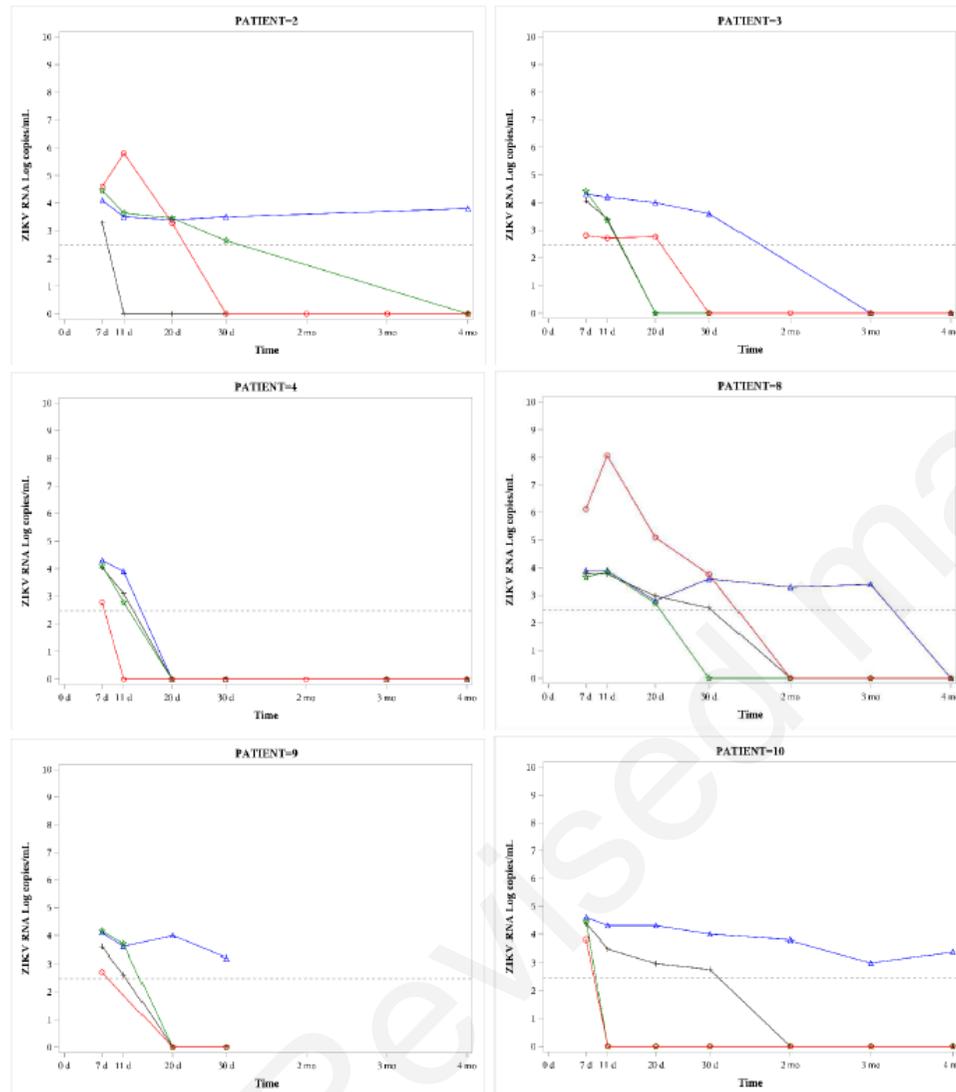
Blue line: whole blood; deep brown medium dash: serum; green short dash: urine; red medium dash: seminal plasma; black large dash: semen cells. d=days; mo=months.

Revised manuscript

A



B



C

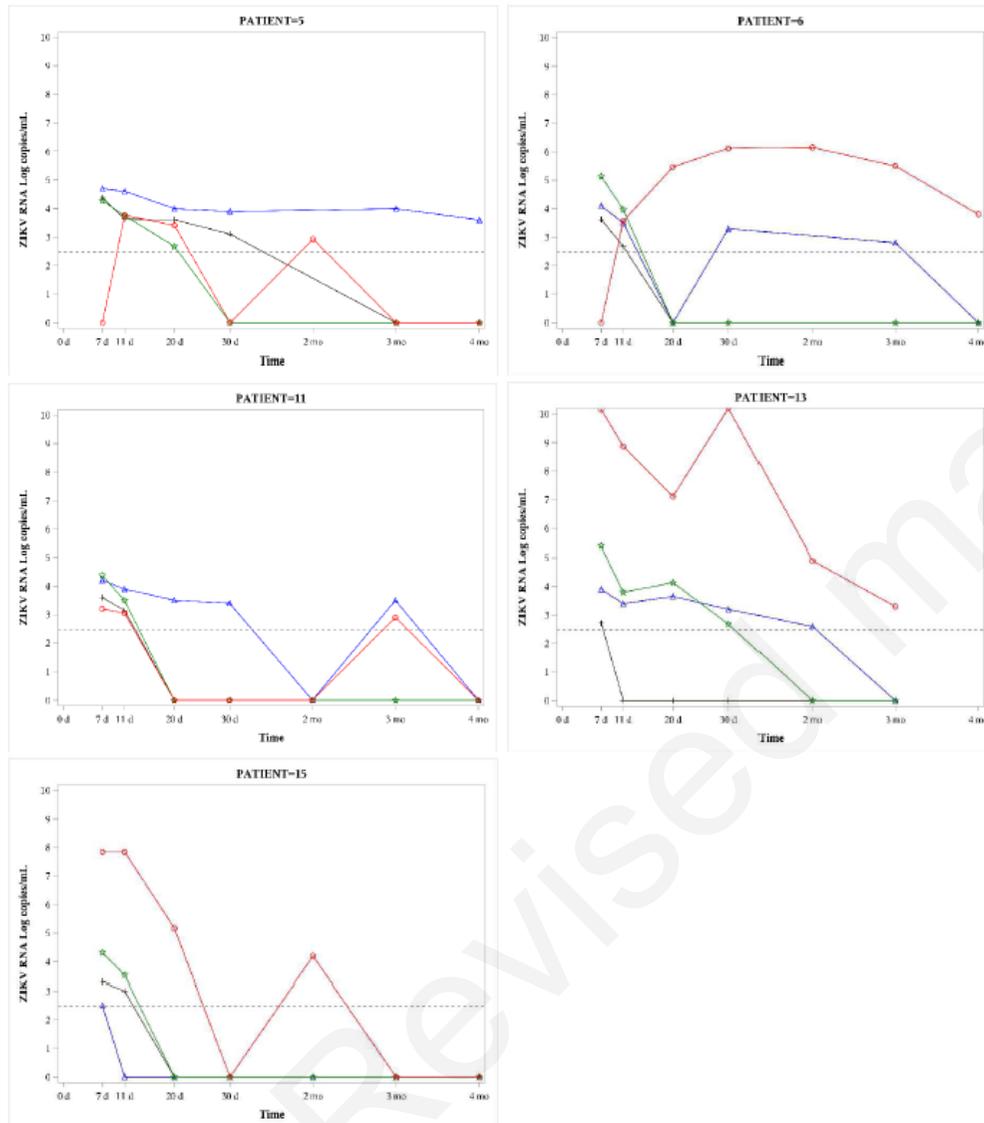


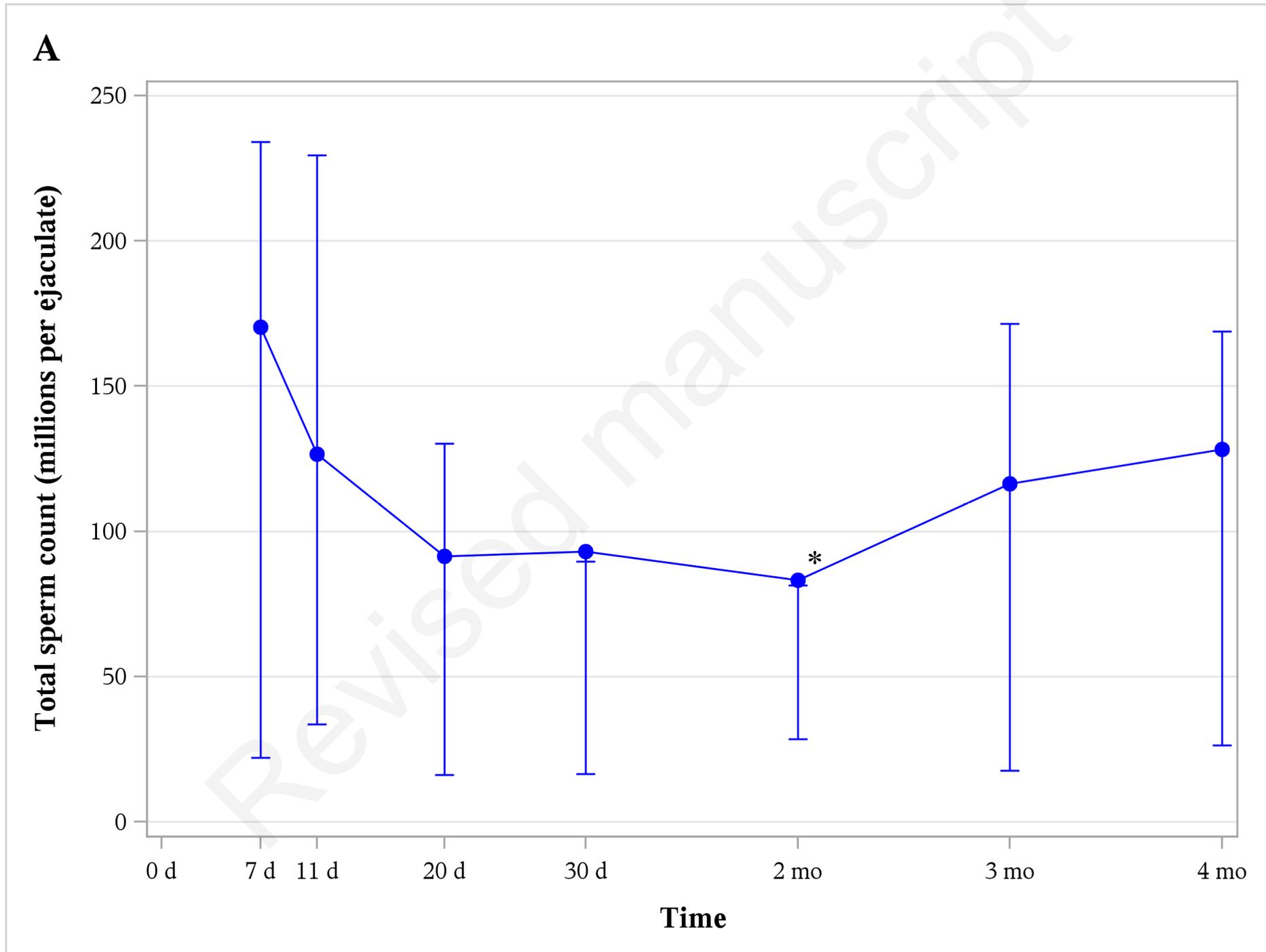
Figure 2. ZIKA viral load (ZIKV RNA log copies/mL) in the Fluids Studied for All 15 Patients.

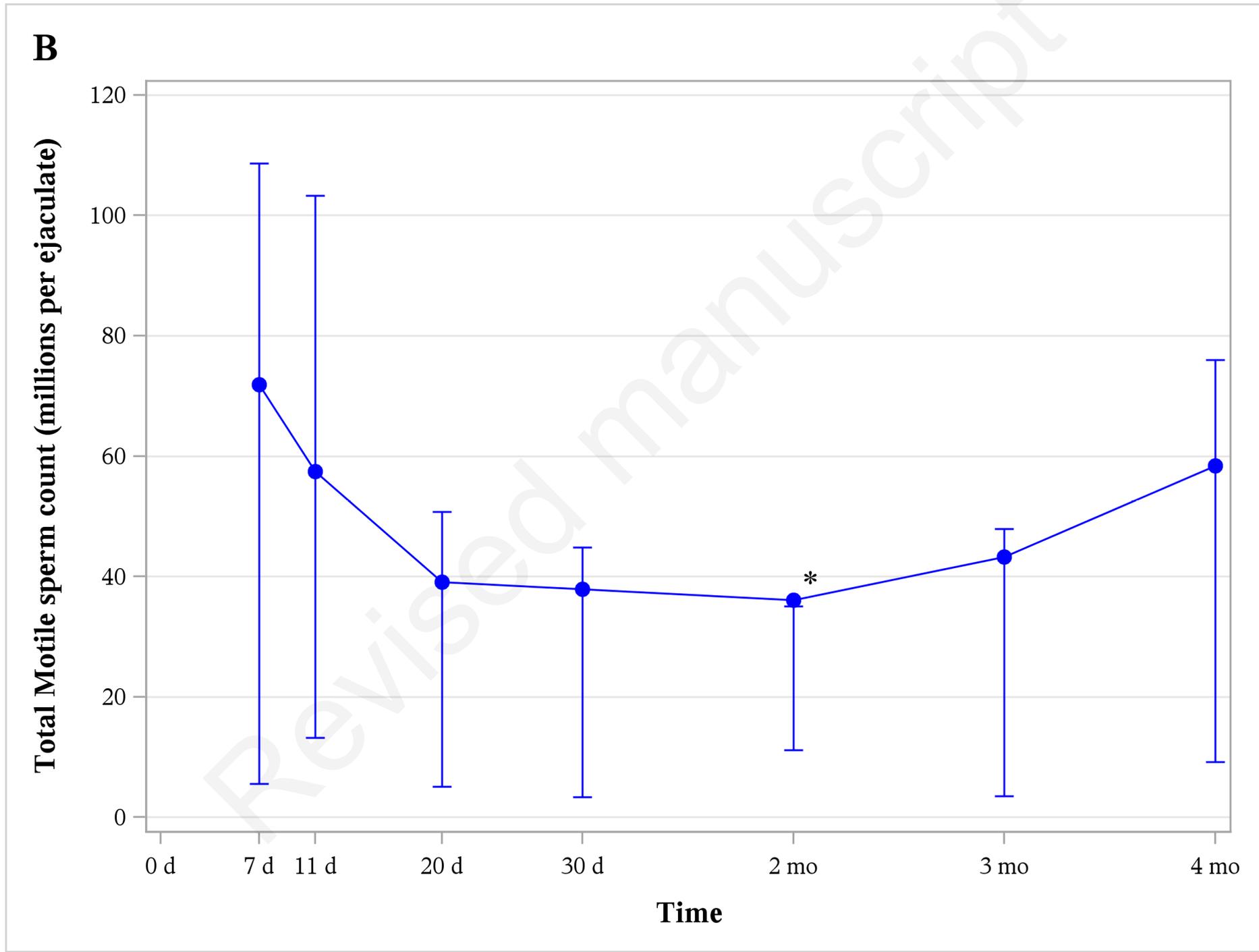
According to the seminal viral load, three patterns were identified: A. Non-shedding patients (patients #1, #7, #12, #14); B. Seminal shedders with concomitant blood and/or urines shedding (patients #2, #3, #4, #8, #9, #10); C. Long-term seminal shedding patients discordant with urine/blood shedding (patients #5, #6, #11, #13, #15).

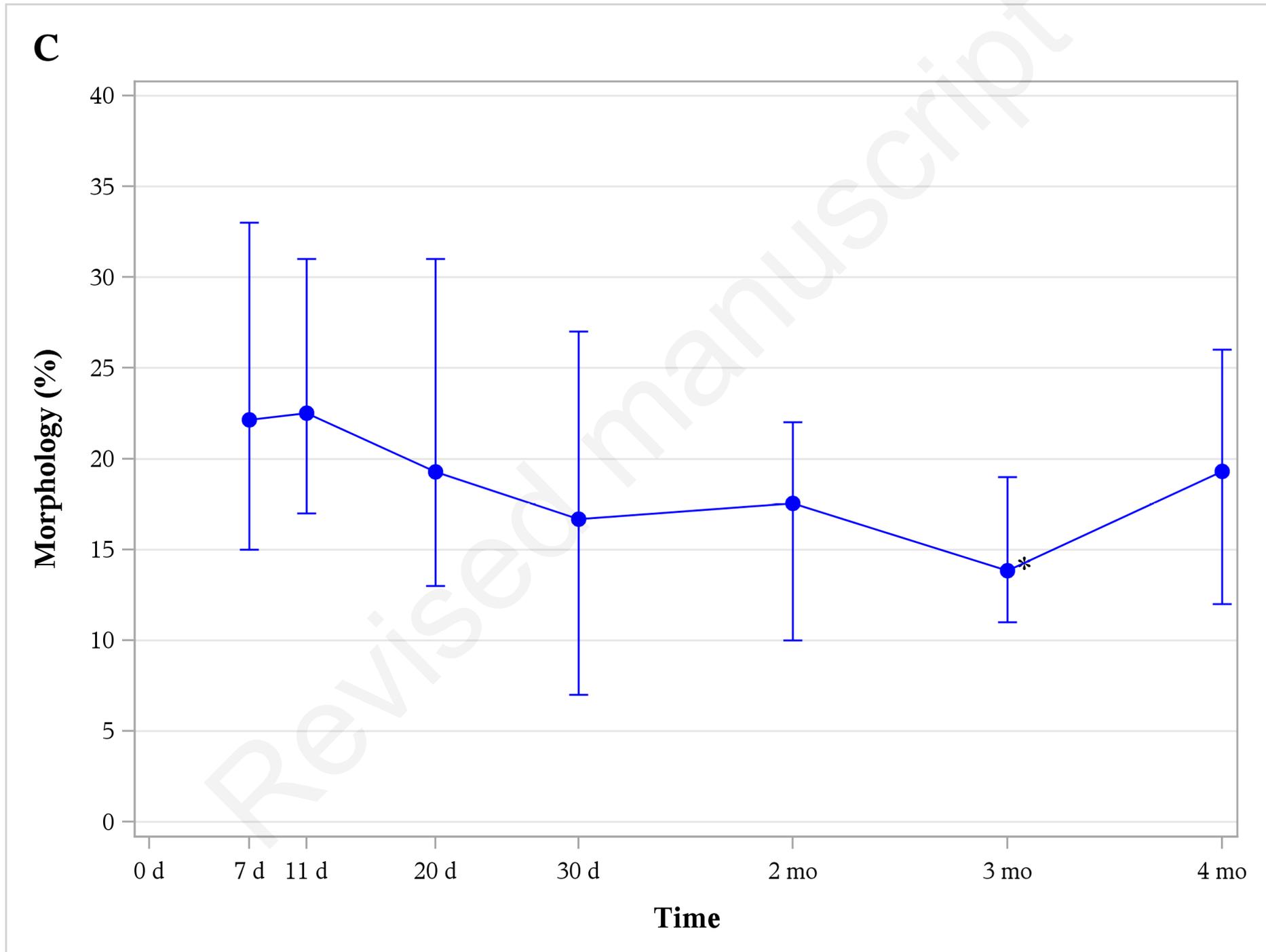
Blue: whole blood; deep brown: serum; green: urine; red: seminal plasma.

The dotted line represents the detection limit of the kit (2.48 log copies/mL).

d=days; mo=months







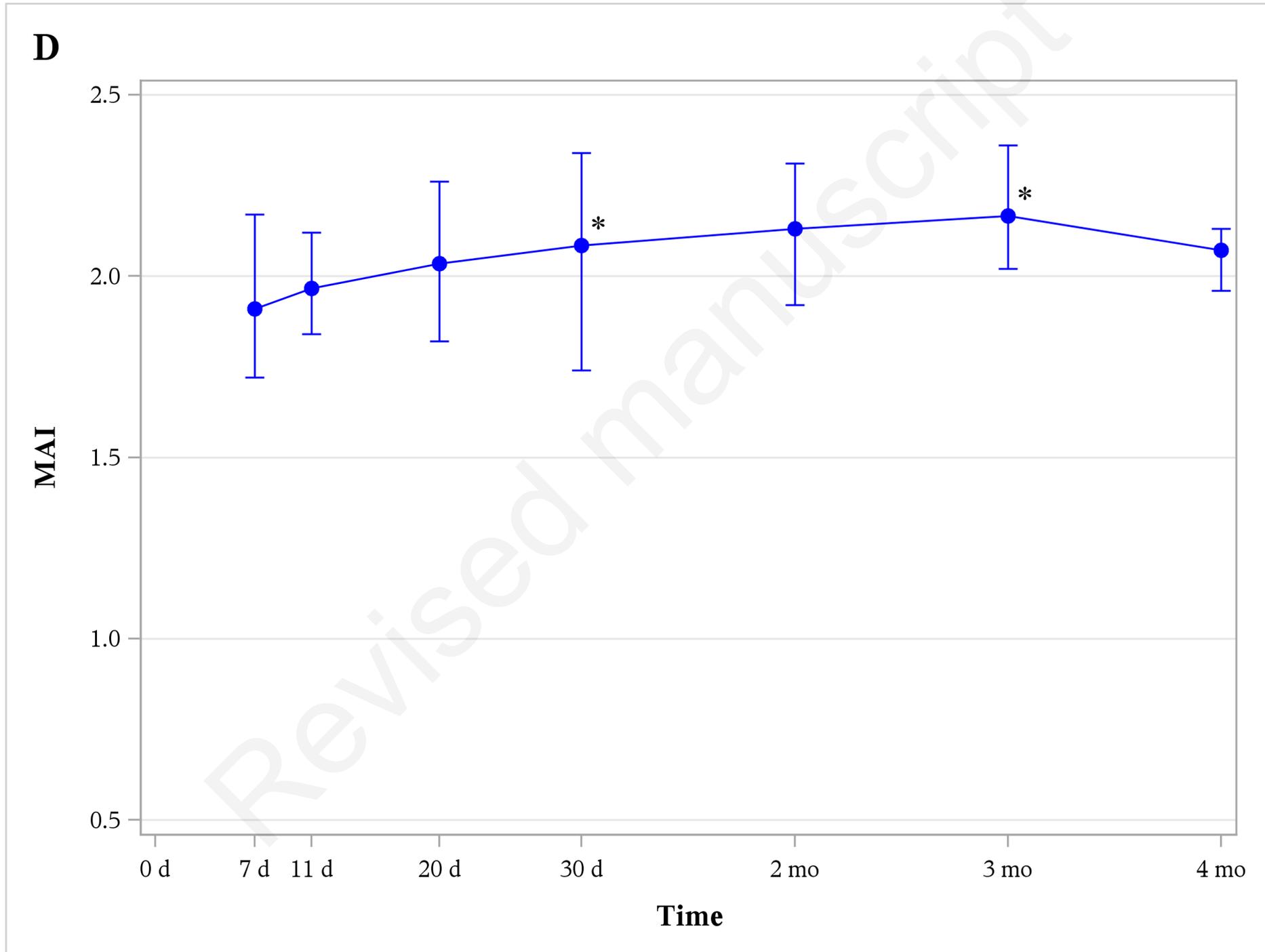


Figure 3. Semen Characteristics of the 15 ZIKV-infected Patients According to Time Points after Infection Symptom Onset.

A. Total sperm count (millions per ejaculate); B. Total motile sperm count (millions per ejaculate); C. Normal sperm (%); D. Multiple anomalies index (MAI).

Data are presented as median [q1–q3] boxplot (median is represented as the line under the boxplot, the mean as the diamond, q1 and q3 as the border of the box and circles represent the outliers)

* $p < 0.083$ between D7 and D11, 20, 30, 60, 90 or 120.

d=days; mo=months.

Table 1. Semen Characteristics of the 15 ZIKV-infected Patients According to Time Points After Infection Symptom Onset*

	Median [Q1–Q3]						
	D 7 n=15	D 11 n=15	D 20 n=15	D 30 n=15	D 60 (2 mo) n=13	D 90 (3 mo) n=14	D 120 (4 mo) n=13
Volume (mL)	2.75 [1.9–4]	2.9 [2–3.6]	2.6 [1.7–4.2]	2.8 [2–4.2]	2.5 [1.9–4]	2.7 [1.7–4]	3.35 [2–4]
Sperm count (millions/mL)	44 [11.5–83]	26.6 [14–63]	14.9 [11.5–47]	13 [5–37]	20.2 [11.5–37]*	21 [7.7–61.5]	31.5 [14.5–67.5]
Total sperm count (millions per ejaculate)	119 [22–234]	52 [33.6–229.5]	80.46 [16.1–130.2]	45.2 [16.5–89.6]	70 [28.5–81.4]*	44.925 [17.6–171.5]	86.9 [26.32–168.75]
Total motile sperm count (millions per ejaculate)	49.01 [5.5–108.61]	19.89 [13.2–103.28]	36.21 [5.04–50.72]	10.12 [3.3–44.8]	24.42 [11.16–35]*	13.81 [3.52–47.88]	34.76 [9.135–75.9375]
Motility (%)	35 [35–45]	40 [35–45]	40 [30–45]	35 [20–45]	40 [30–50]	40 [20–42]	40 [35–45]
Vitality (%)	60 [56–73]	61 [55–66]	62 [51–66]	54 [49–63]	64 [61–69]	54.5 [47–62]	67 [53–70]
Normal sperm (%)	21 [15–33]	20 [17–31]	16 [13–31]	13 [7–27]	16 [10–22]	13 [11–19]*	20 [12–26]
MAI	1.85 [1.72–2.17]	1.995 [1.84–2.12]	2.06 [1.82–2.26]	2.08 [1.74–2.34]*	2.17 [1.92–2.31]	2.22 [2.02–2.36]*	2.09 [1.96–2.13]

Table 2. Hormone Values in Sera of ZIKV-Infected Patients According to Time Points after Infection**Symptom Onset.**

	Median [Q1–Q3]							
	D 7		D 30		D 60 (2 mo)		D 120 (4 mo)	
	(n=14)		(n=8)		(n=7)		(n=13)	
FSH (IU/L)	4.45	[3.6–7.8]	5.85	[2.75–8.75]*	3.3	[2.1–4]*	3.7	[3–5.4]*
LH (IU/L)	6.95	[4.9–7.5]	4.9	[3.55–5.55]*	5	[3.2–6.1]	4.4	[3.2–5.5]*
Inhibin (pg/mL)	93.5	[55–162]	125.5	[53–196.5]*	144	[70–220]*	150	[78–209]*
Testosterone (ng/dL)	344.5	[246–477]	517	[338–543]	515	[365–591]	415	[300–460]

* $p < 0.0166$ between D7 and D 30, 60 or 120

D=days; mo=months