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Reconciling the first epidemiological findings following emerging disease events : examples from Zika and Hepatitis C

Anna Funk

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**THESE DE DOCTORAT DE
SORBONNE UNIVERSITE**

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L'INFORMATION BIOMEDICALE

Présentée par

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Pour obtenir le grade de

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Reconciling the first epidemiological findings following emerging disease events: examples
from Zika and Hepatitis C

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To my mom and dad,

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Preface

I made the decision to do this thesis while on a field epidemiology mission with Médecins sans Frontières in the Democratic Republic of the Congo. A PhD thesis based in Paris, with occasional travel to Cairo to work on a large cohort study, was an attractive and secure situation in comparison to my leaking hut and work/personal encounters with parasites in South Kivu.

Over the first year and a half, I made many trips back and forth to Cairo, setting up a cohort study for 7500 persons with hepatitis C receiving direct acting antivirals. In January 2016, on the cusp of enrolling the first patients, the entire project was halted for reasons outside of our control. This timing coincided with the emergence of Zika virus as a non-benign threat in the Americas, and the subsequent involvement of my supervisor, and then myself, in the pregnant women and infant cohort studies in the Caribbean. We also obtained funding for a Zika-related microcephaly surveillance study, for which I became the coordinator, in sub-Saharan Africa and Asia. Originally this study was in six countries, including: Thailand, Vietnam, China, Sri Lanka, Cameroon, and Ivory Coast.

So, rather than the stable and settled situation I had imagined... four years, three full-passports, and two thesis topics later, I present you with a story of many scientific (and just as many diplomatic) lessons learned. For me, it has been fascinating, and I hope you will agree.

Abstract

Initial epidemiological investigations following emerging disease events sometimes uncover spurious associations and lead to misinterpretations of eventual disease severity. This may occur due to poorly understood biological mechanisms and infection dynamics, or inappropriate study methods and case definitions. In this thesis, this phenomenon will be discussed for two recent examples, including the estimation of Zika-related birth defects and evaluations of the introduction of direct acting antivirals for hepatitis C in Egypt.

In the French Territories of the Americas, we estimated the risk of Zika-related birth defects to be 7% (95%CI: 5.0%-9.5%) through follow-up a prospective cohort of 546 infected pregnant women. When a subset of this cohort was compared to a control group of non-exposed pregnancies from the same region, our estimate of the risk of birth defects attributable to Zika virus decreased to 1.6% (95%CI: 0.4-4.1%). In addition, in a surveillance study in four cities in sub-Saharan Africa and Asia, we found high, and regionally variable, proportions of microcephaly when using a standardised definition - from 3% in China to 35% in Ivory Coast. Difficulties in reconciling our findings with other contemporary estimates on Zika-related birth defects are likely due to variation in study procedures, a lack of appropriate control groups, and use of problematic definitions for key conditions, such as microcephaly.

In Egypt, highly effective direct acting antivirals for hepatitis C virus were introduced on a large scale in 2014. Our research, conducted through 'real-life' cohort studies, found a strong association between liver cancer recurrence and treatment with these new regimens, as well as a non-negligible risk of hepatitis B reactivation following therapy for persons co-infected with both viruses. In comparing these findings with those of other groups outside of Egypt, we encountered difficulties in drawing conclusions due to variability in research methods as well as in the definitions used for the adverse events.

Following an emerging disease event, research priorities and hypotheses can be focused using techniques such as expert opinion elicitation, as well as collaboration through diverse research networks. Transparency in reporting, as well as use of standardised protocols and case definitions will ameliorate delays in drawing a consensus from initial findings.

Resumé

Les enquêtes épidémiologiques initiales effectuées à la suite d'événements épidémiques émergents révèlent parfois des associations fallacieuses et conduisent à des interprétations erronées de la gravité éventuelle de la maladie. Cela peut être dû à des mécanismes biologiques et à une dynamique d'infection mal compris, ou à des méthodes d'étude et à des définitions de cas inappropriées. Dans cette thèse, je discuterai ce phénomène avec deux exemples récents, à savoir l'estimation des anomalies congénitales liées au virus Zika et les évaluations de l'introduction d'antiviraux à action directe contre l'hépatite C en Égypte.

Dans les Districts Français d'Amérique, nous avons estimé le risque d'anomalies congénitales liées au virus Zika à 7% (IC 95%: 5,0-9,5%) grâce au suivi d'une cohorte prospective de 546 femmes enceintes infectées. Lorsqu'un sous-groupe de cette cohorte a été comparé à un groupe témoin de grossesses non exposées de la même région, notre estimation du risque de malformations congénitales imputable au virus Zika a diminué à 1,6% (IC 95%: 0,4-4,1%). En outre, une étude de surveillance menée dans quatre villes d'Afrique subsaharienne et d'Asie et basée sur une définition standardisée de la microcéphalie, a révélé des proportions élevées, et variables d'une région à l'autre, de cette condition - allant de 3% en Chine à 35% en Côte d'Ivoire. Nos difficultés pour réconcilier ces résultats avec d'autres estimations contemporaines des malformations congénitales liées au virus Zika est probablement due aux différences entre les schémas d'étude utilisés, à l'absence de groupes contrôles appropriés, et à l'utilisation de définitions de cas discutables pour des affections clés telles que la microcéphalie.

En Égypte, des antiviraux à action directe très efficaces contre le virus de l'hépatite C ont été introduits à grande échelle en 2014. Nos recherches, menées dans le cadre d'études de cohortes «en vie réelle», ont mis en évidence un lien étroit entre la récurrence du cancer du foie et le traitement par ces nouvelles thérapies, ainsi qu'un risque non négligeable de réactivation de l'hépatite B après le traitement pour les personnes co-infectées par les deux virus. En comparant ces résultats avec ceux d'autres groupes situés en dehors de l'Égypte, nous avons eu des difficultés à tirer des conclusions en raison de la variabilité des méthodes de recherche ainsi que des définitions utilisées pour les effets indésirables.

Après une maladie émergente, les priorités et hypothèses de recherche peuvent être ciblées à l'aide de techniques telles que la collecte d'opinions d'experts et la collaboration via divers réseaux de recherche. La transparence dans les publications, de même que l'utilisation de protocoles standardisés et de définitions de cas, permettra de réduire les délais d'obtention d'un consensus sur les premières conclusions.

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List of Abbreviations

AASLD	American Association for the Study of Liver Disease
AHP	analytical hierarchy process
AIDP	acute inflammatory demyelinating polyradiculoneuropathy
AIDS	acquired immune deficiency syndrome
AMAN	acute motor axonal neuropathy
anti-HBC	hepatitis B core antigen antibodies
anti-HBV	hepatitis B virus antibodies
anti-HCV	hepatitis C virus antibodies
ANRS	Agence nationale de recherche sur le Sida et les hépatites virales
APASL	Asian Pacific Association for the Study of the Liver
APGAR	Appearance, Pulse, Grimace, Activity, and Respiration
BCLC	Barcelona Clinic Liver Cancer stages
CDC	Centers for Disease Control and Prevention
CFR	case fatality risk (or ratio, or rate)
CI	confidence interval
CIC	Centre d'Investigation Clinique
CIRAD	International Centre for Cooperation in Agronomical Research for Development
CJD	sporadic Creutzfeldt-Jakob's disease
CMV	cytomegalovirus
CNS	central nervous system
CONSISE	Consortium for the Standardization of Influenza Seroepidemiology
CONSORT	CONsolidated Standards Of Reporting Trials
CRF	case report form
CT	computed tomography
CW	camel worker
CZS	congenital Zika syndrome
DAA	direct acting antiviral
DENV	dengue virus
DHS	demographic health survey
DNA	deoxyribonucleic acid
EASL	European Association for the Study of the Liver
ECDC	European Center for Disease Control and Prevention
ECLAMC	Latin American Collaborative Study of Congenital Malformations
EDHS	Egyptian Demographic Health Survey
EHIS	Egyptian Health Information Survey
EIA (#)	enzyme immunosorbent assay (if followed by a #, indicates generation)
ELISA	enzyme-linked immunosorbent assay

EOE	expert opinion elicitation
EOT	end of treatment
EUROCAT	European surveillance of congenital anomalies network
F (#)	fibrosis level (the # indicates which level)
FTA	French Territories in the Americas
G (#)	genotype (the # indicates which genotype)
GBS	Guillain-Barré syndrome
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCZ	head circumference Z-score
HI	hemagglutination inhibition assay
HIV	human immunodeficiency virus
IBM	individual-based modelling or multi-agent systems
IFN	interferon
IgM	immunoglobulin M
IgG	immunoglobulin M
IL28B	interleukin 28 B
INSERM	Institut national de la santé et de la recherche médicale
INTERGROWTH-21st	International Fetal and Newborn Growth Consortium for the 21 st century
IPTW	inverse probability of treatment weighting
IRR	incidence rate ratio
ISARIC	International Severe Acute Respiratory and Emerging Infection Consortium
MCDA	multi-criteria decision analysis
MoHP	Ministry of Health and Population
MSM	men who have sex with men
mRNA	messenger ribonucleic acid
MWA	microwave ablation
NANB	non-A non-B hepatitis
NCCVH	National Committee for the Control of Viral Hepatitis
NICHD	National Institute for Child Health and Human Development
PCR -	polymerase chain reaction
peg-IFN	pegylated interferon
PEI	percutaneous ethanol injections
PHEIC	Public Health Emergency of International Concern
PM	person months
PRNT	plaque reduction neutralization test

QRA	quantitative risk assessment
RBV-	ribavirin
RECIST -	response evaluation criteria in solid tumors
RFA	radiofrequency ablation
RM	recruitment method
RNA	ribonucleic acid
RT-PCR	real-time polymerase chain reaction
SARS	severe acute respiratory syndrome
SD	standard deviation
S(E)IR	susceptible (exposed) infectious recovered compartmental models
SNA	social network analysis or contact network analysis
SVR (#)	sustained virological response (# indicates number of weeks post treatment)
TACE	transarterial chemoembolization
TOP	termination of pregnancy
TORCH	toxoplasmosis, other, rubella, cytomegalovirus, HIV
UI	international units
UK	United Kingdom
USD	United States dollars
USZIPR	United States Zika Infant and Pregnancy Registry
vCJD	variant Creutzfeldt-Jakob's disease
WHO	World Health Organization
ZIKV	Zika virus
ZIKA-DFa-FE	Zika - Districts Français d'Amérique - Femmes Enceintes
ZIKA-DFa-BB	Zika - Districts Français d'Amérique - Bébé

**Part 1. Epidemiological investigations of emerging
infectious diseases**

1.1 Epidemiological investigations of emerging disease events

An emerging infectious disease is commonly defined as illness due to a newly discovered pathogen or one for whom the number of persons infected has increased over the past twenty years or so. This can therefore include newly identified pathogens, those that have recently started affecting different populations, and old infections that are re-emerging as threats due to changes in the environment or because of drug resistance (CDC, 1994; Morse and Schluenderberg, 1990; Rosenthal et al., 2015). In this thesis manuscript I will be discussing epidemiological investigations surrounding emerging disease events. While the term ‘event’ has previously been used to describe the origin and identification of a novel emerging diseases (Jones et al., 2008), in this manuscript I will expand this definition to also include occurrences that change drastically the forecast, control, and understanding of an emerging disease. The recent spread of Zika virus (ZIKV) into the Americas and realization that infection can cause severe neurological manifestations in infants (i.e. birth defects) and in adults (i.e. Guillain-Barre syndrome) conforms to the traditional definition of an emerging disease event. The introduction of highly effective direct acting antivirals for the treatment of chronic Hepatitis C virus (HCV) infection, a disease identified approximately 30 years ago and for which worldwide spread dates back from the 1960s, will adhere to my expanded terminology for an ‘event’.

Following an emerging disease event, it can understandably take time to tease out important disease parameters that are key to understand in order to properly implement control measures. Depending on the type of infectious agent, the delay by which we have been able to identify the culprit behind each emerging disease has varied. It took more than 10 years to isolate the cause of non-A non-B hepatitis (i.e. HCV) (Alter, 1999), two years to identify the agent (i.e. human immune deficiency virus (HIV)) responsible for acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983), and only two months to identify the novel coronavirus behind Severe Acute Respiratory Syndrome (SARS) (Drosten et al., 2003). The full range of clinical symptoms related to an infectious illness, and the side effects of the personal or environmental treatments we use to control them, may equally take time to unravel. An example of this is the greater than 50 year delay in recognition of the severe neurological manifestations linked to ZIKV infection, as well as the fact that, for more than

five years around the early 1990s, early antiviral regimens for HIV were administered with little or no benefit to persons who had not yet progressed to AIDS (Concorde Coordinating Committee, 1994).

Even prior to having a full medical and biological understanding of the infectious agent and disease parameters, epidemiological and clinical studies may play a role in guiding control and prevention efforts. Relatively simple and rapid assessments, such as surveillance analyses and case studies, can identify the clinical presentation of infection, characteristics of the populations affected by the disease, and the rate at which the disease is circulating. This allows for inference of the method of transmission, and then, appropriate control measures. However, in this initial period, where some or all of the biological mechanisms behind a disease are still elusive, it is also the case that epidemiological analyses can have incorrect or misinterpreted conclusions. This may lead to any of inappropriate, insufficient, or harmfully over-reactive actions in disease management. To showcase this, below I will briefly describe some recent historical examples of initial emerging disease investigations with inaccurate findings, along with the impact these had on further research direction and the affected populations.

“Poppers” as a strong confounder of HIV infection causing Kaposi’s sarcoma

In 1981, reports of an increase in typically rare opportunistic infections, such as Kaposi’s sarcoma and pneumocystis pneumonia, in men who have sex with men (MSM) in the United States were actually the first published observations AIDS caused by HIV (Friedman-Gottlieb et al., 1981; Kien et al., 1981; Masur et al., 1981; Siegal et al., 1981; Thomasen et al., 1981). At the time, the virus itself had not been identified, and the condition was temporarily defined based on a combination of the populations it seemed to be disproportionately affecting, as well as the unusual clinical manifestations being observed in these groups. The result of this was misleading initial names for AIDS, by both the media and scientists, including “lymphadenopathy” (CDC, 1982a), “Kaposi’s sarcoma and opportunistic infections” (CDC Task Force on Kaposi’s Sarcoma and Opportunistic Infections, 1982), “Gay-related immune deficiency (GRID)” (The New York Times, 1981), “the 4H disease” (indicating

heroin users, homosexuals, hemophiliacs, and Haitians) (Marc et al., 2010). Although the CDC adopted a more objective terminology in 1982, with the first mention of AIDS (CDC, 1982b), these latter labels had lasting stigmatizing effects.

Fairly rapidly, the involvement of a blood-borne pathogen in AIDS was hypothesized based on the populations most affected (Curran et al., 2011). While microbiological attempts to identify a potential underlying infectious agent were underway, epidemiological studies were also being performed to examine the risk factors of persons with this unique pattern of opportunistic infections in order to determine causal relationships. One case-control study published in *The Lancet* in 1982, found a strong and significant association between Kaposi's sarcoma and amyl nitrite use in MSM (Marmor et al., 1982). Amyl nitrite is a recreational drug, also called "Poppers" that was started become popular in the 1970s (Israelstam et al., 1978). The magnitude of the association found through this seemingly robust study, with a risk ratio of 12.3 (95%CI: 4.2-35.8), even after controlling for factors such as sexual activity and other recreational drug use, led to the temporary belief that this may indeed be the cause of AIDS. The hypothesis was that the amyl nitrite could be inducing immune suppression itself, leading to the development of Kaposi's sarcoma; however, the authors of the study could not rule out that a likely alternative explanations was that the drug was a surrogate for exposure to an unknown oncogenic virus (Marmor et al., 1982).

In 1983, a novel retrovirus, to eventually be called HIV, was discovered in a person with lymphadenopathy, and this became known as the true cause of AIDS (Barre-Sinoussi et al., 1983). However, there has now been more than 30 years of research on the subject of poppers and HIV, with findings that amyl nitrite itself could cause immune suppression in mice (Guo et al., 2000; Soderberg, 1999), that its use in humans could lead to sexual inhibition and increased likelihood of seroconversion (Buchbinder et al., 2005; Daskalopoulou et al., 2014; Plankey et al., 2007), but more recently, that there is no association between heavy popper use and cancer risk in HIV-positive men (Dutta et al., 2017). This initial confused relationship between poppers and Kaposi's sarcoma has been considered as a very strong empirical example of confounding (Morabia, 1995), and demonstrates clearly how, in initial emerging disease investigations, even apparently robust correlations may actually be covering a yet-to-be understood biological mechanism.

The expected vCJD disease burden in the United Kingdom

Variant Creutzfeldt-Jakob's Disease (vCJD) is a prion disease that is caused by the same agent that leads to bovine spongiform encephalopathy (BSE or 'mad cow' disease) and was first discovered in the United Kingdom in 1996 (Bruce et al., 1997; Hill et al., 1997; Will et al., 1996). The mean duration of vCJD is 14 months, and is characterized initially by psychological abnormalities (e.g. depression and anxiety) and joint and limb pain, followed by a diminution in cognitive function and an increase in involuntary movements, eventually leading to severe sporadic muscle contractions, abnormal eye movements, speech inability, and death in all cases (Zerr and Poser, 2002).

The prion causing vCJD is thought to be transmitted to humans through the ingestion of certain meat products (e.g. brain, spinal cord, eye) from cows with BSE, and it has been estimated that more than 450000 infected cows were slaughtered for consumption prior to the 1989 bovine specified risk materials ban in the UK (FAO, 2002; Ferguson et al., 1997). This led to considerable concern over the eventual number of persons who would contract the disease, all for whom the result would be fatality. Some factors that complicated the ability to estimate the possible disease burden were the unusual nature of the infectious agent and, related to this, uncertainty on the incubation period in humans. Through modeling studies using varying scenarios for the incubation period and the number of cases appearing over that next year, Cousens and colleagues (1997) and Ghani and colleagues (2000), ended up with a wide range of possible vCJD cases that may occur in the UK, from 74 to 80000, and 63 to 136000, respectively.

By early 2001, there had been a total of 97 reported vCJD cases in the UK (Valleron et al., 2001). One interesting, and unique feature of the vCJD cases that had been reported up until that point, was their young age (mean 28 years) at the time of symptom onset; this varied from sporadic CJD, an already established prion disease, where the mean age of cases is closer to 70 (The National CJD Research & Surveillance Unit, 2001). At this time, Valleron and colleagues (2001) mentioned two possible, and possibly overlapping, hypotheses for this young mean age in cases - the first was that young people were more susceptible to infection, and the second was that the incubation period differed for persons of different ages contracting

the disease (i.e. shorter in younger persons). This study group quickly ruled out the second hypothesis, as there was no obvious correlation between the age of cases and the date of diagnosis. Instead, they fit a model assuming that after 15 years of age, the force of infection decreased exponentially, and using this, estimated that the incubation period was approximately 17 years and that the eventual total number of cases in the UK would be around 200 (upper limit approximately 400).

As of the end of December 2017, a total of 178 cases of vCJD had been reported in the UK (The National CJD Research & Surveillance Unit, 2017), closely aligning with the results of the modeling study performed in 2001 by Valleron and colleagues. Considering that the policy implications for disease management in the UK, not to mention national panic, would differ significantly with an expected 200 versus >100000 cases/deaths due to vCJD, this highlights the impact of analysis assumptions in early emerging disease event research. In this case, the unusual infectious agent involved, and its related unknown disease parameters, exacerbated these complications.

Super-spreaders and perceived aerosolized transmission of SARS

The outbreak of SARS started in late 2002 in Guangdong province, P.R. China; however, for some months, the world was largely uninformed about this event, hearing only rumors of many cases of atypical pneumonia occurring in that region (WHO, 2003a). In February 2003, a doctor who had treated SARS patients in Guangzhou (Guangdong) visited Hong Kong for a wedding and stayed on the 9th floor of the Metropole Hotel, where he infected seven other persons staying on the same floor; this included three from Singapore, one from Vietnam, two from Canada, and one local resident. Subsequent spread of SARS back to the countries of origin of each of these international secondary cases rapidly brought global attention and involvement in the outbreak (Hung, 2003; WHO, 2003). In early March 2003, a physician who contracted the illness while treating a patient in Singapore was taken off of his flight during a layover in Frankfurt and hospitalized there, allowing for isolation of a novel coronavirus (i.e. SARS-CoV) by a German team (Drosten et al., 2003).

Hong Kong was one of the regions most affected by the SARS outbreak, with approximately 1800 infections and a 17% case fatality risk (CFR) (WHO, 2003b). Certain characteristic events that occurred in Hong Kong led to initial misunderstanding about the transmissibility of the virus, which also led to increased, and retrospectively, unwarranted, panic and preventive measures with lasting social and economic impact (Smith et al., 2006; Qiu et al., 2018). In a SARS-naïve population, one infected person will transmit to an average of about 3 others ($R_0=2.7$), which represents low-moderate transmissibility (Riley et al., 2003). However, early in the SARS outbreak, some super-spreader individuals transmitted the infection to between 20-180 persons each (Wong et al., 2015). In Hong Kong, there were multiple important super-spreading events leading to large disease clusters; for example, the initial patient at the Metropole Hotel is assumed to have eventually infected up to 20 others (CDC, 2003a; 2003b), and one of those individuals, admitted to the local Prince of Wales Hospital, is estimated to have infected more than 100 others (Lee et al., 2003; Tomlinson and Cockram, 2003). Another large case cluster occurred at the private residence called Amoy Gardens, where 331 patients were infected, comprising almost a fifth of all cases in Hong Kong (Yu et al., 2014). Here, an index SARS patient with comorbidities visited a relative in Block E of the complex, and had an episode of diarrhea there. Subsequently, many persons in Block E became infected, but the majority of cases in this cluster occurred in adjacent apartment blocks (Government of Hong Kong, 2003). Some initial investigations into this cluster suggested possible aerosolisation of SARS (Yu et al., 2014), others concluded that it occurred because of a poorly functioning sewage system and other environmental factors (Government of Hong Kong, 2003; Lee, 2003), and later, some suggest possible exceptional airborne transmission due to unique environmental factors (i.e. the high concentrations of viral droplets in the sewage system) (McKinney et al., 2006).

In the case of SARS, both the occurrence of super-spreading events, a phenomenon little experienced until that time, as well as the unique case cluster at Amoy Gardens, confused initial estimates of the transmissibility of the novel virus. It could be seen that, positively, confusion surrounding these events initially led to heightened preventive measures as well as global attention on SARS which may have limited its eventual spread, and which has allowed for an increased understanding of super-spreading events as well as the role of environmental factors in transmission of such diseases (McKinney et al., 2006; Wong et al., 2015). On the

other hand, the resulting level of caution and panic, as well as public communication surrounding this, had a disproportionate effect on the economy and social wellbeing in Hong Kong and mainland China, when compared to the absolute morbidity and mortality linked to the outbreak (Smith, 2006; Qiu et al., 2018; Yip et al., 2010).

Estimation of CFR during the 2009 influenza A(H1N1) pandemic

The 2009 influenza A(H1N1) virus pandemic, also called “Swine Flu”, started in April of that year, in Mexico. In order to understand the potential severity of an influenza pandemic, it is necessary to consider both the estimate of the transmissibility of the new or re-emerging virus as well as the risk of fatality for those infected (i.e. the CFR) (Lipsitch et al., 2009; Van Kerkhove et al., 2010). The transmissibility of the 2009 influenza A(H1N1) virus was established relatively early on during the pandemic period; the median reproductive number was 1.5, which is similar to previous influenza pandemics (Boëlle et al., 2011). Conversely, deriving a reliable estimate of the severity of infection, in terms of the proportion of cases who were likely to die, proved much more difficult. Initial approximations of the CFR were greater than 5% in Mexico, approximately 4% in Colombia, and around 14% in Argentina (Castro-Jimenez et al., 2009; Chowell et al., 2011; Nishiura et al., 2010; Wong, Kelly, et al., 2013); for comparison, the 1918 “Spanish” influenza A(H1N1) pandemic had a CFR of roughly 3%, and that of seasonal influenza is <0.1% (Taubenberger and Morens, 2006). These initial high estimates from Latin America led to the first ever Public Health Emergency of International Concern (PHEIC) issued by the WHO, and an increasing of the level of influenza pandemic alert to phase 6 (WHO, 2009a; WHO 2009b).

We now know that an accurate estimate of the CFR for the 2009 influenza A(H1N1) pandemic is up to 10 per 100000 (0.01%), meaning that initial estimates that highly influenced international policy for disease control (e.g. vaccine production and purchase), were grossly overestimated (Wong, Kelly, et al., 2013). A retrospective review by Wong, Kelly, and colleagues (2013) demonstrates the variability in CFR estimates during the entire pandemic period, ranging from as low as 0% to as high as 14%. The case definition for the denominator in each study accounted for some, but not all, of the magnitude of difference across the

estimates. Ideally, CFR is calculated based on an understanding of the total number of persons infected (i.e. infection fatality risk), but this is a measure complicated to determine at the start of an outbreak, as it requires extensive serological testing (Wong, Wu, et al., 2013). A likely reason for initial overestimates of the CFR during the 2009 influenza A(H1N1) pandemic, was the fact that laboratory confirmed and hospitalized cases were often used as the denominator in early-reported studies in Latin America, likely with a severe selection bias on which cases had samples sent for confirmation (Wong, Kelly, et al., 2013). In this case, the wide uncertainty around the seriousness of the influenza pandemic hindered the ability to perform an accurate risk assessment and subsequently to implement an appropriate level of control, and this has led to recommendations for the improvement of defining CFR in the face of a pandemic (WHO, 2011).

1.2 Aims of this thesis

As described earlier, in the preface of this thesis, the aims of my research and what I imagined I would be presenting in this manuscript have changed dramatically over the past four years. The primary objective of my first thesis topic was to demonstrate the ‘real-life’ safety and efficacy of direct acting antivirals for HCV in Egypt. Secondary objectives of that project included a description of adherence to these treatment regimens, as well as an evaluation of methods to improve data collection in large centralized national treatment programs. In my second thesis topic, on ZIKV, my goal was to estimate the risk of ZIKV-related birth defects in both epidemic and non-epidemic regions. This work was meant to include a description of the proportion of ZIKV-related birth defects, and factors related to these abnormalities, in the French Territories in the Americas, as well as an estimate of the proportion of ZIKV-related microcephaly in different settings across Africa and Asia.

I will be able to present you with my findings on the many of the above listed specific objectives in this manuscript; however, its own overall goal will be broader. Here, my aim is to draw your attention to the difficulties in bringing together initial epidemiological findings related to emerging infectious disease parameters in order to draw meaningful conclusions that will responsibly inform policy actions for control.

My specific objectives for each of the next chapters will be:

- To describe how our own findings on the risk of ZIKV-related birth defects in epidemic and non-epidemic regions compare to other contemporary studies, drawing specific attention to differences in study methodologies as well as issues with the case definition used for research on a key birth defect - microcephaly.
- To demonstrate the challenges in drawing a consensus on adverse events linked to new direct acting antivirals for HCV in Egypt due to non-standardization of study methodologies and case definitions used.
- To discuss the utility of methods such as protocol standardization, collaborative research networks, and expert opinion elicitation to generate comparable results and to focus research priorities during emerging disease events.

Part 2: Estimation of ZIKV-related birth defects in epidemic and non-epidemic regions, 2016-2018

In this section, I will focus on the emergence of Zika virus (ZIKV) as a public health threat – specifically with regards to the recent realization that infection with the virus during pregnancy can lead to adverse fetal outcomes and infant birth defects. This has been the subject of my thesis work for the past two years. In early 2016 we started collaborating with a research group working in the French Territories of America (FTA) on ZIKV-infected pregnant women and infant cohorts; personally this has meant my involvement in analyzing and interpreting data on ZIKV-related birth defects. Also in 2016, we secured funding to start a multi-country study doing surveillance for ZIKV-related microcephaly in sub-Saharan Africa and Asia. I have been coordinating this study, which is taking place in Vietnam, Sri Lanka, China, Cameroon, and Ivory Coast.

I will start with some background information on ZIKV, describing its discovery and epidemiological history over the past 60 years. The severe manifestations of ZIKV infection will be presented, as have been made apparent through the more recent French Polynesian (2013-2014) and Americas (2015-2017) outbreaks. I will then describe the methods of the pregnant women cohort in the FTA, along with two articles that use data from this cohort to estimate the risk of birth defects in ZIKV-exposed pregnancies. After this, a short description of our multi-country ZIKV-related microcephaly surveillance study will be given, followed by an article that includes our preliminary findings on the proportion of infants with microcephaly in four of the included countries. Finally I will discuss potential reasons for our findings and their differences or similarities with other reports, with a specific focus on use of a standardized case definition for microcephaly and the importance of control groups.

2.1 Background

2.1.1 Discovery of ZIKV

In 1947, in the Zika forest of Uganda, a flavivirus of the family *flaviviridae* was isolated from a sentinel rhesus monkey showing signs of fever. A year later, another strain of the virus was isolated from wild *Aedes africanus* mosquitoes (Dick et al., 1952). When inoculated into the brains of mice, the first strain caused mortality in all, and the second strain led to transient motor dysfunction and paralysis (Dick et al., 1952; Dick, 1952). This work was done in the well-equipped Uganda Virus Research Institute, situated in the Zika forest near Entebbe, which had been started by the Rockefeller Foundation in 1936 for the study of yellow fever, and later expanded its mandate to study various other viruses in the region (Zhou et al., 2016). A subsequent serosurvey in 45 persons residing in two Western regions of Uganda, Bwamba and West Nile, demonstrated that 6.1% of persons had evidence of past infection of ZIKV, however, near to Zika forest, none of the sera of 54 persons tested contained antibodies (Dick, 1952; Dick, 1953). It is commonly thought that the first reported description of human illness due to ZIKV was in West Africa, reported by Macnamara in 1954 for three persons in Nigeria; this virus was isolated and later inoculated into a human volunteer (Bearcroft, 1956). However, subsequent research has confirmed that the virus isolated by Macnamara was more closely related to Spondweni virus than ZIKV (Boorman and Draper, 1968; Moore et al., 1975; Simpson, 1964; Wikan and Smith, 2017). Therefore, the first official recorded human case of ZIKV was a decade later in (and reported by) David Simpson (1964), a physician who contracted the virus while working in the Uganda Virus Research Institute in Entebbe. Simpson carefully recorded his symptoms, which included fever, headache, fatigue, backache, and leg pain over a period of two days, as well as a full-body maculopapular rash that appeared on the second day of illness but which had faded by the end of the fifth day of illness (Simpson, 1964).

2.1.2 ZIKV circulation 1947-2007

Serological studies indicate that ZIKV circulated widely throughout Africa and Asia in the five decades following its discovery - this has been summarized in a number of reviews (Faye et al., 2014; Kindhauser et al., 2016; Musso and Gubler, 2016; Petersen et al., 2016; Posen et al., 2016;). As mentioned previously, the first serostudies were performed in East Africa, namely Tanzania and Uganda, in the late 1940s (Dick, 1952; Dick, 1953; Smithburn, 1952). Posen and colleagues (2016) and Kindhauser and colleagues (2016) describe well the appearance (i.e. reporting) of ZIKV in other countries in these two continents following that time period, which, in order, occurred in: Nigeria, India, Malaysia, the Philippines, Central African Republic, Chad, Egypt, Republic of Congo, Thailand, and Vietnam (1950-1955); Mozambique (1957); Angola, Ethiopia, Kenya, Senegal, Cote d'Ivoire, Burkina Faso, Cameroon, Guinea Bisau, Mali, and Togo (1960-1965); Niger, Somalia, Benin, Gabon, Morocco, and Liberia (1965-1970); Republic of Seychelles, Sierra Leone, Hong Kong, and Indonesia (1970-1975); Sudan, Madagascar, and Pakistan (1975-1980); Burundi (1980); Djibouti (1991). The seroprevalence reported for some countries, notably Benin, Burkina Faso, Mali, Senegal, and Malaysia were around 40% or higher (Posen et al., 2016). Serological methods for detecting ZIKV and other viruses evolved significantly between the 1950s and early 2000s, and therefore the seroprevalence estimate each region/country obtained through the studies should be interpreted along with the type of test used. In most early studies, hemagglutination inhibition assays (HI) were employed, which, compared to more specific methods, such as plaque reduction neutralization tests (PRNT), are known to overestimate seroprevalence (Omer et al., 1981; Waggoner and Pinsky, 2016).

Interestingly, two studies took place in Spain in the late 1970s, which, via HI testing, revealed a 2.9% seroprevalence of ZIKV (Gonzalez and Filipe, 1977; Lozano and Filipe, 1998). In addition, one laboratory worker in Portugal was reported to have contracted the virus in 1973 (Filipe et al., 1973).

Despite detection of ZIKV in many countries, prior to 2007, only 14 cases of acute human illness with the virus had been documented (Fagbami, 1979; Filipe et al., 1973; Moore et al., 1975; Olson et al., 1981; Simpson, 1964). In all these cases, the disease caused by ZIKV was

described as generally mild, causing symptoms such as fever, headache, as well as joint and eye pain; only in the very first clinical case of ZIKV, that of Simpson in 1964, was rash portrayed (Kindhauser et al., 2016; Simpson, 1964). No deaths, hospitalizations, or ZIKV-related birth defects were reported.

2.1.3 Outbreaks in the South Pacific (2007-2015)

The first significant outbreak of ZIKV ever recorded was in Micronesia (the Yap Islands), lasting for three months from mid-April to mid-July of 2007 (Barboza et al., 2008; Duffy et al., 2009). On this small island with a population of <8000 persons, a subsequent serosurvey demonstrated that 73% (95%CI: 68-77) of persons had been infected. Of those showing evidence of ZIKV infection, approximately one-fifth had symptoms, including rash in 90%, fever, joint pain and conjunctivitis in 55-65% of patients, and muscle pain, headache, and eye pain in 40-50% of persons (Duffy et al., 2009). In mid-October 2013, ZIKV cases were observed in French Polynesia, an archipelago of islands more than 8000 km distance away from Yap (Cao-Lormeau et al., 2014; Mallet et al., 2016). This was the beginning of an outbreak that lasted approximately six months and led to almost 32000 symptomatic cases presenting to health care facilities; a post-epidemic serological survey indicates that 49% of the population had been infected (Aubry et al., 2017; Mallet et al., 2016). Similar symptoms were reported as those seen in the Yap Island outbreak (Duffy et al., 2009; Musso et al., 2018). In French Polynesia, the first severe manifestations of ZIKV were noted; these were cases of Guillain-Barre Syndrome (GBS), an autoimmune condition that causes acute or subacute paralysis (Cao-Lormeau et al., 2016; Musso et al., 2014; Oehler et al, 2014). Following the outbreak in French Polynesia, ZIKV spread through other islands in the South Pacific, including New Caledonia, the Cook Islands, Easter Island, American Samoa, Vanuatu, and the Solomon Islands (Dupont-Rouzeyrol et al., 2015; Healy et al., 2016; INVS, 2014; Musso et al., 2015; Roth 2014; Tognarelli et al, 2016)

2.1.4 ZIKV Outbreak in the Americas (2015 - present)

The virus arrived in South America in early 2015, signaled by notifications of a large outbreak of rash-causing illness in Bahia State, as well as transmission noted in Rio Grande de Norte State, Brazil (Campos et al, 2015; Cardoso et al, 2015; Zanluca et al, 2015). By December 2015, the outbreak had spread across most of Brazil (Secretaria de Vigilância em Saúde, 2016), with the Ministry of Health estimating that there had been between 440 000 and 1 300 000 cases (Ministério da Saúde (Brazil), 2015a). Furthermore, the virus had also spread, at that time, to seven other countries in the Americas, including Colombia, El Salvador, Guatemala, Mexico, Panama, Paraguay and Venezuela (Gomez, 2015; Instituto Nacional de Salud, 2015; Ministry of Health of the Republic of Panama, 2015, Ministry of Public Health and Social Welfare Paraguay, 2015; WHO, 2015a; WHO, 2015b; WHO, 2015c). At that time, ZIKV cases had also been detected in the Cape Verde Islands in Africa (Ministério da Saúde (Capo Verde), 2015). By the end of the following year, 2016, autochthonous transmission of ZIKV had been confirmed in 48 countries and territories of the Americas, including in the southern United States. To date, in all of the Americas, only Canada, Chile, and Uruguay have not reported any locally acquired cases (PAHO, 2017a).

In March of 2016, the WHO declared the outbreak in the Americas to be a Public Health Emergency of International Concern (PHEIC), due to the surfacing associations between ZIKV infection and microcephaly in infants whose mothers were infected during pregnancy, as well as other severe neurological manifestations in adults, such as GBS (Heymann et al., 2016; Oehler et al., 2016; PAHO, 2016; WHO, 2016a). A more detailed description of these findings, as well as an update on ZIKV-related birth defects, will be given later in this background section. At present, the outbreak situation in the Americas had declined, with limited circulation being recorded (PAHO, 2017b; Siedner et al., 2018). The PHEIC was declared over in November of 2016, with a recommendation for sustained research (WHO, 2016b)

2.1.5 Diagnosis of ZIKV infection

Molecular diagnosis and persistence of ZIKV RNA in body fluids

ZIKV infection can be definitively diagnosed by molecular methods (e.g. real-time polymerase chain reaction (RT-PCR)); however, depending on the body fluid used and the time since initial infection, the likelihood of detecting ZIKV ribonucleic acid (RNA) differs. One study by Paz-Bailey and colleagues observed 150 participants with confirmed symptomatic ZIKV infection and detected RNA in 88% of serum samples with a median duration of positivity of 14 days, with no detection in 95% of participants after 54 days. For urine, ZIKV could be detected in 62% of participants; the median duration of positivity was 8 days, with no detection in 95% of participants after 39 days (Paz-Bailey et al., 2017). These results are in contrast to some previous smaller studies that reported detecting ZIKV RNA in urine more frequently (i.e. in up to 100% of patients) and for longer median durations than in serum (Bingham et al., 2016; Gourinat et al., 2015). Saliva presents another possibility for molecular diagnosis of ZIKV infection, but the reported sensitivities of this sample when compared to serum vary. Of the larger studies that have evaluated this topic, two have reported detection of ZIKV RNA more frequently in saliva when compared to serum (19% vs 8% and 81% vs 51%) (Bingham et al., 2016; Musso et al., 2015); however, Paz-Bailey and colleagues report detection in only 10% of saliva samples when compared to 88% of serum samples (Paz-Bailey et al., 2017). ZIKV RNA has been seen to persist for longer periods in whole blood samples when compared to serum, and has therefore been proposed as an alternative and superior sample for diagnosis (Barzon et al., 2018; Baud et al., 2017).

In ZIKV infected men, semen is frequently positive for ZIKV RNA, and the virus is detectable for longer durations than in other body fluids. In the study by Paz-Bailey et al., ZIKV RNA was detected in semen samples from 81% of 68 male participants, with a median disappearance time of 34 days (Paz-Bailey et al., 2017). In a prospective cohort study of 184 men in Puerto Rico, ZIKV RNA could be detected in 60 (33%) of participants, with a median time until non-detection of 35 days (Mead et al., 2018). It is also possible to detect ZIKV RNA in vaginal secretions and conjunctival fluid, however, these samples are rarely positive (Paz-Bailey et al., 2017; Tan et al., 2017). Semen, vaginal secretions and conjunctival fluid are

not typically proposed as samples for diagnosis, but rather for monitoring and research purposes.

ZIKV RNA has been seen to persist for long periods in pregnant women, up to three times longer than in non-pregnant women in the same age range (Lozier et al., 2018; Meaney-Delman et al., 2016). The reason for this is not completely understood, although an altered immune state, or replication of the virus in additional reservoirs such as the placenta or the fetus itself, have been proposed as possible explanations (Aagaard et al., 2017; Bhatnagar et al., 2017; Lozier et al., 2018; Meaney-Delman et al., 2016).

Serological diagnosis

While detection of RNA is the only way to definitely confirm acute infection with ZIKV, this type of diagnosis has limited use due because of short duration of positivity, as well as the need for symptomatic infection to prompt testing. Serological diagnosis of ZIKV, on the other hand, provides a more flexible window of time to define exposure. Serum can be tested for immunoglobulin M (IgM) using an enzyme-linked immunosorbent assay (ELISA); IgM appears within a week but typically wanes after a few months and is therefore not recommended for diagnosis after 12 weeks of presumed infection (Landry and St. George, 2017; Rabe et al., 2016). In a study of 29 travelers returning to Italy, ZIKV IgM was detectable as early as five days (median 12 days) following estimated time of infection (Barzon et al., 2018). Another study in 15 persons in Guadeloupe found that IgM was detectable for the majority of patients within one week of infection and for all patients by the eleventh day after infection; in this study, two different commercial immunoassays were used and sensitivities at various time-points differed for each. In the same study in Guadeloupe, IgM had decreased to the point of being undetectable by both immunoassays in the majority of patients within two months of infection (Pasquier et al, 2018).

ZIKV immunoglobulin G (IgG) appears shortly after IgM, and is detectable for long periods using commercial immunoassays (Barzon et al., 2018; Pasquier et al., 2018). Experience with other flaviviruses indicates that these neutralizing antibodies could remain for

years and may confer lifelong immunity, however, this is not yet certain for ZIKV (Baud et al., 2017; Busch et al., 2008; Poland et al., 1981; Whitehead et al., 2007). A follow-up survey in French Polynesia, 18 months after the original outbreak in 2013-2014, demonstrated a decrease in seroprevalence that, although non-significant, may indicate that IgG may wane to undetectable levels over time (Aubry et al., 2017)

While serological diagnosis provides a time-advantage when compared to molecular methods for detection of ZIKV, this method is complicated by flavivirus cross-reactivity (Lanciotti et al., 2008). Therefore, in the case of any positive or uncertain serological test result, whether or not ZIKV has been the most recent infection, cannot be confirmed unless plaque reduction neutralization test (PRNT) for ZIKV and other flaviviruses (e.g. Dengue virus (DENV)) are performed and compared against each other (Rabe et al., 2016). Unfortunately, in the case of a secondary flavivirus infection (e.g. recent infection with either ZIKV or DENV with past infection of the other), PRNT is not able to conclude the culprit of the recent infection. This results from the phenomenon of the ‘original antigenic sin’ where IgM results by ELISA testing may be negative for ZIKV, or have equally high results for both ZIKV and DENV by PRNT, because of a more rapid and exponential increase in DENV antibodies if infection has occurred with it in the past (Barzon et al., 2018; Halstead et al., 1983; Morens et al., 2010; Rabe et al., 2016). Further complicating the use of PRNT for ZIKV confirmation is that it is typically only available in specialized and well-equipped laboratories, and therefore not accessible in all settings.

2.1.6 Transmission

- **Vector-borne**

ZIKV is primarily a vector-borne illness, transmitted mainly by mosquitoes of the genus *Aedes* (*Ae.*), with the role of *Culex* mosquitoes still debated (Boyer et al., 2018; Guedes et al., 2017). Transmission can occur through a sylvatic cycle where infection is passed between non-human primates and forest-dwelling mosquitoes, eventually leading to infection of a dead-end human host. This cycle involves a range of zoophilic *Aedes* and other

mosquitoes, and has been observed in some countries in Africa, with proposed potential for occurring in the Americas (Althouse et al., 2015; Althouse et al., 2016; Boyer et al., 2018; Bueno et al., 2016). Alternatively, transmission can occur through an urban cycle, where mosquitoes move the virus from human to human. This urban cycle is the culprit of large human outbreaks of ZIKV, such as those seen recently in the Pacific Islands and the Americas; three species of mosquito, *Ae. aegypti*, *Ae. albopictus*, and *Ae. hensilii* are involved in this transmission. (Boyer et al., 2018). The efficiency of different mosquitoes as vectors of ZIKV varies by region as well as viral genotype, as has been seen previously with DENV (Boyer et al., 2018; Lambrechts et al., 2009). However, of the mosquitoes implicated in urban transmission of ZIKV, *Ae. aegypti* has been shown to be the most competent vector in the recent epidemics, likely due to an acquired infectivity-increasing mutation (Liu et al., 2017). *Ae. aegypti* is present worldwide in tropical regions; this distribution corresponds to places where DENV is endemic, and serves to highlight areas where ZIKV may, or already is, occurring (Boyer et al., 2018, Messina et al., 2016). *Ae. albopictus*, an invasive species and another competent vector, is partially responsible for autochthonous transmission that occurred in the southern United States, as well as elsewhere, in the recent epidemics (Boyer et al., 2018). This mosquito is seen to be a potential threat for eventual endemicity of the virus in Europe; however, vector competence studies that have taken place in Italy and France so far have indicated minimal threat (Di Luca et al., 2016; Jupille et al., 2016).

- **Mother to child**

Vertical transmission of ZIKV, from mother to child during pregnancy, was not realized until the recent epidemics in the Americas. In early 2016, the virus was detected in the amniotic fluid of Brazilian pregnant women whose fetuses had evidence of microcephaly (Calvet et al., 2016; Melo et al., 2016). Around the same time, ZIKV was detected in brain and placental tissues for two infants with microcephaly who died within 20 hours of birth as well as two miscarried fetuses, all from symptomatic mothers, which indicated the ability of the virus to cross the placental barrier and infect the fetus (Martines et al., 2016). In addition, ZIKV was detected in the brain of a terminated fetus from a pregnant woman returning to

Slovenia from Rio Grande de Norte State in Brazil; she had experienced symptomatic infection at the end of the first trimester of pregnancy, and microcephaly and other brain malformations in the fetus initially detected by ultrasound were later confirmed via autopsy (Mlakar et al., 2016). Noronha and colleagues (2016) further evidenced vertical transmission of ZIKV through description of placental barrier destruction, similar to that occurring with other harmful mother-child transmitted viruses, using tissue samples from exposed pregnancies. The risk of vertical transmission of ZIKV, and identification of demographic and clinical factors that may increase it, are not yet known. One study following 301 ZIKV exposed pregnancies prospectively estimated the proportion of cases with vertical transmission to be approximately 11% (Pomar et al. 2017). More studies on this topic are needed, but it is eventually possible that risk of mother-child transmission of ZIKV in-utero will be similar to analogous teratogenic infections, such as cytomegalovirus, for which the likelihood of the virus breaching the placental barrier is approximately 30% (Benoist et al., 2013; Panchaud et al., 2016; Revello and Gerna, 2002; Yinon et al., 2010).

Perinatal transmission of ZIKV infection was seen in two live born infants in French Polynesia where mothers had symptomatic infection at or within one week of delivery. The serum of both neonates showed evidence of ZIKV RNA within 1 week of birth, however, only one infant presented with symptoms (rash), and neither of the breast-milk samples was positive by cell culture (Besnard et al., 2014). Three case reports have described identification of infectious ZIKV particles in breast milk when women are infected close to the time of delivery, this includes one case in New Caledonia in 2015, one case in Brazil in early 2017, and one case from Venezuela in late 2017 (Blohm et al., 2018; Blohm et al., 2017; Dupont-Rouzeyrol et al., 2016; Sotelo et al., 2017). However, only for the most recent case in Venezuela has mother to child transmission through this means been proposed (Blohm et al., 2017; Blohm et al., 2018).

- **Sexual**

ZIKV RNA has been detected in human semen, vaginal fluid, and saliva and possible sexual transmission of the virus has been reported through penile-vaginal intercourse as well

as, limitedly, through both anal and oral intercourse. Sexual transmission of, and the susceptibility of different reproductive organs for, ZIKV has been well evidenced through *in vitro* studies, *in vivo* animal models, and also through observational studies in humans. A thorough summary of these studies has been given by various reviews, including in a living systematic review (continuously updated online at: <http://zika.ispm.unibe.ch/stf/>) by Counotte and colleagues that was published in May 2018 (Counotte et al., 2018; Moreira et al., 2017; Sherley and Ong, 2018).

Sexual transmission of ZIKV was first evidenced in 2008 after a field-researcher working in Senegal returned to the United States and transmitted the virus to his wife (Foy et al., 2011). Then again in the 2013 outbreak in French Polynesia, one possible case of sexual transmission was described (Musso et al., 2015). Although sexual transmission is difficult to confirm in areas with ongoing circulation, following the large outbreak in the Americas and increased circulation/detection in Asia and Africa, the United States CDC and European Center for Disease Prevention and Control (ECDC) have reported more than 50 and 20 cases of confirmed sexual transmission in returning travelers, respectively (CDC, 2018a; Spiteri et al., 2017). Most of these reports signify male to female infection, however, there has also been one case of potential female-to-male, and one case of male-to-male, transmission noted (Counotte et al., 2018; Davidson et al., 2016; Deckard et al., 2016). In 2016, a report of ZIKV persistence (detection of viral RNA) in semen for greater than 6 months prompted interim guidelines from the WHO for use of protection for prevention of sexual transmission for at least this length of time (Nicastri et al., 2016; WHO, 2016c). However, the review from Counotte and colleagues indicates that when bringing together many observational studies in humans, ZIKV RNA and ZIKV infectious viral particles are present for a median of 40 days (95%CI: 30-49) and 10 days (95%CI: 1-20), respectively (Counotte et al., 2018). Within this review, a clear demonstration of the rapid reduction of infectious virus shedding (indicating risk of transmission) comes from a study of 184 men, which found that infectious virus was only present in three participants in whom semen was collected within 30 days of illness onset (Mead et al., 2018). These findings have led to proposed changes (i.e. to decrease the time period protection is needed) to the guidelines on prevention of sexual transmission (Vouga et al., 2018).

- **Transfusion associated**

Blood transfusion was first proposed as a possible route of ZIKV transmission following the outbreak in French Polynesia in 2013, where 3% of asymptomatic blood donors were found to be RNA-positive during the acute phase of the epidemic (Musso et al., 2014). This possibility was confirmed with the reports of three cases in Brazil that were potentially linked to platelet transfusion in 2016 (Barjas-Castro et al., 2016; Motta et al., 2016). This risk has led to various agencies releasing guidelines for prevention of transfusion associated ZIKV transmission through screening (FDA, 2016a; FDA, 2016b; WHO, 2016d; AABB, 2016), as well as research towards new strategies to inactivate ZIKV in plasma and platelet components (Blumel et al., 2017; Santa Maria et al., 2017).

- **Other**

Finally, the occurrence of one non-sexual secondary case has been reported in the United States. In this case, a 73-year old man with comorbidities, who had recently returned from Mexico, fell fatally ill with ZIKV; quantitative tests for ZIKV RNA indicated that the patient was highly viremic. A healthy 38-year old man who visited the case in hospital came down with rash and other ZIKV symptoms five days later, with urine analysis showing positivity for ZIKV IgM. It has therefore been proposed that transmission may have occurred through either sweat or tears (Swaminathan et al., 2016).

2.1.7 Clinical and neurological manifestations of ZIKV in humans

- **Symptomatic infection**

The incubation period of ZIKV in humans following mosquito-borne transmission is estimated to be a median 6 days; this was found by both a meta-analysis including data for 25 persons, as well as a study of 79 returning travelers (Krow-Lucal et al, 2017; Lessler et al., 2016;). There is not yet a reliable estimate of incubation period for cases of sexual

transmission (Counotte et al., 2018). Symptoms of ZIKV infection last for approximately one week and typically include maculopapular rash, fever, headache, itching, joint pain, muscle pain, conjunctivitis, pain behind the eyes, fatigue, and edema of hands or feet. Rash is typically the most commonly experienced symptom, followed sometimes by fever (self-reported), arthralgia, or headache (Brasil et al., 2016; Cerbino-Neto et al., 2016; Duffy et al., 2009; Hoen et al., 2018; Li et al., 2017; Mallet et al., 2016; Read et al., 2018;). Some example symptom distributions from studies in the general adult population, pregnant women, and children can be seen in Table 1. A slightly itchy rash associated with ZIKV infection typically appears within a few days of symptom onset, can be specific to certain parts of the body or be generalized, and fades after approximately one week (He et al., 2017).

Table 1: Sample symptom distributions for persons with ZIKV infection in the general adult population, pregnant women cohort studies, and children

	General Adult Population			Pregnant Women		Children	
	Yap, Micronesia	French Polynesia	Brazil	Brazil	FTA	Puerto Rico (≤17 yrs)	Singapore (≤16 yrs)
# Persons	31	297	57	134	546	351	14
Maculopapular rash	90%	93%	98%	100%*	95%	80%	100%
Itching	NR	NR	56%	90%	48%	59%	NR
Fatigue	NR	78%	NR	52%	NR	67%	NR
Fever	65%*	72%*	67%*	27%	23%	99%†	93%†
Arthralgia	65%	65%	58%	62%	55%	37%	14%
Conjunctivitis	55%	63%	39%	58%	36%	58%	29%
Myalgia	48%	44%	49%	41%	23%	37%	21%
Headache	45%	46%	67%	54%	30%	64%	21%
Retro-orbital pain	39%	16%	40%	41%	19%	38%	NR
Edema	19%	47%	23%	55%	19%	NR	NR
<i>Publication</i>	<i>Duffy et al., 2009</i>	<i>Mallet et al., 2016</i>	<i>Cerbino-Neto et al., 2016</i>	<i>Brasil et al., 2016</i>	<i>Hoen et al., 2018</i>	<i>Read et al., 2018</i>	<i>Li et al., 2017</i>

*Fever is self-reported or self-reported/measured by a clinician mix **Rash was an inclusion criteria

A recent meta-analysis combined results from 23 studies to report that the proportion of asymptomatic cases of ZIKV infection was approximately 62%; however, the authors concluded that due to considerable heterogeneity in studies included, this estimate is not likely robust (Haby et al., 2018). In the same meta-analysis, three cross-sectional seroprevalence studies were included; these were performed in Yap Island, French Polynesia, and Puerto Rico and found asymptomatic proportions of 82% (95%CI: 81-83), 49% (95%CI: 43-55), and 57% (48-66) (Aubry et al., 2017; Duffy et al., 2009; Haby et al., 2018; Lozier et al., 2017). A study in 114 ZIKV positive persons in Puerto-Rico found that being female, younger than 40, and having asthma, were all factors significantly associated with having symptomatic infection (Lozier et al., 2018).

- **Severe manifestations**

In the first mouse models of ZIKV infection, performed by Dick in the late 1940s, marked neurotropism and mortality were seen when the brains of mice were inoculated repeatedly with strains of the virus recovered from infected rhesus monkeys and mosquitoes (Dick, 1952). However, the first severe manifestation of ZIKV infection in humans that was recorded was a case (female, early 40s) of GBS observed during the 2013-2014 outbreak in French Polynesia (Oehler et al., 2014). GBS is a severe autoimmune condition that affects peripheral nerves, causing paralysis and often requiring respiratory support in intensive care units; while most patients recover within a period of months, it can be fatal where adequate supportive care is not available (Hughes and Cornblath, 2005). In total, 42 cases of GBS were reported after the outbreak in French Polynesia; this was considered unexpectedly high, and was retrospectively linked directly to ZIKV exposure through a case-control study (Cao-Lormeau et al., 2016; Watrin et al., 2016). Following the establishment of GBS as a severe clinical manifestation of ZIKV infection, descriptions of linked cases and increased incidence were reported in many places in the Americas during the outbreak period there (Araujo et al., 2016; Dirilkov et al., 2017; Dos Santos et al., 2016; Parra et al., 2016; Roze et al., 2017). A multi-country report, including studies from South America as well as Yap Island and French Polynesia, estimated the risk of GBS to be 2 per 10000 cases (0.02%) (Mier et al., 2018).

There are four subtypes of GBS (Hughes and Cornblath, 2005), and two of these, including acute inflammatory demyelinating polyradiculoneuropathy (AIDP) and acute motor axonal neuropathy (AMAN), have been indicated in ZIKV infection, however, so far, the relative frequency of each varies depending on the case series being considered (Cao-Lormeau et al., 2016; Parra et al., 2016).

During this recent outbreak period, other severe manifestations, such as autonomic disorders, cardiac anomalies, encephalopathy, meningoencephalitis, have also been linked to ZIKV infection in very rare cases (Abdalla et al., 2018; Brito Ferreira et al., 2017; Carteaux et al., 2016; Malta et al., 2017; Rodriguez et al., 2018; Roth et al., 2017; Roze et al., 2016). Fatal cases of ZIKV have also been reported (Azevedo et al., 2016; Sarmiento-Ospina et al., 2016; Swaminathan et al., 2016).

- **ZIKV-related birth defects**

One of the most concerning manifestations of ZIKV infection is the occurrence of neurological defects in fetuses and infants whose mothers are infected with the virus during pregnancy. This was first noticed in Brazil in 2015, signalled by an unprecedented rise in incident cases of neonatal microcephaly (a small head for gestational age) that coincided regionally and temporally with the ZIKV epidemic (Ministério da Saúde (Brazil), 2015b; Kleber de Oliveira et al., 2016). As described in the previous part of this thesis that outlined mother to child transmission of ZIKV, discovery of ZIKV RNA in amniotic fluid as well as in brain and placental tissues of pregnancies/foetuses/infants where there was evidence of microcephaly, further evidenced this potential link (Calvet et al., 2016; Martines et al., 2016; Melo et al., 2016; Mlakar et al., 2016). In late 2015, the Brazilian Ministry of Health declared a possible connection between the birth defect and ZIKV infection (Ministério da Saúde (Brazil), 2015c). The World Health Organisation declared a Public Health Emergency of International Concern in early 2016, with the aim to encourage further clarification (through research) on this and other severe side effects of ZIKV (Heymann et al., 2016; WHO, 2016a).

Since that time, some robust case-control studies examining the association between ZIKV infection during pregnancy and microcephaly have been carried out, and a causal link between the two has been solidified (de Araujo et al, 2018; Krow-Lucal et al., 2018; Rasmussen et al., 2016; Santa-Rita et al, 2017). Case series and imaging studies performed during the ZIKV outbreak in the Americas identified a series of neurological and ocular abnormalities that could be linked to infection during pregnancy. Moore and colleagues (2017) brought these findings together to give a working definition of ‘Congenital Zika Syndrome’ (CZS), which includes: severe microcephaly with collapsed skull, specific structural brain abnormalities (i.e. calcifications, lissencephaly, ventriculomegaly, corpus callosum anomalies, underdeveloped cerebellum, decreased white matter, thin cerebral cortex), congenital contractures (i.e. club foot, arthrogyrosis), eye abnormalities (i.e. structural ocular anomalies, cataracts, posterior eye anomalies), early-life presentation of neurological sequelae (i.e. increased or decreased muscle tension, irritability, swallowing problems, motor and cognitive disabilities, hearing and visual impairment, epilepsy) (CDC, 2018b). This describes the pattern of *unique* features that can be seen in fetuses/infants that are affected in-utero by ZIKV, to aid in differential diagnosis, but does not include all possible effects of ZIKV infection on the fetus. Other reported conditions in infants with ZIKV exposure in-utero include things such as cardiac anomalies, abnormal sleep findings, neurogenic bladder, respiratory problems, digestive disorders, and hydrocephalus (Angelidou et al., 2018; Carvalho et al., 2017; Cavalcanti et al., 2017; Costa Monteiro et al., 2018; Moura da Silva et al., 2016; Pinato et al., 2018; van der Linden, Pessoa et al., 2016; van der Linden, Filho, et al., 2016). Features of CZS, such as structural brain abnormalities and consequences of central nervous system dysfunction can also appear in the absence of microcephaly (Cardoso et al., 2018; van der Linden, Pessoa et al., 2016). It was previously thought that neural tube defects were linked to ZIKV infection, but this is no longer considered so (Delaney et al., 2018; Rice et al., 2018).

The proposed risk of birth defects following ZIKV infection, as investigated by prospective cohort studies, varies greatly, and unfortunately, there has been limited use of control groups in order to estimate the baseline risk of similar defects in the absence of ZIKV infection. Initially, a cohort study of 125 pregnant women with symptomatic (i.e. rash) ZIKV infection in Brazil estimated a 46% risk of overall adverse foetal outcomes, which was significantly higher than the 11.5% found in their group of 61 ZIKV non-infected women

(Brasil et al., 2016). In the United States Zika Pregnancy and Infant Registry (USZPIR), a 5% overall prevalence of birth defects possibly linked to ZIKV infection was determined through follow-up of 2549 completed pregnancies (Shapiro-Mendoza et al, 2017). In our own study, in the FTA, we reported on foetal/infant outcomes up until the time of delivery for 546 ZIKV-infected pregnant women and concluded a 7% risk of neurological and ocular birth defects (Hoen et al., 2018); this paper will be presented in full later in this thesis. In both the USZPIR and our own cohort study in the FTA, there are no prospective ZIKV negative control groups with which to compare. One ultrasound-based study in French Guiana followed 301 ZIKV exposed and 399 non-exposed pregnancies to examine the risk of foetopathy. This study found central nervous system abnormalities to be 9% and 4% in the ZIKV exposed versus unexposed groups, respectively (Pomar et al., 2017). In Colombia, a retrospective cohort study of 86 ZIKV-exposed pregnancies, without a control group, found a 2% risk of adverse neurological outcomes; all of these were microcephaly cases without any other neurological signs (Rodriguez-Morales et al., 2018).

So far, only one study, a cohort follow-up of infants from the USZPIR, has evaluated the risk of presentation of neurological, visual, and auditory sequelae in the first year of life. Here, Rice and colleagues (2018) evaluated outcomes for 1450 infants whose mothers had possible ZIKV infection during pregnancy, and found that 8% of infants who had no apparent ZIKV-associated defect at birth presented with a possible or confirmed neurodevelopmental abnormality. Multiple longer term cohort studies following infants up until 5-7 years of age, ideally with control groups, will be needed to draw a consensus on the proportion of infants who have consequences of ZIKV infection in-utero.

The risk of having an infant with birth defects appears to be highest in the first, followed by the second and third, trimesters of pregnancy (Brasil et al, 2016; Cauchemez et al, 2016; Hoen et al, 2018; Pacheco et al, 2016; Pomar et al, 2017; Shapiro-Mendoza et al, 2018).

2.1.9 Prevention and Treatment of ZIKV

Currently, the main protective strategies against ZIKV infection are the prevention of mosquito bites and use of protection with sexual partners with recent or current infection. The vector control strategies recommended by the WHO include employment of traditional methods such as insecticide spraying (both targeted residual and space), larvicide use and reduction of mosquito breeding sites, and personal protection against bites (WHO 2016e). In addition, some newer control methods, such as introduction of *Wolbachia* spp bacteria into populations of *Ae aegypti* mosquitoes to reduce their ability to transmit ZIKV, and the release of mosquitoes with dominant lethal genes to reduce mosquito population density through limiting the number that survive until adulthood, have both undergone some field testing in Latin America (Carvalho et al., 2015). Deployment of these novel methods are recommended, following carefully planned pilot phases, by both the WHO and others (WHO, 2016e; Yakob and Walker, 2016a; 2016b). For prevention of sexual transmission, it was originally recommended that there should be use of physical (e.g. male or female condom) protection during intercourse for 6 months following confirmed or probable ZIKV infection in men, however, due to recent findings that indicate that infectious virus is found for a much shorter period of time, this recommendation will likely change to be less conservative (Counotte et al., 2018; Mead et al., 2018; Nicastri et al., 2016; Vouga et al., 2018; WHO, 2016b)

Besides management of mild symptomatic infection through taking painkillers and antihistamines (Valentine et al., 2016), there is currently no approved treatment for ZIKV infection. Many studies have been performed which evaluate the repurposing of already approved drugs for treatment of ZIKV; Masmajan and colleagues (2018) have summarized this in a recent review. They describe that while many treatments that have been approved by the United States Food and Drug Administration (FDA) have demonstrated activity against ZIKV, few have been adequately tested and proven to be safe in pregnant women, which is a necessary consideration for a treatment that may eventually be recommended for large-scale use. There is equally no current treatment for CZS, although, early diagnosis through pre-natal diagnostic techniques and imaging, or screening at birth, may help to confer a better prognosis through allowing appropriate and rapid referrals to appropriate therapists. (Karoly et al., 2005;

Rice et al., 2018 ; Sonksen et al., 1991, Ventura et al., 2017; Wheeler, 2018; Yoshinaga-Itano et al., 2017)

As with treatments against ZIKV, an important, but complicating, factor for development of a vaccine is its eventual use and safety in pregnant women. Various types of ZIKV vaccines are currently being evaluated, including deoxyribonucleic acid (DNA), inactivated virus, recombinant protein subunit, messenger RNA (mRNA), virus-like particles, and live-attenuated. Most of these candidates have only undergone testing in mice or macaques, with very few results from early-phase clinical trials being available. Mamejan and colleagues (2018) have summarized progress in vaccine research in a systematic review, for the period up until June 2018. Multiple DNA vaccines have and are being evaluated in phase one clinical trials and two of these have demonstrated adequate safety to move to phase II trials (Gaudinski et al., 2018; Makhluף and Shresta, 2018; Tebas et al., 2017). Multiple inactivated virus vaccines, and at least one ZIKV mRNA lipid nano-particle vaccine is undergoing phase one clinical trials (Makhluף and Shresta, 2018; Modjarrad et al., 2017). Finally, in mid-August, 2018, the National Institutes for Health in the United States of America announced the first live-attenuated vaccine undergoing a phase one trial in 28 healthy, non-pregnant, persons; however, live-attenuated vaccines are typically contraindicated in pregnant women (Mamejan et al., 2018; NIH, 2018). Phase one clinical trial results for vaccines are typically only available after at least one year, and these will need to be followed by phase II-IV trials. The length of time needed for proper evaluation, coupled with the increasingly limited ability to test vaccines in real-life populations due to decreasing ZIKV transmission, means that it will be quite some time before a vaccine for ZIKV is available for large-scale use (Cohen, 2018; Mamejan et al., 2018; PAHO, 2017b).

2.1.10 ZIKV in Africa and Asia

Circulation of ZIKV has been reported in many Southeast Asian countries as well as India, over the past years (Gu et al., 2017; Khongwichit et al., 2018; Ngwe et al., 2018; Perkasa et al., 2016; Pettersson et al., 2018; Sapkal et al., 2018; Tappe et al., 2015), and notable outbreaks occurred in Singapore and Vietnam in 2016 (Chu et al., 2017; Singapore

Zika Study Group, 2017). Besides the Cape Verde Islands off the western coast, no large outbreaks have been reported in recent years in Africa, even though seroprevalence studies indicate that transmission has, and still is, occurring in many countries across the continent (Gake et al., 2017; Herrera et al., 2017; Kraemer et al., 2017; Lourenco et al., 2018; Mathe et al., 2018; Rosenstierne et al., 2018; Sherman et al., 2018). Due to a possibly high at-risk population in these regions, there is a need for increased surveillance of ZIKV and related manifestations (Bogoch et al., 2016; Lucey, 2016; Siraj et al., 2017).

There are two distinct lineages of ZIKV, an African and an Asian one. Descendants of the Asian lineage have led to the strain of ZIKV that circulated in French Polynesia and Latin America during the recent outbreaks (Pettersson et al., 2016). Differences in the pathogenicity of the older Asian strains and their descendants that led the Pacific islands and American outbreaks, has been proposed as the reason for the lack of large outbreaks and ZIKV-related birth defects seen in Asia over the past six decades. Additional research shows that the recent descendants of the Asian strain that have been implicated in the South Pacific and Americas outbreaks have specific mutations which lead to increased infection rates in mosquitoes as well as augmented cytopathic effects and ability to cause foetal brain defects in mice (Alpuche-Lazcano et al., 2018; Xia et al., 2018; Yuan et al., 2017; Zhange et al., 2017). However, there have been recent reports of microcephaly and CZS linked to earlier Asian isolates found in Thailand and Cambodia (Chu et al., 2018; Wongsurawat et al., 2018). In contrast, both Shao and colleagues (2017), and Smith and colleagues (2018), have demonstrated that the African ZIKV strain is more lethal in mice than older Asian isolates. In addition, Sheridan and colleagues (2018) reported that the African strains caused increased placental destruction, with the conclusion that infection with this strain early in pregnancy would likely lead to miscarriage rather than foetal defects. Some cases of microcephaly possibly linked to infection with the African ZIKV strain have been reported in Guinea-Bissau (Rosenstierne et al., 2018). One case of congenital ZIKV syndrome was recently described through infection in Angola, although this was caused by a newer Asian lineage of ZIKV, which would contain recent pathogenic mutations (Sasseti et al., 2018).

2.2 Investigations within the ZIKA-DFA cohort studies

2.2.1 Description of the ZIKA-DFA-FE cohort study

ZIKA-DFA-FE is a French acronym that stands for ‘Zika - Districts Francais d’Amerique - Femmes Enceintes’, which translates to ‘Zika - French Territories in the Americas - Pregnant Women’. Below is a description of the methods of this cohort study, taken from the first supplement our paper published in the *New England Journal of Medicine* (Hoen et al., 2018). A link to the full version of the supplement can be found here at link 1 in Appendix 4 at the end of this manuscript. This cohort study is registered with ClinicalTrials.gov (NCT02916732) and received ethics approval by the Comité de Protection des Personnes Sud-Ouest et Outremer III (CEBH2016/03). Funding for this study comes from the by the French Ministry of Health (Soutien Exceptionnel à la Recherche et à l’Innovation), Laboratoire d’Excellence Integrative Biology of Emerging Infectious Diseases project (ANR-10-LABEX-62-IBEID), the European Union’s Horizon 2020 Research and Innovation Program through the ZikAlliance consortium, and by the Institut national de la santé et da la recherche médicale (INSERM).

- **Background**

After the start of its rapid spread throughout South America in late 2015, it appeared obvious that an outbreak of ZIKV infection would be unavoidable in the Caribbean region. A study group headed by the Centre d’Investigation Clinique (CIC) Antilles therefore decided to implement a prospective observational study on ZIKV infection in pregnant women in the FTA, which include the islands of Guadeloupe, Martinique, Saint-Martin, and Saint-Barthelemy and the territory of French Guyana in South America. The overall population of these territories is about 1.1 million persons. The purpose of these cohorts would be to follow enrolled women until their pregnancy outcomes (ZIKA-DFA-FE), and then to enroll their infants (ZIKA-DFA-BB – to be described very briefly at the end of this section). The Gynecology and Obstetrics departments of the public hospitals of Pointe-à-Pitre, Basse-Terre,

and Saint-Martin in Guadeloupe, Cayenne and Saint-Laurent-du-Maroni in French Guyana, and Fort-de-France in Martinique participated in this study.

- **Research questions**

The main objectives of the ZIKA-DFA-FE study were to describe the clinical manifestations of ZIKV infection during pregnancy, estimate the proportion of microcephaly and other neurological defects among fetuses and infants whose mothers had been infected with ZIKV infection during pregnancy, identify other complications not yet known as possible complications of ZIKV infection, identify factors associated with birth defects /other complications, with a focus on the roles of gestational age at the time of ZIKV infection, symptomatic ZIKV infection, and environmental cofactors.

- **Cohort Recruitment and Sample Size Considerations**

ZIKA-DFA-FE

There was no predetermined sample size for the pregnant women cohort, rather, the aim was reach and enroll the highest proportion of ZIKV infected pregnant women during the epidemic period in the FTA. There were four recruitment methods used. Each recruitment method applied to all women living in the FTA whose pregnancy overlapped with the ZIKV epidemic period for any amount of time. Figure 1 shows the various recruitment methods used in ZIKA-DFA-FE and ZIKA-DFA-BB. The first recruitment method (RM1) was the identification of pregnant women who presented with clinical symptoms of acute ZIKV infection and/or tested positive for ZIKV through serology or RT-PCR, at any time during their pregnancy, either at a local hospital or during their routine antenatal visits. In a second recruitment method (RM2) study enrollment was proposed to all pregnant women in whom a fetal defect was detected during routine pregnancy ultrasound monitoring in the FTA during the epidemic period; this was independent of whether or not they had been symptomatic or tested positive for ZIKV during their pregnancy. If not already enrolled through RM1 or RM2, then any woman who had been pregnant during the epidemic period would still be proposed enrollment on the day of their pregnancy outcome, whether this outcome was delivery of a

live birth (RM3), or delivery of a still birth, experiencing a miscarriage or undertaking a medically indicated abortion (RM4). In order to maximize enrolment, extensive efforts were made by the study team to sensitize all of the local obstetrician/gynecologists to the study objectives, and include all of the large hospitals in the study regions. In this way, doctors throughout the regions could propose enrolment to all women as early as possible in their pregnancy when they presented for routine antenatal care.

As part of ZIKA-DFA-FE, a serum biobank was also established, which aimed to collect samples from all pregnant women in the FTA during the ZIKV epidemic period once per trimester of pregnancy. This would allow eventual confirmation of the trimester of seroconversion for each pregnant woman included in the study. Constitution of this biobank was made possible through referral by all locally sensitized gynecologists/obstetricians and hospital doctors. Indeed a validated tool for ZIKV serology was not available when ZIKA-DFA-FE study began. Therefore, the tubes that were collected by the laboratories in each region were transferred to the Centres de Ressources Biologiques of Martinique and Guadeloupe, which served as biobanks in these territories. In French Guyana, an in-house serological test was made available from the beginning of the ZIKV outbreak by the local Institut Pasteur, which provided results in real time.

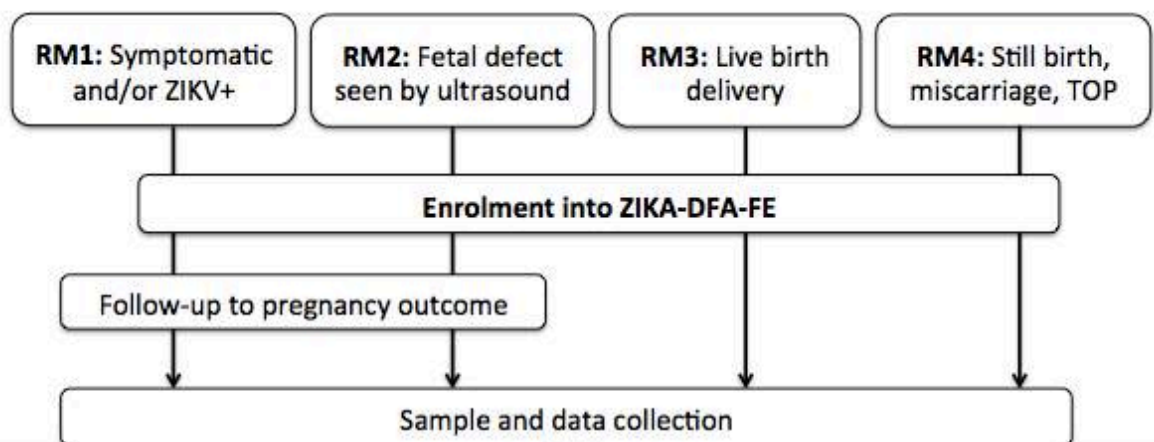


Figure 1: Methods of recruitment into ZIKA-DFA-FE. Note: TOP = termination of pregnancy

- **Follow-up of enrolled participants and data collection**

Through RM1 and RM2 of ZIKA-DFA-FE, women could be enrolled at any moment during their pregnancy. During their enrollment visit, women were interviewed for 30 minutes regarding their life-style practices (e.g. smoking, alcohol intake, mosquito bite exposure) and medical and obstetric history, followed by collection of samples for laboratory testing. Following this, a dedicated obstetrician performed one ultrasound per month on participants and asked the women at each visit about any complications experienced, biological tests and examinations done, and treatments received between visits. These visits lasted approximately 15 minutes each. This meant that there was one enrollment visit and 0-6 ultrasound/follow-up visits per participating woman, depending on the moment in her pregnancy at which she was recruited. If the woman had been enrolled through RM2 (fetal defect had been detected by ultrasound), it was possible that the parents would opt for termination of pregnancy prior to term; in this case follow-up visits would also occur monthly until the day of termination of pregnancy, at which point, further information and samples would be collected if the mother agreed. If recruited into ZIKA-DFA-FE at the time of delivery through RM3, then all data were collected at the time of recruitment (i.e. one visit); this included retrospective data about events occurring/exposures during pregnancy as well as the pregnancy outcome and collection of biological samples. Similarly if women were being recruited into the study due to experiencing a miscarriage, stillbirth or termination of pregnancy (RM4) all data and sample collection was done at one moment on the day of recruitment. The specific information collected for each pregnant woman enrolled and at the timing of this, is indicated in the supplementary material (see link 1 in Appendix 4)

- **Data management and statistical analysis**

Paper forms were used to collect data of participants in hospitals, clinics, and maternities, which were then input into an electronic case report form (CRF) implemented under Ennov Clinical (Clinical Data Management System). Data was systematically checked,

with original records being retrieved to answer queries. The statistical analysis performed on the database varies by topic of analysis and is/will be described separately for each paper produced from these cohorts.

- **Infant cohort (ZIKA-DFA-BB)**

Women with live born infants were proposed enrolment in a follow-up cohort study entitled ZIKA-DFA-BB (BB = bébé = baby). This included infants with or without abnormalities present at birth, as well as a control group of infants born to ZIKV-uninfected mothers. Enrolled infants would be followed for two years in order to observe the clinical and developmental evolution of those with congenital CZS identified at birth and evaluate any late-appearing abnormalities in infants whose mothers were infected with ZIKV during pregnancy, but who were apparently normal at birth. This cohort study is ongoing and will not be discussed further in this thesis.

2.2.2 Original research (full article): Estimation of ZIKV-related birth defects in an epidemic region

In the paper described below, we demonstrate the risk of fetal abnormalities following confirmed ZIKV infection during pregnancy in a large prospective cohort of 546 women from the FTA. The population from this analysis was drawn from the first recruitment method (i.e. proposal of the study to ZIKV symptomatic pregnant women presenting at hospital or clinics) of the ZIKA-DFA-FE cohort study, which has been described in the previous section. At the time that we produced this analysis, two large prospective cohort studies had already been published on the same topic. These two studies were from Brazil and the United States, and the estimates that had been presented by each differed greatly. In Brazil, 125 ZIKV symptomatic pregnancies were followed, with an eventual risk of adverse outcomes of 46%. Alternatively, in the first report of the USZIPR, a Zika-related birth defect risk estimate of 6% was derived. Considering this stark variation, further studies on this subject were (and still are) warranted.

One of our aims in formulating this manuscript was to be as comparable as possible with the case-definitions and abnormalities-categories used by the prospective studies that had been published before us. Ideally this would facilitate bringing the results of studies on these topics together to aid in creating consensus estimates; this would be useful for clinical practice and policy. This also included my taking an in-depth look at birth defects classifications as presented by the research group in Brazil in their supplementary materials of their article, and creating our own supplement (see link 1 in Appendix 4) with a similar format. The idea behind this was to enable detailed comparison of the individual conditions for each infant that were considered as birth defects in order to see whether or not this would explain the differences being seen.

Eventually, our own estimate of an approximate 7% risk of Zika-related birth defects was closely aligned with that of the USZIPR. The reasons for the large differences between the estimates of the FTA and USZIPR studies, when compared to the original study performed in Brazil, remain largely elusive, but will be featured in some discussions later in this thesis manuscript. The PDF of this article can be found in Appendix 5.

Pregnancy outcomes after Zika infection in the French territories of America

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- **Abstract**

Background: The risk of ZIKV-related congenital neurological defects varies from 6% to 42% in the two largest studies of pregnant women published so far. The aim of this study was to estimate this risk in pregnant women with symptomatic ZIKV infection in the French territories of America. *Methods:* From March 2016 onwards, pregnant women diagnosed with symptomatic and PCR-positive ZIKV infection were enrolled in this prospective cohort study. The analysis included all data up to April 27, 2017, date of the last delivery in the cohort. *Results:* Among the 546 women included in the analysis, there were 28 (5.0 %) pregnancy losses and 527 live births. Neurological and ocular defects potentially associated with ZIKV infection were seen in 39 (7.0%, 95% confidence interval = [5.0% - 9.5%]) fetuses and infants: 10 terminations of pregnancy, 1 stillbirth, and 28 live births. Microcephaly was detected in 32 (5.8%) infants and fetuses, of which 9 (1.6%) were severe (-3 standard deviations (SD)). Neurological and ocular defects were more common when ZIKV infection occurred during the first trimester (24/189, 12.7%), compared to infection during second (9/252 = 3.6%) or third trimester (6/114 = 5.3%) (P = 0.001). *Conclusions:* Among pregnant women with PCR-confirmed symptomatic ZIKV infection, birth defects potentially associated with ZIKV infection were present in 7% of fetuses and infants. They were more frequent in fetuses and infants whose mothers had been infected early in pregnancy. Longer-term follow-up of infants is required to assess any manifestations not detected at birth.

- **Introduction**

It has been recognized recently that ZIKV infection during pregnancy can cause severe birth defects (Baud et al., 2017), including microcephaly (Melo et al., 2016), other brain defects (Brasil et al., 2016), and the Zika congenital syndrome (Moore et al., 2017). However, the magnitude of this risk is not clearly defined. It was estimated above 40% in a prospective observational study of women who developed symptomatic ZIKV infection during pregnancy in Brazil (Brasil et al., 2016). In the USZIPR, it was 6% overall and 11% when ZIKV exposure occurred during the first trimester (Honein et al., 2017). The latter risk has been updated recently to 15% (Reynolds et al., 2016). The ZIKV epidemic in the French Territories of America began in early 2016 and presented another opportunity to assess the risk of ZIKV-related congenital neurological defects in a population of pregnant women living in a territory exposed to a ZIKV outbreak. The centralized antenatal and maternal facilities enabled enhanced surveillance of all pregnancies during the ZIKV epidemic. This study presents the pregnancy outcomes of a cohort of women living in the French Territories of America, who developed symptomatic, laboratory-confirmed ZIKV infection during pregnancy.

- **Patients and methods**

Overview of the ZIKA-DFA-FE study (see link 1 in Appendix 4)

ZIKA-DFA-FE is a cohort study that uses four different recruitment methods in an attempt to capture all women whose pregnancies have overlapped with the ZIKV epidemic period in the French Territories of America. This study is registered with ClinicalTrials.gov (NCT02916732) and received ethics approval by the Comité de Protection des Personnes Sud-Ouest et Outremer III (CEBH2016/03). All participants provided written informed consent.

A key component of ZIKA-DFA-FE cohort is the prospective follow-up, until the end of pregnancy, of women who developed clinical symptoms of ZIKV infection during pregnancy. In accordance with the guidelines by the French High Council of Public Health and the National College of Gynecologists and Obstetricians that were released on January 25, 2016 and February 5, 2016, respectively, whenever a pregnant woman presented to the

outpatient clinic or emergency room of a participating center with symptoms consistent with acute ZIKV infection, she was examined clinically and had blood and urine sampled for confirmation of recent ZIKV infection (Haut Conseil de la Santé Publique, 2017; Collège National Professionnel de Gynécologie et Obstétrique, 2016).

Enrolment criteria

Pregnant women with suspected ZIKV infection were referred to the prenatal diagnosis center in each territory, where they were tested for ZIKV infection and invited to consent to participate in ZIKA-DFA-FE. Women were included in this analysis if they met all of the following criteria: 1) ongoing pregnancy at any gestational age; 2) development of clinical symptoms consistent with acute ZIKV infection, with at least one of pruritic skin rash, fever, conjunctival hyperemia, arthralgia, and myalgia; and 3) laboratory confirmation of recent ZIKV infection, based on a positive ZIKV RT-PCR test on serum and/or urine. The date of ZIKV infection was considered to be the date of the first ZIKV-related symptom onset.

Follow-up and cohort endpoints

Once enrolled, women underwent monthly clinical and ultrasound examinations until reaching a pregnancy outcome. During the monthly follow-up visits, the clinician also inquired as to events that may have occurred (e.g. pregnancy complications, treatments received) since the previous visit. If a fetal defect was identified during ultrasound follow-up, foetal magnetic resonance imaging (MRI) was performed and a close laboratory and ultrasound follow-up was conducted, as reported elsewhere (Pomar et al., 2017; Schaub, Gueneret et al., 2017; Schaub, Vouga, et al., 2017). The endpoint for each woman enrolled in the cohort was the pregnancy outcome: delivery of live born infants with or without birth defects, miscarriage, medical termination of pregnancy (TOP), or stillbirth.

Data and sample collection in mothers

During the enrolment visit, sociodemographic data were collected for each woman. These included age, ethnic origin, residence, education, professional activity, and lifestyle factors. Clinical information including the number of previous pregnancies and live births, prior history of adverse pregnancy outcomes, significant medical history, body mass index, symptoms of ZIKV, gestational age, and any clinically significant medical event during pregnancy was collected during this baseline visit, as well as a blood and urine sample.

Laboratory tests included detection of ZIKV by RT-PCR (Real Star[®] Zika Virus RT-PCR Kit 1.0, Altona diagnostics) in blood and urine at baseline in all women and at the end of pregnancy in case of fetal death, TOP, or stillbirth. In addition, results of TORCH serologic tests routinely performed during pregnancy in the French territories of America were recorded, which included syphilis, toxoplasmosis, rubella, and HIV (Tolan, 2008; de Jong et al., 2013). Cytomegalovirus (CMV) serology was performed only on an elective basis, when fetal abnormalities were detected.

Data collection in infants

For live born infants, maternal and cord blood samples were collected on the day of delivery and sera were frozen. From these infants, the following information was also collected on the day of birth: gestational age, length, weight, and head circumference, Appearance, Pulse, Grimace, Activity, and Respiration (APGAR) score at 5 minutes of life, and a standardized clinical examination.

Pregnancy outcomes

Pregnancy outcomes included live birth delivery (with or without abnormalities) or pregnancy loss through miscarriage, TOP, or stillbirth. For purpose of comparability, miscarriage was defined as intrauterine fetal death earlier than 20 weeks gestational age. Stillbirth was defined as intrauterine fetal death at or after 20 weeks gestational age or intrapartum death during delivery (Honein et al, 2017).

Likewise, we summarized our data into the two mutually exclusive categories of birth defects potentially associated with ZIKV infection: 1) brain abnormalities with or without microcephaly regardless of the presence of additional birth defects; and 2) neural tube defects and other early brain malformations, eye abnormalities, and other consequences of central nervous system dysfunction among those who had neither evident brain abnormalities nor microcephaly (Honein et al, 2017). Consequences of central nervous system dysfunction included conditions such as arthrogyriposis, clubfoot, congenital hip dysplasia, and congenital deafness. In case of live birth, microcephaly was defined as moderate when head circumference was between -3 SD and -2 SD and severe when head circumference was < -3 SD, based on the International Fetal and Newborn Growth Consortium for the 21st century (INTERGROWTH-21st) standards (<http://intergrowth21.ndog.ox.ac.uk/>) for gestational age and sex. Moderate microcephaly was further defined as proportionate or disproportionate depending on whether the neonate was small for gestational age (von der Hagen M, 2014). Small for gestational age was defined as having a weight < -1.28 SD according to the INTERGROWTH-21st standards for gestational age and sex. In case of pregnancy loss or medical TOP, autopsy measurements when available and the last ultrasound data were used to assess microcephaly, which was defined as a head circumference of < -3 SD when ultrasound data were considered.

In addition, we specified the number of cases who had any of the severe neurological birth defects that are included in the currently proposed definition of CZS, i.e. one or more of severe microcephaly (< -3 SD), brain abnormalities with a specific pattern of damage (e.g. calcifications, ventriculomegaly, cortical malformations), damage to the back of the eye, joints with limited range of motion (e.g. clubfoot), or hypertonia that restricts body movement (e.g. arthrogyriposis) (Moore et al, 2017).

Statistical analysis

The proportion of fetuses/infants with birth defects potentially associated with ZIKV infection was estimated by trimester of ZIKV infection for pregnant women, and compared across the three groups using Fisher's exact test. Data were analyzed by using Stata 13

(StataCorp LP Lakeway, Texas, United States of America). All authors vouch for the completeness and accuracy of the data and analyses presented.

- **Results**

From March 2, 2016 onwards, 1152 women were evaluated at prenatal diagnosis centers for suspicion of acute ZIKV infection. Of these, 108 were not enrolled in ZIKA-DFA-FE (36 refusals, 48 lost during the recruitment process, 16 less than 18 years, and 8 living outside the French territories of America), 458 had a negative ZIKV RT-PCR, and 25 had no symptoms of the pre-specified list, leaving 561 women with symptomatic, PCR-confirmed, ZIKV infection for analysis in this study (Figure 2). Of these, six (1.1%) women were excluded after updated information on eligibility criteria and nine (1.6%) women were lost to follow-up. For the latter 9 women, fetal ultrasound follow-up was normal, with the last available ultrasound from the 3rd trimester for 5 women and from the 2nd trimester for 4 women. Among the 546 women whose pregnancy outcome was known, there were 9 twin pregnancies. We were therefore able to describe pregnancy outcomes for 555 infants and fetuses (Figure 2).

The 9 twin pregnancies resulted in 17 live births and one miscarried fetus. No abnormalities were detected in any of the live born neonates from twin pregnancies. In the twin pregnancy that resulted in one live birth and one miscarried fetus, the mother had been infected with ZIKV during the sixth week of pregnancy, and the loss of one fetus occurred at 10 weeks of gestation, with the other fetus being carried until 41 weeks of gestation and born healthy without any abnormalities. Table 2 shows the main characteristics of the 546 women (mean age, 29.7 years) with known pregnancy outcomes. Table 3 shows the main characteristics of ZIKV infection in these women. Co-infections with TORCH microorganisms are summarized in Table 4.

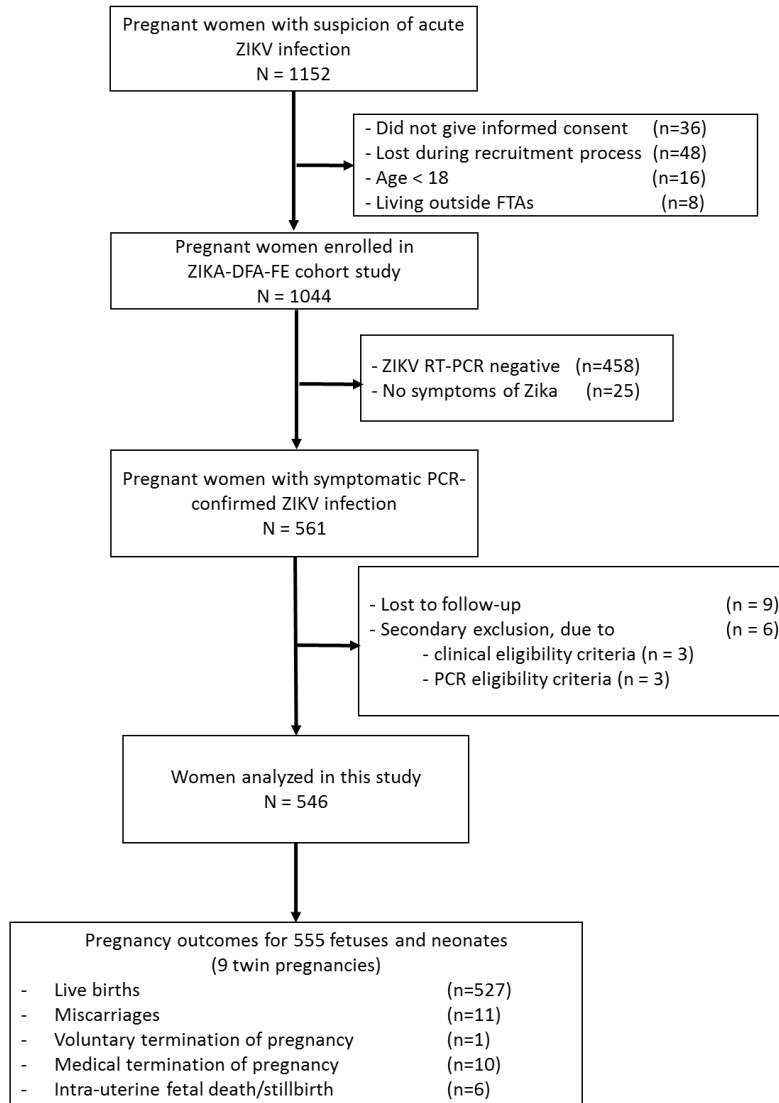


Figure 2: Disposition of the 561 pregnant women enrolled in ZIKA-DFA-FE with symptomatic laboratory-confirmed ZIKV infection during the Zika outbreak in the 3 French territories of America in 2016

Overall, the mean number of fetal ultrasound examinations performed between the date of ZIKV infection and pregnancy outcome was 3.5 and 2.2 when ZIKV infection occurred during the first and the second trimester, respectively. There were 28 (5.0%) pregnancy losses, including 11 miscarriages, 10 medical TOP, 6 stillbirths, and 1 voluntary abortion. Among the 527 live births, 75 neonates (14.2%) were delivered through emergency caesarian section. There were 31 (5.9%) infants hospitalized immediately after birth, 7 of

whom (1.3%) were admitted into the neonatal intensive care unit. There were 8 (1.5%) infants with an APGAR score <7 at 5 minutes after birth. These proportions were not different by trimester of infection.

Neurological and ocular abnormalities potentially associated with ZIKV infection were observed in 39 (7.0%, 95% confidence interval = [5.0%-9.5%]) infants/fetuses (28 live births, 10 medical TOP, and 1 stillbirth). Microcephaly was detected in 32 (5.8%) infants/fetuses, with 9 (1.6%) severe, 9 (1.6%) moderate-disproportionate, and 14 (2.5%) moderate-proportionate. Additional defects were observed in only one of the 23 infants with moderate microcephaly; this was a case of medical TOP with moderate-disproportionate microcephaly. Severe microcephaly or other brain abnormalities described in CZS were seen in 17 (3.1%) fetuses/infants. Neurological and ocular abnormalities were more frequent when ZIKV infections had occurred during the first trimester (n=24, 12.7%), compared to second (n=9, 3.6%) or third trimester (n=6, 5.3%) (P = 0.001). The same was true for severe microcephaly (3.7%, 0.8%, 0.0%, respectively, P= 0.02), and Zika congenital syndrome (6.9%, 1.2%, and 0.9%, respectively, P =0.002). The risk of birth defects potentially associated with ZIKV infection and those included within Zika congenital syndrome in Guadeloupe and Martinique were both similar, with 7.2% and 7.5%, and 3.6% and 2.8%, in each territory respectively. There was no statistical association between any potentially identifiable toxic pre-natal exposures (i.e., larvicides, repellants, alcohol, tobacco, illicit drugs) and birth defects.

Table 2: Demographic, social, and pregnancy characteristics of the 546 women with symptomatic and PCR-confirmed ZIKV infection

	n	%
Residence		
French Guyana	24	4.4
Guadeloupe	245	44.9
Martinique	277	50.7
Age in years, mean (SD; Range)	29.7	(6.2; 18-46)
Occupation		
Student	23	4.2
Artisan, merchant, business owner	30	5.5
Highly qualified professional	55	10.1
Intermediate professions	56	10.3
Employee	177	32.4
Labourer/Factory worker/Farmer	5	0.9
Unemployed	187	34.2
Missing/Does not wish to respond	13	2.4
Medical history		
Arterial hypertension	23	4.2
Diabetes	8	1.5
Sickle cell disease	4	0.7
Number of previous pregnancies		
0	131	24.0
1	153	28.0
2	126	23.1
≥3	136	24.9
Previous pregnancy adverse outcomes		
Congenital abnormalities	6	1.1
Still births	10	1.8
TOP	10	1.8
BMI prior to pregnancy, mean (SD)	26.1*	(6.3)
Lifestyle practices during this pregnancy		
Alcohol consumption	2	0.4
Drug use	6	1.1
Smoking	23	4.2
Use of mosquito repellents	445	81.5
Use of larvicides	337	61.7

Data are displayed as n (%) unless otherwise stated. *BMI missing in 90 women (16.5%)

Table 3: Characteristics of infection in the 546 women with symptomatic and PCR-confirmed ZIKV infection

		n	%
Trimester of symptomatic ZIKV infection			
	First	185	33.9
	Second	249	45.6
	Third	112	20.5
Number of symptoms at time of Zika diagnosis			
	1	66	12.1
	2	111	20.3
	3	121	22.2
	4	95	17.4
	5+	153	28.0
Zika symptoms			
	Rash	519	95.1
	Arthralgia	300	54.9
	Itching	263	48.2
	Conjunctival hyperemia	199	36.4
	Headache	161	29.5
	Myalgia	128	23.4
	Fever	123	22.5
	Limb swelling	104	19.0
	Pain behind eyes	102	18.7
	Petechiae	38	7.0
	Bleeding	1	0.2

No fetus abnormality or birth defect was observed in any of the cases of co-exposure to ZIKV and syphilis (n=4), HIV (n=2), toxoplasmosis (n=3), or cytomegalovirus (n=1). Thirty-one women had an amniocentesis performed during the course of their pregnancy, with 27 karyotypings and 20 ZIKV RT-PCR assays. All karyotypes were normal except for one pericentric inversion of chromosome 2 and 7 ZIKV positive cases detected through RT-PCR. Additionally, 6 (1.1%) non-neurological birth defects were detected through this cohort and are described in the third supplementary appendix (see link 1 in Appendix 4) where a detailed description of all birth defects can be found.

Table 4. Results of ZIKV and TORCH testing in the 546 women with symptomatic and PCR-confirmed ZIKV infection

	Time of Zika infection					
	1st Trimester		2nd Trimester		3rd Trimester	
ZIKV RT-PCR Positive	185	100.0	249	100.0	112	100.0
ZIKV RT-PCR						
Blood and urine positive	121	65.4	159	63.9	66	58.9
Blood only positive*	40	21.6	63	25.3	23	20.5
Urine only positive**	24	13.0	27	10.8	23	20.5
Syphilis						
# Women tested	150	81.1	206	82.7	87	77.7
Positive	4	2.7	0	0	0	0
HIV						
# Women tested	161	87.0	210	84.3	97	86.6
Positive	1	0.6	1	0.4	0	0
Toxoplasmosis (IgM)						
# Women tested	165	89.2	235	94.4	105	93.8
Positive	1	0.6	0	0	2	1.9
Rubella (IgM)						
# Women tested	152	82.2	222	89.2	97	86.6
Positive	0	0	0	0	0	0
Cytomegalovirus (IgM)						
# Women tested	20	10.8	30	12.0	14	12.5
Positive	0	0	1	3.3	0	0
Any TORCH positive	6	3.2	2	0.8	2	1.8

*Urine results are negative, unknown, or not done **Blood results were negative, unknown, or not done

Note: in highly febrile women, DENV RT-PCR was performed on blood samples. Of the 267 tests performed, only 1 was positive.

Table 5: Pregnancy outcomes, by trimester of ZIKV infection, in the 546 pregnant women and 555 fetuses and neonates (9 twin pregnancies)

	Trimester of Zika infection		
	1 st	2 nd	3 rd
NUMBER FETUSES AND NEONATES	189	252	114
PREGNANCY OUTCOMES			
Pregnancy losses	24 (12.7)	4 (1.6)	0
Miscarriage	11 (5.8)	0	
Voluntary termination of pregnancy	1 (0.5)	0	
Medical termination of pregnancy (TOP)	9 (4.8)	1 (0.4)	
Stillbirth	3 (1.6)	3 (1.2)	
Live births	165 (87.3)	248 (98.4)	114 (100)
Live births with no prenatal ultrasound examination after ZIKV infection	13 (7.9)	28 (11.3)	55 (48.3)
ABNORMALITIES IN ANY FETUS/ INFANT			
Neurological or ocular birth defects⁺⁺	24 (12.7)	9 (3.6)[*]	6 (5.3)
Severe microcephaly	7 (3.7)	2 (0.8)	0
Moderate microcephaly (disproportionate) ^{**}	4 (2.1)	2 (0.8)	3 (2.6)
Moderate microcephaly (proportionate) ^{**}	8 (4.2)	4 (1.6)	2 (1.8)
Intracranial calcifications	8 (4.2)	0	0
Ventriculomegaly	7 (3.7)	1 (0.4)	0
Lissencephaly	2 (1.1)	0	0
Other brain abnormalities	8 (4.2)	1 (0.4)	0
Neural tube defects	1 (0.5)	0	0
Eye abnormalities	0	0	0
Consequences of CNS dysfunction	1 (0.5)	0	1 (0.9)
Other birth defects	2 (1.1)	3 (1.2)	1 (0.9)
Chromosomal	0	1 (0.4) ⁺	0
Skeleton abnormalities	2 (1.1)	1 (0.4)	1 (0.9)
Other	0	1 (0.4)	0
ZIKA CONGENITAL SYNDROME	13 (6.9)	3 (1.2)	1 (0.9)

*One of these infant's mothers was co-infected with Parvovirus B19 **Moderate microcephaly (less than -2SD) if detected by autopsy + This infant had Down's syndrome with severe microcephaly ++ Potentially associated with Zika infection. CNS = central nervous system dysfunction

- **Discussion**

The main findings of this cohort study are two-fold. Firstly, it showed that in the FTA the overall risk of neurological/ocular defects potentially associated with ZIKV infection in the offspring of women who developed acute symptomatic PCR-confirmed ZIKV infection during pregnancy was 7.0%. Similarly, the overall risk of birth defects included in the current definition of CZS and severe microcephaly were 3.1% and 1.6%, respectively. Secondly, although birth defects could be observed as a consequence of ZIKV infection at any pregnancy trimester, consistent with previous findings (Honein et al, 2017; Shapiro-Mendoza et al, 2017), it confirmed that the risk of birth defects and CZS was higher when ZIKV infection occurred early in pregnancy, with proportions of 12.7%, 3.6% and 5.3%, and 6.9%, 1.2%, and 0.9% when ZIKV infection occurred in the 1st, 2nd, and 3rd trimesters respectively.

The proportion of fetuses/infants with neurological birth defects (7.0%) in this study is similar to the 6% observed in the US women cohort (Honein et al, 2017) and the 5% more recently reported in the US territories (Shapiro-Mendoza et al, 2017), but is much lower than the 42% proportion observed in the Brazilian cohort (Brasil et al, 2016). The difference does not come from the proportion of infants/fetuses with microcephaly, which is similar in the three studies (5.8%, 3.4%, and 4.1%, respectively). Rather it is in the identification of wider neurological birth defects. The proportion of small for gestational age infants was similar in the FTA and in the Brazilian cohort (13.7% and 9%, respectively), but differences between the FTA and Brazilian cohorts appear when examining the proportion of infants needing admission to neonatal intensive care immediately after birth (1.3% and 21%, respectively), and the proportion of infants with abnormal neurological findings at clinical examination at birth (0.5% and 26.5%). The termination of 10 (1.8%) pregnancies in the FTA (compared to 0% in Brazil) may have led to a decreased rate of neurological abnormalities detected at birth in the FTA compared to Brazil, but this cannot explain the entire difference between the two cohorts. In addition, the intensive use of MRI in the Brazilian cohort may have led to isolated abnormal imaging findings that have not been observed in other studies where the use of MRI has been less frequent. The clinical implications of these findings in Brazil are not yet known and will only be determined through longer-term follow-up of infants.

The strengths of our study include the size and homogeneity of the cohort of pregnant women living in a territory exposed to an outbreak of ZIKV and prospectively followed from acute symptoms and PCR-confirmed ZIKV infection. The diagnosis of ZIKV infection was made by PCR-based ZIKV detection in patients' blood, and the date of infection could be ascertained by consistent dates of clinical symptoms and ZIKV PCR testing. The study was conducted in well-defined geographical areas with high standards of care available to all pregnant women living in these territories. Linkage to care of pregnant women who developed ZIKV infection was effective with a low rate of loss to follow-up of 1.6%. In addition, the results were consistent across the two territories that recruited the largest number of women, Martinique and Guadeloupe.

We acknowledge that our study may have some limitations. Firstly, it focused only on pregnant women who developed acute symptomatic ZIKV infection. Although the rate of complications would be expected to be higher among women with symptomatic compared to asymptomatic infections, an observational study of US women did not find any difference in the rate of birth defects in the offspring of women who had symptomatic compared to asymptomatic ZIKV infection during pregnancy (Honein et al, 2017). A recent study also showed the absence of association between disease severity or viral load and adverse outcomes (Halai et al, 2017). Secondly, we were not able to fully assess the presence of birth defects potentially associated with ZIKV infection in the eleven miscarriages, as well as in two of the six stillbirths, the only case of voluntary abortion, and 96 (18.2%) of the 527 live births who did not have prenatal ultrasound examination after ZIKV infection. While missing ultrasounds may have led to under-diagnosis of ZIKV-related birth defects, it is noteworthy that in our cohort, only one live birth had an isolated brain abnormality (ventriculomegaly) following an infection during the second trimester of pregnancy, detected by MRI, in the absence of clinical abnormalities. All other live births with ZIKV-related defects had at least one abnormality that would have been picked up by the clinical examination at birth, whether it was microcephaly, clubbed feet, or neural tube defect (spina bifida). Also, the majority of missing ultrasounds implicated pregnancies in which infections took place during the third trimester, where the consequences of infection were shown to be very limited in the other children of the same cohort. Thirdly, our endpoint was based on pregnancy ultrasound and neonatal clinical examinations and did not include postnatal ultrasound or specialized hearing

and ophthalmological examinations. We believe that it had limited impact on the rate of birth defects that could have been identified if all neonates had been subject to brain imaging early after birth. Indeed, it has been shown that when ZIKV infection occurs during the first or early second trimester, all brain abnormalities can be detected by ultrasound before 28 weeks of gestation (Schaub et al, 2017). Another study showed that none of 103 infants with normal prenatal ultrasound and clinical examination at birth had anomalies attributable to ZIKV at brain MRI performed after birth (Mejdoubi et al, 2017). Still the absence of microcephaly at birth does not exclude the delayed development of microcephaly or other ZIKV-related brain and other abnormalities (van der Linden, Pessoa, et al, 2016). This information is now being collected as part of a cohort study of the infants, including regular clinical examinations with specialized hearing and ophthalmological testing. Only the longer-term follow-up of the children born to these women, initiated as a follow-up to this study, will be able to identify full spectrum of ZIKV-related complications.

In conclusion, among pregnant women with PCR-confirmed symptomatic ZIKV infection, birth defects potentially associated with ZIKV infection were present in 7% of infants and fetuses. They were more common in fetuses and infants whose mothers had been infected early in pregnancy. Longer-term follow-up of infants is required to assess for late-onset manifestations not detected at birth.

2.2.3 Original research (full article): Comparison of ZIKV-related birth defects in ZIKV exposed and unexposed pregnancies

In our work on ZIKV-related birth defects in the FTA we had derived an overall risk of 7.2%, with a 12.7%, 3.6% and 5.3%, risk per trimester of pregnancy for women with symptomatic ZIKV infection, respectively. However, many of these abnormalities (59%) were cases of moderate microcephaly, defined, as in similar cohort studies, as having a head circumference of less than -2SD according to a pooled international growth standard for gestational age and sex (in our case, this was the INTERGROWTH-21st standards). In the birth records for 96% of the infants (i.e. 22/23) with an eventual classification of moderate microcephaly, there were no abnormalities noted (i.e. no diagnosis of microcephaly); this infant was defined as abnormal not by a pediatrician, but rather, by our analysis program. This left us with a feeling that, although technically ‘microcephaly’ according to the internationally accepted definition at the time as well as by the definition used in other cohort studies on the subject, it may be incorrect to label these infants as being abnormal due to ZIKV exposure. In that publication, we were able to distinguish between these infants, and those with more severe abnormalities, based on a further narrowed down classification using the proposed definition of CZS. However, in that report, without a ZIKV-negative control group to match our prospective cohort of symptomatic ZIKV-positive women, we were unable to link moderate microcephaly with being affected by the virus.

In the paper that will be presented below, we compare the proportion of live born infants with birth defects that are deemed ‘potentially linked to ZIKV infection’ in a retrospective cohort of ZIKV non-exposed pregnancies from Guadeloupe during the epidemic period, to the subset of women with confirmed ZIKV infection who had live born infants from the same region out of our previously published symptomatic prospective cohort. Considering that no prospective ZIKV negative cohort could be identified, comparison with this retrospective cohort was a compromise, rather than the perfect solution to understanding the baseline of adverse pregnancy outcomes and birth defects in the population. Through a retrospective cohort we were limited to comparing abnormalities in live born infants, as we were unable to capture miscarriage, stillbirth, and pregnancy termination due to abnormalities

in the ZIKV non-exposed pregnancies. The restraints of this approach will be discussed in the article.

Our findings, as you will see, lead to a significant adjustment of our original estimate of the proportion of infants with birth defects that can be confidently linked to ZIKV infection. This research highlights the importance of having a control group in cohort studies examining the risk of ZIKV-related birth defects.

Note: At the time of submitting this thesis manuscript to the referees on October 17th 2018, the manuscript below is submitted at *BMJ*. The PDF of this article can be found in Appendix 5.

Comparison of the risk of birth defects in live births of pregnant women infected and not infected by Zika virus in Guadeloupe, 2016-2017

Anna L Funk, Bruno Hoen, Ingrid Vingadassalon, Catherine Ryan, Philippe Kadhel, Kinda Schepers, Stanie Gaete, Benoit Tressières, Arnaud Fontanet

- **Abstract**

Objectives: To compare the risk of ZIKV-related congenital abnormalities among exposed and non-exposed live born infants in the FTA. Design: Cross-sectional study of pregnant women and live born infants without exposure to ZIKV, compared to those from a previously reported prospective cohort with confirmed ZIKV exposure. Setting: Guadeloupe (France) during the 2016 ZIKV epidemic period. Participants: 484 ZIKV negative pregnant women and their 490 live born infants. Main outcome measures: ZIKV-related congenital abnormalities as measured by clinical examination at birth and foetal ultrasound imaging during pregnancy Results: Of the 490 live born infants without in-utero exposure to ZIKV, 42 infants (8.6%) had indication of neurological abnormalities known as ‘potentially linked to ZIKV infection’; all but one of these were microcephaly without any other brain or clinical abnormalities. When compared to the 241 live born infants from pregnancies with ZIKV exposure, the proportion of such abnormalities was similar (6.6%, $p=0.36$). Conclusions: Isolated microcephaly and other mild neurological conditions were as prevalent among infants with and without ZIKV exposure in-utero. As a result, when considering 249 fetuses and infants of women with confirmed ZIKV infection in Guadeloupe, only one (0.4%) live born infant and three (1.2%) medically-aborted fetuses had birth defects that could be linked to ZIKV infection. Trial registration: This study is registered at ClinicalTrials.gov (NCT02916732).

- **What is already known**

- Based on different studies, the risk of birth defects following ZIKV infection during pregnancy varies from as low as 5 to as high as 46%.

- The most precise estimate obtained through a prospective cohort study with PCR-confirmed diagnosis of infection in the pregnant mother comes from French territories in the Americas, with a risk estimated at 7.0% (95%CI: 5-10%).
 - However, in the absence of a control group of ZIKV non-infected pregnant women, it is difficult to attribute all observed birth defects to ZIKV infection.
- **What this study adds**
 - This study found no difference in the prevalence at birth of anthropometric and other mild abnormalities that may be potentially associated with ZIKV infection when comparing a group of ZIKV-infected and ZIKV non-infected women.
 - In both exposure groups, most abnormalities described at birth were isolated microcephaly, i.e. without any additional clinical or imaging abnormalities.
 - This study leads to a reduction of our estimate of the risk of ZIKV-related birth defects among women infected during pregnancy down to 1.6% (95% CI: 0.4% - 4.1%), with no risk related to ZIKV infection in the 3rd trimester of pregnancy.

- **Introduction**

Since the first evidence surfaced that linked ZIKV to foetal microcephaly and other brain abnormalities (Calvet et al., 2016; Cordeiro et al., 2016), key research priorities have been to define the range of defects associated with ZIKV infection during pregnancy, as well as to establish the risk of a foetus or infant being affected by them following infection during pregnancy. A multitude of case-series and case-control studies of foetuses and infants exposed to ZIKV in-utero have now been summarized to establish a preliminary definition of CZS that includes a range of ocular abnormalities and neurological defects, such as microcephaly, structural brain abnormalities (e.g. calcifications, lissencephaly, ventriculomegaly), consequences of central nervous system dysfunction (e.g. congenital contractures, abnormal muscle tension, hearing impairment), swallowing disorders, irritability, seizures, neurodevelopmental issues, and others (de Araujo et al., 2018; de Oliveira Dias et al., 2018; Krow-Lucal et al., 2018; Moore et al., 2017; Oliveira-Filho et al., 2018; Santa-Rita et al.,

2017; Wheeler, 2018). In addition, studies from Brazil, the United States of America, and the FTA, have attempted to answer the question of how likely it is for the foetus or infant to be affected by any of these negative outcomes after infection during pregnancy, deriving differing risks of 46% (95%CI: 37-56%), 5% (95%CI: 4-6%), and 7% (95%CI: 5-10%), respectively (Brasil et al., 2016; Hoen et al., 2018; Shapiro-Mendoza et al., 2017). In addition, a retrospective cohort study of 86 pregnancies in Colombia found a 2.4% (95%CI: 0.3-8%) risk of adverse foetal outcomes; all of these were microcephaly cases without indication of brain abnormalities or clinical signs (Rodriguez-Morales et al., 2018).

For the purpose of determining those birth defects that can actually be attributed to ZIKV in an exposed population, the estimation of the baseline level of birth defects in an appropriate ZIKV non-infected control group is necessary. In Brazil, a prospectively followed control group of 61 symptomatic pregnant women negative for ZIKV by RT-PCR was compared to 125 symptomatic pregnant women showing evidence of ZIKV infection; they found that total adverse outcomes were significantly less in women without evidence of ZIKV (11.5% versus 46.0%, $p < 0.001$), although for some specific outcome categories, such as foetal demise and proportion of infants with microcephaly, there were no differences between the two groups (Brasil et al., 2016). Two other prospective studies have used control groups: a prospective ultrasound study examined foetopathy in French Guiana and found central nervous system abnormalities in 9.0% and 4.3% of the ZIKV-exposed and non-exposed foetuses, respectively (Pomar et al., 2017), and a prospective study of 29 ZIKV exposed pregnancies compared to 518 ZIKV non-exposed in the United States found no difference in outcomes between the two (Adhikari et al., 2017).

In the French Territories in the Americas, in the absence of co-circulation of viral infections with similar symptoms at the time of the ZIKV epidemic, it was not possible to enrol a non-ZIKV symptomatic control group as done in Brazil. We therefore enrolled women and their live born infants at the time of delivery, known for not being infected with ZIKV during pregnancy. Thus, we were able to compare the proportion of live births with anthropometric abnormalities, including microcephaly and small weight for gestational age, and other neurological abnormalities in the recently published prospective cohort of ZIKV symptomatic women with that of a control group of ZIKV non-infected pregnant women and

their live born infants (Hoen et al., 2018). To optimize the comparability of the two groups and study environment, the analysis was restricted to the women from Guadeloupe where sufficient numbers were available.

- **Methods**

Study design and participants

The ZIKA-DFA-FE cohort study (a French acronym representing “Zika in the French Territories in the Americas in Pregnant Women”), which has been described elsewhere (Hoen et al., 2018), used four different recruitment methods in an attempt to capture all women whose pregnancies overlapped with the ZIKV epidemic period, 2016-2017, in the FTA (Guadeloupe, Martinique, French Guyana). These included: enrolment of women presenting to hospital with symptoms consistent with ZIKV infection, enrolment of pregnant women for which a foetal abnormality was detected during routine ultrasound, enrolment of pregnant women for which there was foetal demise, and finally, enrolment of pregnant women not yet included through other methods who presented at participating hospitals to deliver during and up until nine months following the ZIKV epidemic period. Those final women recruited at delivery in Guadeloupe, and their live born infants, were used for the study presented in this article.

Procedures

At the time of admission to hospital for labour, each eligible woman was informed of the study and invited to participate; oral consent was obtained before delivery and written informed consent was obtained before delivery whenever possible or within 24 hours after delivery otherwise. A questionnaire including socio-demographic data, such as age, ethnic origin, residence, education, professional activity, and lifestyle factors, was administered. Clinical information, including the number of previous pregnancies, history of adverse pregnancy outcomes, significant medical history, symptoms of ZIKV experienced during

pregnancy, and any clinically significant medical event during pregnancy, was also collected at this time. From the live born infants of participating women, clinical data such as gestational age, length, weight, and head circumference, APGAR score at 1, 5 and 10 minutes of life were collected on the day of birth, and a standardized clinical examination was carried out in the first four days of life. After enrolment, the medical files of participating women were retrospectively reviewed and data were collected on clinical and ultrasound examinations that had been performed during the pregnancy.

Blood samples were collected from all participating women recruited at the time of delivery. These were tested for serological presence of ZIKV, including IgG, using the Euroimmun ZIKV IgG immunoassay (Euroimmun, Medizinische Labordiagnostika AG, Lübeck, Germany). In addition, some women had had other biological samples collected during the pregnancy that were tested for the presence of ZIKV by serological tests and/or by RT-PCR; when available these results were also taken into account. Results of TORCH serologic tests that were routinely performed during pregnancy in the French territories in the Americas were recorded, which included syphilis, toxoplasmosis, rubella, and HIV (Tolan, 2008; de Jong et al., 2013). CMV serology was performed only on an elective basis, for high-risk pregnancies or when foetal abnormalities were detected.

Statistical analysis

Out of the women enrolled in the study, data was included in this analysis if they gave birth in Guadeloupe, had a confirmed negative IgG serology test for ZIKV from maternal blood taken at time of delivery as well as no other positive ZIKV tests during pregnancy, and if their infant was live born. Evidence (i.e. seen and reported by a clinician) or recollection of symptoms evocative of ZIKV infection during the pregnancy was not an exclusion criterion.

Microcephaly was defined as moderate when head circumference was between -2 SD and -3 SD and severe when head circumference was less than -3 SD, based on the INTERGROWTH-21st standards (<http://intergrowth21.ndog.ox.ac.uk/>) for gestational age and sex. Moderate microcephaly was further defined as proportionate or disproportionate

depending on whether the neonate was small for gestational age (Brasil et al., 2016; Hoen et al., 2018; von der Hagen et al., 2014). Small for gestational age was defined as having a weight less than -1.28 SD according to the INTERGROWTH-21st standards for gestational age and sex (Brasil et al., 2016; Hoen et al., 2018).

In addition to determination of anthropometric abnormalities, we reviewed clinical examination records and ultrasound files of participants for evidence of birth defects that are considered to be potentially associated with ZIKV infection according to the current definition of CZS, including: structural brain abnormalities (e.g. calcifications, ventriculomegaly, lissencephaly), neural tube defects and other early brain malformations, eye abnormalities, hearing impairment, and other consequences of central nervous system dysfunction (e.g. arthrogyriposis, clubfoot) (Moore et al., 2017; Hoen et al., 2018). Other birth defects that are not currently considered to be associated with ZIKV infection during pregnancy (e.g. skeletal and other malformations) were also noted.

Baseline characteristics of women with ZIKV infection during pregnancy were compared to those of women without ZIKV infection during pregnancy using the Student's t-test for continuous variables and chi-square or Fisher's exact test for categorical variables. The proportion of infants with anthropometric abnormalities and other birth defects was compared to data of live born infants whose mothers had RT-PCR confirmed ZIKV infection during pregnancy within the same setting and timeframe in Guadeloupe (Hoen et al., 2018), using Fisher's exact test. Data were analysed using Stata 13 (StataCorp LP Lakeway, TX, USA).

The ZIKA-DFA-FE study is registered with ClinicalTrials.gov (NCT02916732) and received ethics approval by the Comité de Protection des Personnes Sud-Ouest et Outremer III (CEBH2016/03).

Patient involvement

The pregnant women and their infants were not involved in the development of the research question or design of the study. Each woman was notified of her ZIKV status

following testing by her attending physician. Women have been given information to able them to contact the study investigators to receive information on the results of the study.

- **Results**

Participants

Of 1484 women enrolled at delivery in Guadeloupe, 1088 had available ZIKV test results. Of these, 588 showed evidence of ZIKV infection through either serological or RT-PCR testing, 16 had negative RT-PCR results but indeterminate ZIKV serological tests, and 484 were confirmed negative by serological tests at the time of delivery with no other positive test seen during pregnancy. Of these 484 ZIKV non-infected women, 6 had twin pregnancies; 490 live born infants were therefore considered in this analysis. In the previously published prospective cohort by Hoen and colleagues (2018) of the 254 pregnant women in Guadeloupe who had symptomatic RT-PCR confirmed ZIKV infection during pregnancy and were enrolled into the prospective cohort, nine pregnancies were excluded either because of loss to follow-up (n=5) or for mistaken inclusion in the cohort (n=4). Of the remaining 245 pregnancies, there were four sets of twins, and eight cases of foetal demise, including: two miscarriages and three stillbirths all without evidence of neurological birth defects, and three cases of medical abortion all with evidence of neurological abnormalities potentially linked to ZIKV infection. The lack of neurological birth defects was confirmed via autopsy for one of the two miscarriages, and all three of the stillborn infants. Therefore, we were able to compare 490 ZIKV-unexposed live born infants with 241 ZIKV exposed live born infants (with ZIKV exposure confirmed by RT-PCR), all born to mothers living in Guadeloupe during the ZIKV outbreak. See Figure 3.

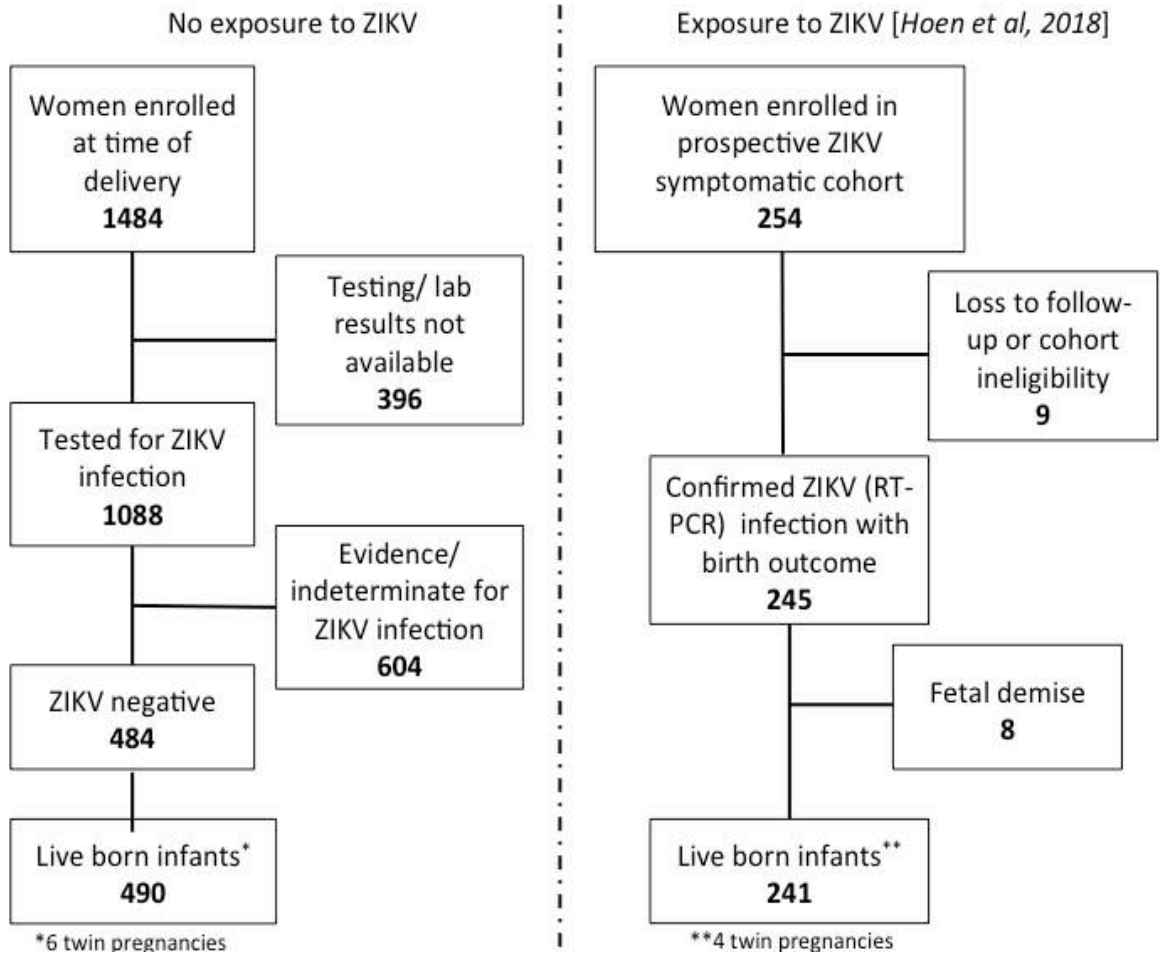


Figure 3: Flow chart of inclusion of ZIKV un-exposed and exposed pregnant women for inclusion of live births in this analysis

The mean age of ZIKV non-infected women was 30.7 years (SD=6.4), and that of ZIKV infected women was 30.0 years (SD=6.3). There was a higher proportion of reported smoking in the ZIKV-infected cohort during pregnancy compared to the ZIKV non-infected: 4.2% (95%CI: 2.0-7.6%) versus 0.6% (95%CI: 0.4-1.8%). There were also more unemployed women in the ZIKV non-infected group compared to the ZIKV-infected one: 48.1% (95%CI: 43.3-52.4) versus 34.6% (95%CI: 28.6-41.0). See Table 6.

Anthropometric and other birth defects in live born infants

Of the 490 live born infants of women without ZIKV infection during pregnancy, 66 (13.5%, 95%CI: 10.6-16.8) were small for gestational age, and 41 (8.4%, 95%CI: 6.1-11.2) had microcephaly. One of these moderate microcephaly cases had a possible genetic aetiology (Adams Oliver syndrome). Of the remaining infants with microcephaly, 29 (5.9%, 95%CI: 4.0-8.4%) had either moderate-proportionate or moderate-disproportionate microcephaly with no other structural brain or clinical abnormalities. Eleven (2.2%, 95%CI: 1.1-4.0) infants had severe microcephaly, seven of which were proportionate. One infant (0.2%, 95%CI: 0.01-1.1) had an abnormality that could be a consequence of central nervous system (CNS) dysfunction, which was clubfoot. Five infants (1.0%, 95CI: 0.3-2.4) had skeletal or other abnormalities that are not currently classified as potentially linked to ZIKV infection, including: skeletal abnormalities of the fingers or toes (n=2) anal imperforation (n=1), and urinary tract abnormalities (n=2). Besides two small-for-gestational-age infants whose mothers were HIV-positive, there were no other abnormalities (including microcephaly) identified in the remaining four infants of TORCH-positive mothers. See Table 7.

There were no significant differences in the anthropometric or other birth defects in live born infants whose mothers had a symptomatic PCR-confirmed ZIKV infection during pregnancy compared to those whose mothers had no evidence of prior ZIKV infection at the time of delivery in Guadeloupe. In two categories, 'neurological or ocular abnormalities without microcephaly' and 'neural tube defects', there were no cases seen in live born infants of women without ZIKV infection during pregnancy, and one case each in infants born to women infected with ZIKV during pregnancy. See Table 8.

Table 6: Baseline characteristics of ZIKV non-infected and infected (Hoen et al., 2018) women from Guadeloupe who delivered live born infants

Characteristic	ZIKV non-infected (N=484)	ZIKV infected (N=237)
Age — yr (mean and range)	30.7 (18-46)	30.0 (18-46)
<i>Missing</i>	0	0
Occupation — no. (%)		
Student	13 (2.7)	6 (2.5)
Artisan, merchant, or business owner	14 (2.9)	17 (7.2)
Professional	75 (15.5)	39 (16.5)
Employee	148 (30.4)	91 (38.4)
Laborer, factory worker, or farmer	1 (0.2)	1 (0.4)
Unemployed	233 (48.1)*	82 (34.6)*
<i>Missing data or declined to respond</i>	0	1 (0.4)
Medical history — no. (%)		
Arterial hypertension	12 (2.5)	7 (3.0)
Diabetes	12 (2.5)	4 (1.7)
Sickle cell disease	7 (1.5)	2 (0.8)
Previous pregnancies — no. (%)		
0	110 (22.7)	65 (27.4)
1	133 (27.5)	57 (24.1)
2	109 (22.5)	52 (21.9)
>=3	130 (26.9)	63 (26.6)
<i>Missing</i>	2 (0.4)	0
Previous adverse pregnancy outcomes — no. (%)		
Congenital abnormalities	2 (0.4)	3 (1.3)
Stillbirth	6 (1.2)	2 (0.8)
Termination of pregnancy for medical reasons	4 (0.8)	4 (1.7)
Lifestyle practices during this pregnancy — no. (%)		
Alcohol consumption	0	0
Drug use	1 (0.2)	2 (0.8)
Current smoker	3 (0.6)*	10 (4.2)*

*Comparison between Zika non-infected and infected women with p=0.001

Table 7: TORCH results in ZIKV non-infected and ZIKV-infected (Hoen et al., 2018) women giving birth in Guadeloupe during the ZIKV epidemic period 2016-2017

		ZIKV non-infected (N=484)	ZIKV infected (N=237)
Positive results on any TORCH test		6 (1.2)	5 (2.1)
Toxoplasmosis	Tested	468 (96.9)	219 (92.4)
	Positive	3 (1.0)	0
Syphilis	Tested	249 (51.6)	184 (77.6)
	Positive	0	2 (0.8)
HIV	Tested	449 (93.0)	188 (79.3)
	Positive	3 (0.6)	2 (0.8)
Rubella	Tested	464 (96.1)	199 (84.0)
	Positive	0	0
Cytomegalovirus	Tested	17 (3.5)	36 (15.2)
	Positive	0	1 (0.4)

Table 8: Abnormalities in *live births* of ZIKV non-infected and infected (Hoen et al., 2018) women in Guadeloupe during the epidemic period in 2016-2017

	ZIKV non-infected (N=490)	ZIKV infected (N=241)
Any neurological or ocular abnormalities	42 (8.6)	16 (6.6)
Microcephaly (<-2SD)	41 (8.4)	12 (5.0)
<i>Severe microcephaly alone</i>	11 (2.2)	1 (0.4)
<i>Moderate-disproportionate alone</i>	10 (2.0)	6 (2.5)
<i>Moderate-proportionate alone</i>	19 (3.9)	4 (1.7)
<i>Severe or moderate microcephaly with other neurological abnormalities</i>	0	0
<i>Severe or moderate microcephaly with a genetic or chromosomal syndrome</i>	1 (0.2)	1 (0.4)
<i>Missing</i>	6 (1.2)	5 (2.1)
Structural brain abnormalities	0	1 (0.4)
Ocular abnormalities	0	0
Neural tube defects	0	1 (0.4)
Consequences of CNS dysfunction	1 (0.2)	2 (0.8) [^]
Other abnormalities	5 (1.0)	2 (0.8)
Skeletal abnormalities	2 (0.4)	2 (0.8) [^]
Other	3 (0.6)	0
Small for gestational age (weight <-1.28 SD) <i>(with or without any of the above abnormalities)</i>	66 (13.5)	33 (13.7)
<i>Missing</i>	1 (0.2)	3 (1.2)

[^] One infant represented in each category as they had both club-foot and polydactyly. **Note:** No significantly different values

- **Discussion**

We found no statistically significant difference in the risk of neurological birth defects in live born infants of ZIKV infected and non-infected women in Guadeloupe during the epidemic period in 2016-2017. These findings have important implications for the estimation of the number of congenital birth defects that can be attributed to ZIKV when the mother is infected during pregnancy. Originally, through a prospective cohort of 249 RT-PCR confirmed ZIKV infected pregnant women in Guadeloupe, we reported a total of 18 (7.2%) neurological or ocular birth defects that were potentially linked to ZIKV infection; 13 (72%) of these were isolated anthropometric abnormalities (i.e. microcephaly) or isolated mild CNS dysfunction defects (i.e. clubfoot) in live born infants (Hoen et al., 2018), which on the basis of the current study cannot be linked to ZIKV exposure. In live born infants, we are left then only with two severe neurological abnormalities (i.e. ventriculomegaly and spina bifida) that have no known alternative etiologies; this is, however, reduced to one abnormality when we remove the neural tube defect (i.e. spina bifida), which is no longer considered as linked to ZIKV infection (Rice et al., 2018; Delaney et al., 2018). As our ZIKV non-exposed study looked back retrospectively from the time of delivery, we are unable to compare the proportion of foetal demise with that of the full 249 ZIKV-exposed foetuses in the prospective cohort. However, the two (0.5%) miscarriages and three (1.2%) stillbirths of the ZIKV-exposed cohort are within the generally expected historical range (Serfaty, 2014; Delabaere et al., 2014), and had no evidence of neurological abnormalities during autopsy for the four that could be examined. There were three (1.2%) further cases of foetal demise in the prospective cohort, which were all medically indicated terminations of pregnancy due to ultrasound detection of severe structural brain abnormalities with microcephaly.¹² If combining the severe neurological abnormalities in foetuses from medically terminated pregnancies (n=3) and live born infants (n=1) our new adjusted estimate of birth defects that could be attributed to ZIKV exposure in Guadeloupe would be 1.6% (95%CI: 0.4-4.1%) (See Figure 4). This would translate to a 4.1% (95%CI: 0.9-11.5%), 0.8% (95%CI: 0.02-4.6%), and 0% (one-sided 97.5%CI:0–6.3%) risk of birth defects per first, second, and third trimester, respectively.

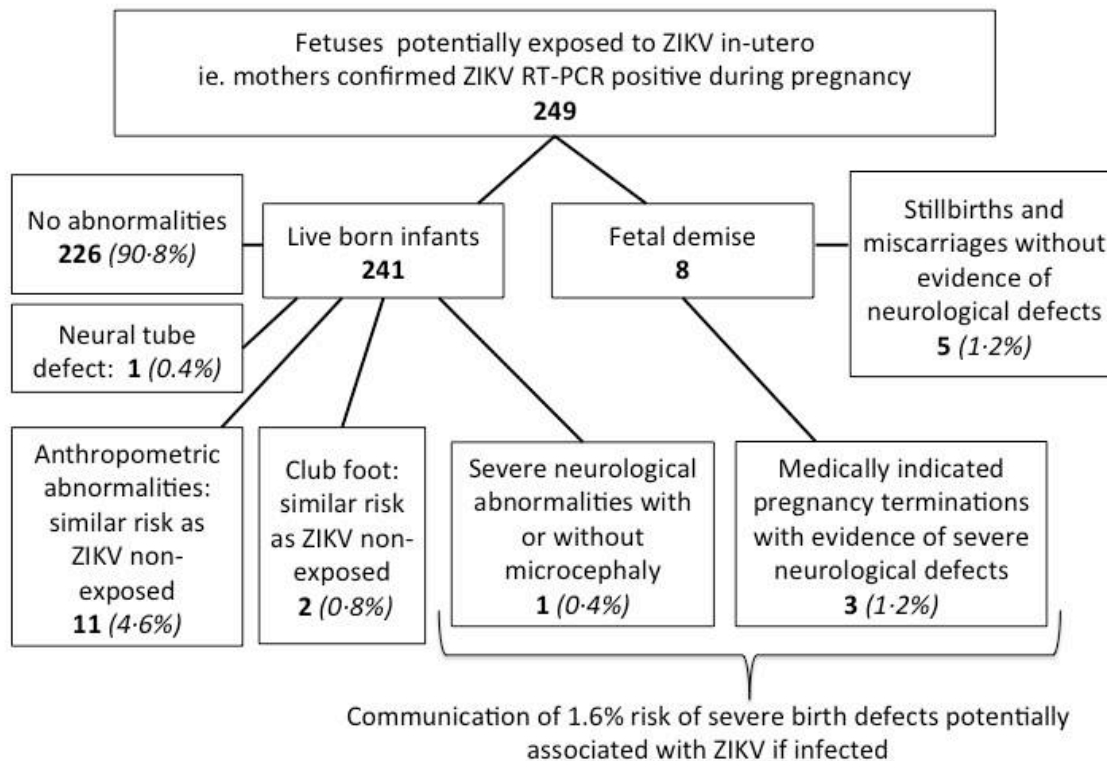


Figure 4: Determination of excess risk of birth defects associated with ZIKV infection, based on autopsy findings and comparison with the ZIKV non-infected group

This is the largest study of ZIKV non-infected women from a defined epidemic region that has been used as a comparative control group against ZIKV-infected pregnant women followed up during pregnancy. This study was conducted in a resource-rich setting where the standard of care for pregnant women is high. The exposure statuses of each of the two groups included in this study were well defined. The ZIKV infected women from Guadeloupe were confirmed via RT-PCR within days of infection (Hoen et al., 2018), and the ZIKV non-infected group was defined so based on the absence of IgG at the time of delivery. Recent studies support a rapid appearance of ZIKV IgG after infection; it was 100% detectable within 11 days following infection for 15 subjects in Guadeloupe and within 26 days following infection for 29 returned travelers to the United States of America (Pasquier et al., 2018; Barzon et al., 2018). The Guadeloupe study also showed consistently positive IgG results throughout follow-up for all subjects, which was up to at least 120 days for around 90% of

subjects included. This evidence suggests that subjects infected with ZIKV, even early in pregnancy, would still have detectable IgG at delivery and would have been excluded from our ZIKV non-infected control group.

This study has several limitations. As we had no directly comparable prospective cohort to that of recently published ZIKV confirmed infected women, we used a group of ZIKV non-infected women delivering at the same hospitals and in the same time period. The prevalence of TORCH infections did not differ significantly between the two groups and in terms of most baseline characteristics, the two groups were similar, although ZIKV non-infected women were more likely to be unemployed, and ZIKV infected women were more likely to report smoking during pregnancy. This may reflect recall ability and employment situation differences according to the timing of data collection, as ZIKV non-infected women were all recruited at the time of delivery and ZIKV infected women were recruited at various earlier time points during their pregnancy. Furthermore, the quality of follow-up and collection of data on the course of pregnancy was likely of a higher quality in the ZIKV-exposed group as this was a prospective cohort where women had been symptomatic during pregnancy. Highlighting this is the fact that the study team was able to retrieve ultrasound records for 88.4% of ZIKV infected women and only 51.6% for ZIKV non-infected women. However, such a difference would only lead to an underestimation of birth defects in the ZIKV non-infected group. Furthermore, the completeness of data at the time of delivery for live births, which was used to determine anthropometric and clinically apparent abnormalities, was very high in both ZIKV-exposed (97.9%) and non-exposed (98.8%) infants. Recruitment of ZIKV non-exposed pregnant women at delivery also prevented us from determining the number of expected miscarriages, stillbirths, and abortions that may occur in the ‘baseline’ population, but this also indicates a potential under rather than over-estimation of birth defects in this group.

Most of the originally reported ‘potentially linked to ZIKV’ abnormalities seen in both the exposed and non-exposed pregnancies of our study represent identification of microcephaly in live births; these cases were defined based only on anthropometric measurements, with known clinical and radiological findings for each infant being normal. This diagnostic approach to microcephaly, which does not require clinician judgment on the

appearance of microcephaly, but relies solely on the comparison of a head circumference measurement against a normalized birth curve, has been used in all of the cohort studies describing the risk of birth defects following maternal ZIKV exposure during pregnancy, to date (Adhikari et al., 2017; Brasil et al., 2016; Hoen et al., 2018; Pomar et al., 2017; Rodriguez-Morales et al., 2018; Shapiro-Mendoza et al., 2017). However, defining microcephaly based on ‘metrics’ does not reflect the real-life clinical diagnosis of this condition, and can lead to a false surge in cases if applied to an entire population for surveillance purposes (Orioli et al., 2017). Registries using more stringent definitions (e.g. -3SD) and/or clinician specific criteria indicate that true disease-related microcephaly is very rare; the European Surveillance of Congenital Anomalies (EUROCAT) recently estimated the prevalence of microcephaly in Europe to be 1.53 per 10,000 births (~0.02%) with data from 2012-2016 (Morris et al., 2016). However, as infant growth is approximately normally distributed, the INTERGROWTH-21st study itself prescribes that approximately 2% and 0.1% of healthy infants should have a head circumference at birth that falls below -2 and -3SD, respectively (Villar et al., 2014). Corroborating this, a recent study applying this definition of microcephaly to birth cohorts from two Brazilian cities, Ribeirão Preto and São Luís, estimated the baseline prevalence of microcephaly in 2010 to be of 2.5% and 3.5%, respectively (Silva et al., 2018). Our own estimates of the prevalence of moderate and severe microcephaly and small weight for gestational age in ZIKV exposed infants was similar to that which the INTERGROWTH-21st standards prescribes. However, in our larger ZIKV non-infected control group, our estimates for each of these anthropometric abnormalities, while not different from those in the ZIKV-exposed cohort, were higher than what is prescribed by the INTERGROWTH-21st growth standards. This may be due to a ‘non-perfect-fit’ of the Guadeloupian population to this international pooled growth standard. Other authors (Albert and Grantz, 2014; Cheng et al., 2016; Liu et al., 2017), as well as the INTERGROWTH-21st study themselves (Villar et al., 2014), have noted varying levels of fit for individual populations when compared to this pooled standard.

This study highlights the importance of a control group to estimate the baseline risk of anthropometric and other birth defects when determining the risk of severe congenital abnormalities that can be attributed to a given infection during pregnancy. This is particularly true for anthropometric measurements where regional variations may exist (Albert and Grantz,

2014; Cheng et al., 2016; Liu et al., 2017; Villar et al., 2014). Pre-Zika microcephaly baseline prevalence estimates are increasingly being reported (de Magalhaes-Barbosa et al., 2017; Hoyt et al., 2018; Orioli et al., 2017; Rick et al., 2017; Silva et al., 2018). While such reports can be used to give general clues as to the magnitude of birth defects seen through cohort studies that can be attributed to ZIKV infection, the definition of microcephaly and CZS used, and whether or not clinician expertise was considered, will be key to their interpretation. As with other congenital infections that cause neurological abnormalities, such as cytomegalovirus and rubella (De Santis et al., 2006; Manicklal et al., 2013), longer-term studies that postnatally follow-up infants that are exposed to ZIKV in-utero but who are apparently healthy at birth are needed in order to understand the true overall risk of defects. However, in terms of the risk of immediate severe congenital defects that are potentially linked to ZIKV infection during pregnancy, and that may impact pregnancy outcome, our findings have further diminished our own estimate for pregnant women in Guadeloupe from 7.2% to 1.6% overall. Communication of the most possibly accurate estimate of the risk of severe birth defects linked to this infectious exposure, as well as the likelihood that such abnormalities will be detected early in pregnancy, will have an important influence on the family planning decisions of pregnant women with ZIKV-positive test results.

2.3 ZIKV-related birth defects in sub-Saharan Africa and Asia

2.3.1 The “Surveillance of ZIKV-related microcephaly in sub-Saharan Africa and Asia” study

- **Background**

As discussed in the introduction to this thesis, after its discovery in Uganda in the late 1940’s, seroprevalence surveys indicate that ZIKV seems to have circulated for decades in sub-Saharan Africa and Asia. However, until very recently there has been no reported excess of ZIKV-related complications, e.g., microcephaly and GBS in these regions (Chu et al., 2018; Rosenstierne et al., 2018; Sasseti et al., 2018; Wongsurawat et al., 2018). This may be related to a historically low circulation of the virus in these regions, a lack of systematic testing for the virus when those complications occurred, or possibly recent mutations in the virus that have increased its pathogenicity. As interventions against ZIKV and its complications are being developed (e.g., vaccine, vector control), we consider it important to document the public health impact of ZIKV in these regions of the world through improved surveillance of its main complication: microcephaly.

- **Objectives**

This study will explore whether ZIKV is currently responsible for neurological complications, and particularly microcephaly, in *Aedes*-infested regions of sub-Saharan Africa and Asia. This may inform regional public health strategies, such as vaccination of women of childbearing age. It will also demonstrate the public health impact of ZIKV infection and increase understanding of other regional infectious (e.g. CMV) causes of microcephaly.

- **Study design**

This study will last for 2 years, and will include only new cases of microcephaly. Surveillance will take place in large maternities in urban areas (see Table 9). In each city,

approximately 3000 live births per month will be monitored, so that on average one disease-related microcephaly case will be diagnosed per month per city (i.e., 24 per city in 2 years); this is based on estimates from birth defects registries, with allowance for a possible increased proportion due to differences in nutritional and environmental factors as well as variation in circulation of infectious agents (e.g. rubella).

Table 9: Participating maternities with estimated number of births per day that will be examined for microcephaly

Country	City	Maternities	# Births/day
Cameroun	Yaoundé	Central Hospital Maternity	11
		Essos Hospital Centre Maternity	7
China	Guangzhou	Guangzhou Women and Children's Medical Centre	55
		Guangzhou Liwan Maternal and Child Health Hospital	9
		Guangzhou Huadu Maternal and Child Health Hospital	22
Côte d'Ivoire	Abidjan	General Hospital of Yopougon-Attie	37
		General Hospital Abobo-Sud	17
Sri Lanka	Colombo	Castle Street Hospital for Women (CSHW)	35
		De Soyza Hospital for Women (DSHW)	20
Vietnam	Ho Chi Minh City	Tu Du Hospital	180-200

For the purpose of this study, microcephaly will be defined as a head circumference of less than -3SD, according to the INTERGROWTH-21st standards for gestational age and sex AND having an abnormal pre or postnatal ultrasound and/or clinical examination. If an infant is disproportional in terms of its weight and/or length by greater than 2SD when compared to its head circumference Z score, this will be considered a clinical examination abnormality. Exclusion criteria include the mother being less than 18 years of age and unwillingness/inability to provide informed consent.

Note: Originally, in September 2016, the microcephaly case definition that we envisaged for this study was simply having a head circumference of less than -2SD according to the INTERGROWTH-21st standards for gestational age and sex. However, due to preliminary results from surveillance using this definition in four of the study sites, as will be presented, this definition was refined to be more specific.

- **Data collection**

A questionnaire that collects information on demographic characteristics, teratogenic exposures, and historical and current pregnancy details will be administered to all mothers whose infants have met the case definition for microcephaly.

- **Laboratory testing and clinical examination**

Various biological samples (e.g. blood, placenta) will be collected from mothers and newborns/fetuses and tested for presence of ZIKV, and for other infectious (e.g. rubella, cytomegalovirus) causes of microcephaly. Table 10 provides an example of this testing for live born infants. In the case of a stillborn infant or a microcephaly case detected via prenatal ultrasound where the pregnancy outcome is termination, the samples collected will differ slightly. Physical, neurological, hearing and visual examinations for all live births will be performed where possible in the first week of life.

- **Study partners**

This study has been designed and implemented in partnership with each participating country/organization, including:

- *Paris, France:* Emerging Disease Epidemiology Unit, Institut Pasteur
- *Lausanne, Switzerland:* University Hospital of Lausanne
- *Yaoundé, Cameroon:* Institut Pasteur of Cameroon

- *Abidjan, Cote d'Ivoire*: Institut Pasteur of Cote d'Ivoire
- *Guangzhou, China*: Guangzhou Women's and Children's Hospital
- *Ho Chi Minh City, Vietnam*: Tu Du Hospital; Institut Pasteur of Ho Chi Minh City
- *Colombo, Sri Lanka*: Perinatal Society of Sri Lanka

Table 10: Laboratory examinations for live born cases

	Zika virus	Cytomegalovirus	Rubella	Toxoplasmosis
<i>Serological testing for all infectious agents on maternal sample</i>				
Venous blood	RT-PCR, IgG & IgM	IgM	IgM	IgM
<i>If possible, testing should be performed on one sample below, depending on availability and hospital standard procedure*, in the preferred order as shown</i>				
Cord blood	RT-PCR	PCR	RT-PCR	PCR
Placenta	RT-PCR	PCR	RT-PCR	PCR
Infant saliva	RT-PCR	PCR	RT-PCR	PCR

* In some countries CMV and Rubella viral load may need to be tested using urine sample.

- **Current study progress**

Although the first meeting with study partners from each country took place through a workshop in Paris in September 2016, the implementation of this research has been delayed due to various regulatory processes, mostly in France, but also sometimes in the study countries. As of September 2018, all regulatory procedures have been completed, and authorizations to officially start the study have been given, for two countries- China and Cote d'Ivoire. For two other study countries, Cameroon and Sri Lanka, all regulatory procedures have been completed besides final notice of local ethical approval, which should be completed by October 2018. In Vietnam, the study implementation has faced considerable delays and will likely start in early 2019.

2.3.2 Original research (full article): Surveillance of microcephaly in four cities of sub-Saharan Africa and Asia

Prior to starting the study described above, we needed to understand how many cases of microcephaly we might eventually observe in each location; this would be important for budget and logistical considerations. At our disposal were recent estimates of a 0.02% prevalence at birth of microcephaly in Europe, published just days prior to our international study group meeting in Paris in late 2016 (Morris et al., 2016). We imagined we might find higher estimates than this, due to variance in environmental and infectious factors that may cause the condition – so then, perhaps 0.04% or 0.06%? At this point in our group meeting, one of our Chinese colleagues presented some preliminary data from a birth cohort in Guangzhou; if applying a -2SD cut-off with the INTERGROWTH-21st standards, about 1.4% of infants would be labeled as having microcephaly. This was our first experience with the perils of a ‘metric’ microcephaly definition.

Unsure of what this would look like in each collaborating country, each study partner returned home from Paris with the aim to conduct a ‘pilot’ microcephaly surveillance in participating hospitals in their region. This would be done, where possible, both by reviewing data for 3000 infants in birth registers retrospectively and then, using non-stretch headbands and reinforced methods, moving forward prospectively looking at 3000 births. The comparison of these two methods would give a further indication of data quality. The eventual proportion of both moderate and severe microcephaly would give us more guidance on which cut-off we should use for our over-arching study.

At least, we expected to find around 2-3% of moderate microcephaly (<-2SD) and <0.05% (<-3SD) of severe microcephaly, as predicted by the normal distribution under the INTERGROWTH-21st growth standards. In this case, we could go with a more specific definition of microcephaly (i.e. severe microcephaly) for our study and, possibly with need for consideration of imaging and clinical evaluations for each infant. Our actual results showed highly varied proportions of both moderate and severe microcephaly by region. We also were able to see that the data we had collected produced head circumference Z-score distributions that were fairly normal, but shifted either right or left when compared to the reference

standard. These results highlighted a potentially more serious problem with a measurement-based microcephaly definition – extreme variation by region, likely due to diverse socioeconomic and environmental factors common in real-life settings, when using a pooled international growth standard.

At the time of submitting this thesis manuscript to the referees on October 17th, 2018, this article is submitted at *Pediatrics*. The PDF of this article can be found in Appendix 5.

Microcephaly surveillance in Africa and Asia using a -2SD cut-off and international growth charts

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- **Abstract**

Background and Objectives: Little data on microcephaly baseline estimates exists in sub-Saharan Africa and Asia. We aimed to estimate the proportion of live births with microcephaly from four cities in sub-Saharan Africa and Asia using the INTERGROWTH-21st growth charts and a -2 standard deviation (SD) cut-off. *Methods:* Large maternities in Yaoundé (Cameroon), Abidjan (Ivory Coast), Colombo (Sri Lanka) and Guangzhou (China) collected data for live born infants through retrospective and/or prospective surveillance, as feasible in each setting. Head circumference, gestational age, and sex were compared to the INTERGROWTH-21st standards to obtain head circumference Z-scores (HCZ) for each infant. Moderate and severe microcephaly were defined as between -2 and -3SD, and less than -3SD, respectively. *Results:* Sufficient data for 19914 live births across all study sites were analysed. The proportion of infants with microcephaly, according to the definition, was between 2.9% and 34.8% through retrospective surveillance in three countries. Prospective surveillance in Guangzhou, Colombo, and Abidjan, labelled 3.8%, 7.5%, and 18.6% of infants as having microcephaly, respectively. The retrospective data from Abidjan differed the most from the INTERGROWTH-21st distribution, with a shifted mean HCZ -1.41 SD, whereas the HCZ for infants from Guangzhou, following prospective surveillance, differed the least (mean HCZ - 0.02SD). *Conclusions:* A cut-off of -2SD will most likely lead to labelling large numbers of healthy babies as having microcephaly, with important regional variations. For the purpose of microcephaly surveillance, we suggest using regional growth charts, increasing consideration of infant body size, and defining the cut-off at -3SD (severe microcephaly).

- **What's known on this subject?**

In sub-Saharan African and Asian regions suitable for Zika transmission, little information on the baseline prevalence of microcephaly in live born infants is available. However, the prevalence in Europe and the United States was recently estimated at less than 0.1%.

- **What this study adds?**

A -2SD cut-off with pooled international growth standards classifies many infants (3 to 35%) as having microcephaly in sub-Saharan Africa and Asia. Consideration of infant body size and regional growth differences are needed in the microcephaly definition used for surveillance.

- **Introduction**

Microcephaly is a congenital anomaly that can appear in neonates following abnormal brain development due to infectious, genetic or environmental causes (Gilmore and Walsh, 2013; von der Hagen et al., 2014). This birth defect has attracted increased attention recently, following the rapid spread of Zika Virus (ZIKV) throughout the Americas in 2015-2016 and the realisation that microcephaly due to abnormal brain development can occur in the infants of women infected with ZIKV during pregnancy (de Araújo et al., 2016). Although few recent cases have been reported so far outside of the South-Pacific and Americas, a large proportion of sub-Saharan Africa and Asia is equally suitable for ZIKV transmission (Messina et al., 2016). The lack of understanding of the effects of current or impending transmission of ZIKV in these latter regions further emphasizes the need for strong surveillance systems and clear case definitions for microcephaly (Wetsman, 2017).

From 2003 to 2012, the European Surveillance of Congenital Anomalies (EUROCAT) registry estimated the prevalence of microcephaly in Europe to be 1.5 per 10,000 births

(~0.02%) (Morris et al., 2016). In the United States, between 2009 and 2012, pooled results from 30 birth defects registries, estimated the prevalence of microcephaly to be 9 per 10,000 births (0.09%) (Cragan et al., 2016). To our knowledge, there are no recent microcephaly registries or causality profiles for most regions of sub-Saharan Africa and Asia. In these regions we may expect different figures than in Europe due to environmental factors, differences in nutrition, and increased circulation of and lower vaccine coverage for infectious agents such as rubella. For the purpose of screening live neonates born to women at risk of ZIKV exposure during pregnancy, at the beginning of the recent outbreak in the Americas, the Centres for Disease Control and Prevention (CDC) (2016) and the World Health Organisation (WHO) (2016f) recommended defining moderate and severe microcephaly as a head size of less than -2SD (or < 3rd percentile) and -3SD, respectively, for gestational age and sex using the INTERGROWTH-21st or WHO growth standards. The same definition has been used by many of the key cohort and case-control studies defining Zika related birth defects (Brasil et al., 2016; de Araújo et al., 2016; Hoen et al., 2018; Shapiro-Mendoza et al., 2017), as well as in some recent estimations of the pre-Zika (i.e. <2015) microcephaly prevalence in South America (de Magalhaes-Barbosa et al., 2017; Rick et al., 2017; Silva et al., 2018). Therefore, we sought to understand the distribution of newborn head sizes, and in particular the prevalence of microcephaly at birth, that could be expected using data from real-life settings in sub-Saharan Africa and Asia, according to a -2SD cut-off with a pooled international growth standard. Such results could be used as a baseline reference in these regions, for interpretation of disease-related microcephaly surveillance following a Zika epidemic.

- **Methods**

Eight hospitals across four countries, two in sub-Saharan Africa and two in Asia, participated: Essos Hospital Maternity in Yaoundé, Cameroon; the General Hospitals of Yopougon-Attie and Abobo-Sud in Abidjan, Ivory Coast; Guangzhou Women and Children's Medical Center, Guangzhou Huadu Women and Children Health Care Hospital and Guangzhou Liwan Women and Children Health Care Hospital; the Castle Street and De Soyza Hospitals for Women in Colombo, Sri Lanka. These are large hospitals in urban areas suitable

for ZIKV transmission (Messina et al., 2016), and have many births per day (mean: 22, range: 7-55) in relation to other regional hospitals. Most regions in which the study sites are situated would be considered low-income, besides Guangzhou.

Data from birth registers was collected retrospectively, with an aim to record data for at least 3000 births consecutively moving back from the day at which the collection started, in the participating hospitals in Yaoundé (January 1st 2015 – December 31st 2016), Abidjan (August 14th, 2016 – November 2nd 2016), and Colombo (June 13th 2016 –December 14th 2016). Head circumference in these maternities is measured within the first 24 hours of life using vinyl-measuring tapes which are crossed over at the front of the head to get a reading of the head circumference. Birth register entries that were missing one or more data points were equally recorded and missing values noted in the data collection form.

In Guangzhou (February 10th 2017 to March 13th 2017), Colombo (December 15th 2016 to April 5th 2017), and Abidjan (April 4th 2018 to 14th July 2018), the participating maternities collected data prospectively with the aim to review 3000 consecutive births moving forward from the date of the collection start. Prior to this prospective data collection, the participating maternities reinforced their standard procedures for head measurement and birth register data collection (Harris, 2015). They also introduced use of non-stretch Teflon seca 212 head measuring bands, which remain in a loop format with a viewing window in order to read head circumference measurements to the nearest millimetre (<https://us.secashop.com/products/pediatric-measuring-systems/seca-212>).

Gestational age calculation

According to standard procedure at each participating maternity, gestational age is calculated based on the first date of the last menstrual period; for varying proportions of patients within each country, this estimate is confirmed using ultrasound examination carried out in the first trimester of pregnancy. In Ivory Coast, women frequently first present at the hospital at a stage very late in pregnancy (e.g. onset of labour or first prenatal visit in the third

trimester); in this case, the calculation of gestational age based on the date of last menstruation is still attempted and is often complemented by symphysis fundal height.

Data analysis

A newborn was included in the analysis if they had all of sex, gestational age and head circumference recorded in the birth register; this data is needed to calculate the infant's head circumference Z-score (HCZ). Moderate microcephaly was considered as having a HCZ of less than or equal to -2SD & greater than -3SD and severe microcephaly was considered as having a HCZ of less than or equal to -3SD (Brasil et al., 2016; CDC, 2016; de Araújo et al., 2016; Hoen et al., 2018; Shapiro-Mendoza et al., 2017; WHO, 2016f;). Data was uploaded into the open access INTERGROWTH-21st online application retrieved at: <http://intergrowth21.ndog.ox.ac.uk>, which gave an exportable HCZ for each infant. We calculated a mean HCZ for each country, by type (retrospective/prospective) of data collection, to compare with the pooled mean from the INTERGROWTH-21st standards (i.e. 0SD). The Student's t-test and the Chi-squared test were used to compare continuous data and categorical data, respectively. We excluded stillborn and very preterm (< 33 weeks gestational age) measurements as these were excluded in the elaboration of the growth standards (Villar et al., 2013; Villar et al., 2014). To facilitate the visual comparison between our data and the INTERGROWTH-21st standards for Figure 5, we recreated the distributions of newborn head circumferences by sex of the INTERGROWTH-21st charts (see PDF of submitted article in Appendix 5). Data analysis and graphics were produced using R version 3.4.0.

Ethical considerations

In Sri Lanka and Ivory Coast, national ethical committee approval was not required in order to publish the aggregated results of the routinely collected data used for this analysis, however, institutional approval from participating hospitals was obtained. In Cameroon and China, institutional ethics committee clearance and approval of hospital authorities was obtained.

- **Results**

In total, data was collected for 21426 births that occurred between January 2015 and July 2018 across eight hospitals in the four participating countries. After exclusion of stillbirths (n=298, 1.4%), very preterm births (n=472, 2.2%), and birth records with missing variables (n=742, 3.5%), data from 19914 (92.9%) live births was analyzed (*Table 11*).

Table 11: Number of births collected and used in this analysis by participating country

	Cameroon		Ivory Coast		Sri Lanka		China
Data collection method	Retrospective	Retrospective ^e	Prospective	Retrospective	Prospective	Prospective	
Total births recorded	5031	3212	3526	3147	3281	3229	
Still births	84 (1.7%)	83 (2.6%)	112 (3.2%)	3 (0.1%)	4 (0.1%)	12 (0.4%)	
Very preterm (<35 weeks gestation)	208 (4.1%)	14 (0.4%)	26 (0.7%)	74 (2.4%)	114 (3.5%)	36 (1.1%)	
Live births (missing data)	312 (6.2%)	158 (4.9%)	81 (2.3%)	11 (0.3%)	21 (0.6%)	159 (4.9%)	
Live births (sufficient data ^a)	4427 (88.0%)	2957 (92.1%)	3307 (93.8%)	3059 (97.2%)	3142 (95.8%)	3022 (93.6%)	
<i>of which were male^b</i>	<i>2242 (50.6%)</i>	<i>1542 (52.1%)</i>	<i>1698 (51.3%)</i>	<i>1592 (52.0%)</i>	<i>1561 (49.7%)</i>	<i>1621 (53.6%)</i>	

^aWith sufficient data for analysis, including sex, gestational age estimation, head circumference measurement ^bPercentage of live births with sufficient data

The distribution of HCZ from each country differed from that of the INTERGROWTH-21st standards by varying degrees (see Table 12). Figure 5 compares the HCZ distribution for each country with the HCZ distribution that would be expected based on the INTERGROWTH-21st standards. The prospective data from Guangzhou was the most similar (mean HCZ = -0.02SD, 95%CI: -0.06 – 0.02) to the INTERGROWTH-21st standards, whereas the retrospective data from Abidjan was the least similar (mean HCZ = -1.41SD, 95%CI: -1.45 - -1.37). We found a significant difference in mean HCZ between male and female infants in the retrospectively collected data in Cameroon and Ivory Coast, as well as in the prospectively collected data in Ivory Coast and Sri Lanka (Table 12, Figure 5).

Overall, in the retrospectively collected data, the prevalence of moderate microcephaly ranged from 2.4% (Cameroon, 95%CI: 2.0-2.9%) to 25.8% (Ivory Coast, 95%CI: 24.2-27.4%), and the prevalence of severe microcephaly ranged from 0.5% (Cameroon, 95%CI: 0.3-0.8%) to 9.0% (Ivory Coast, 95%CI: 8.0-10.1%). In the prospectively collected data, the prevalence of moderate microcephaly ranged from 3.6% (China, 95%CI: 2.9-4.3%) to 14.6% (Ivory Coast, 95%CI: 13.4-15.8%), and the prevalence of severe microcephaly ranged from 0.2% (China, 95%CI: 0.1-0.5%) to 4.1% (Ivory Coast, 95%CI: 3.4-4.8%) (See Table 12).

In Abidjan, where the mean HCZ was the furthest from zero (HCZ=-1.41SD, 95%CI: -1.45 - -1.37), a sensitivity analysis demonstrated a more shifted HCZ distribution (i.e. discord with the INTERGROWTH-21st standards) when mothers presented late in pregnancy (62.4% of women) and uterine measurements were used to confirm the gestational age estimation, compared to when this was not needed: -1.48SD (95%CI: -1.53 - -1.42) vs -1.28SD (95%CI: -1.37 - -1.23, $p<0.001$). The prospectively collected data from the maternities in Colombo showed less microcephaly and a mean HCZ closer to 0 (-0.22SD, 95%CI: -0.26 - -0.18) when compared to the retrospectively collected data from the same site (-0.49SD, 95%CI: -0.54 - -0.44, $p<0.001$). Similarly, the prospectively collected data from the maternities in Abidjan showed less microcephaly and a mean HCZ closer to 0 (-0.85SD, 95%CI: -0.89 - -0.81) when compared to the retrospectively collected data (-1.41SD, 95%CI: -1.45 - -1.37, $p<0.001$).

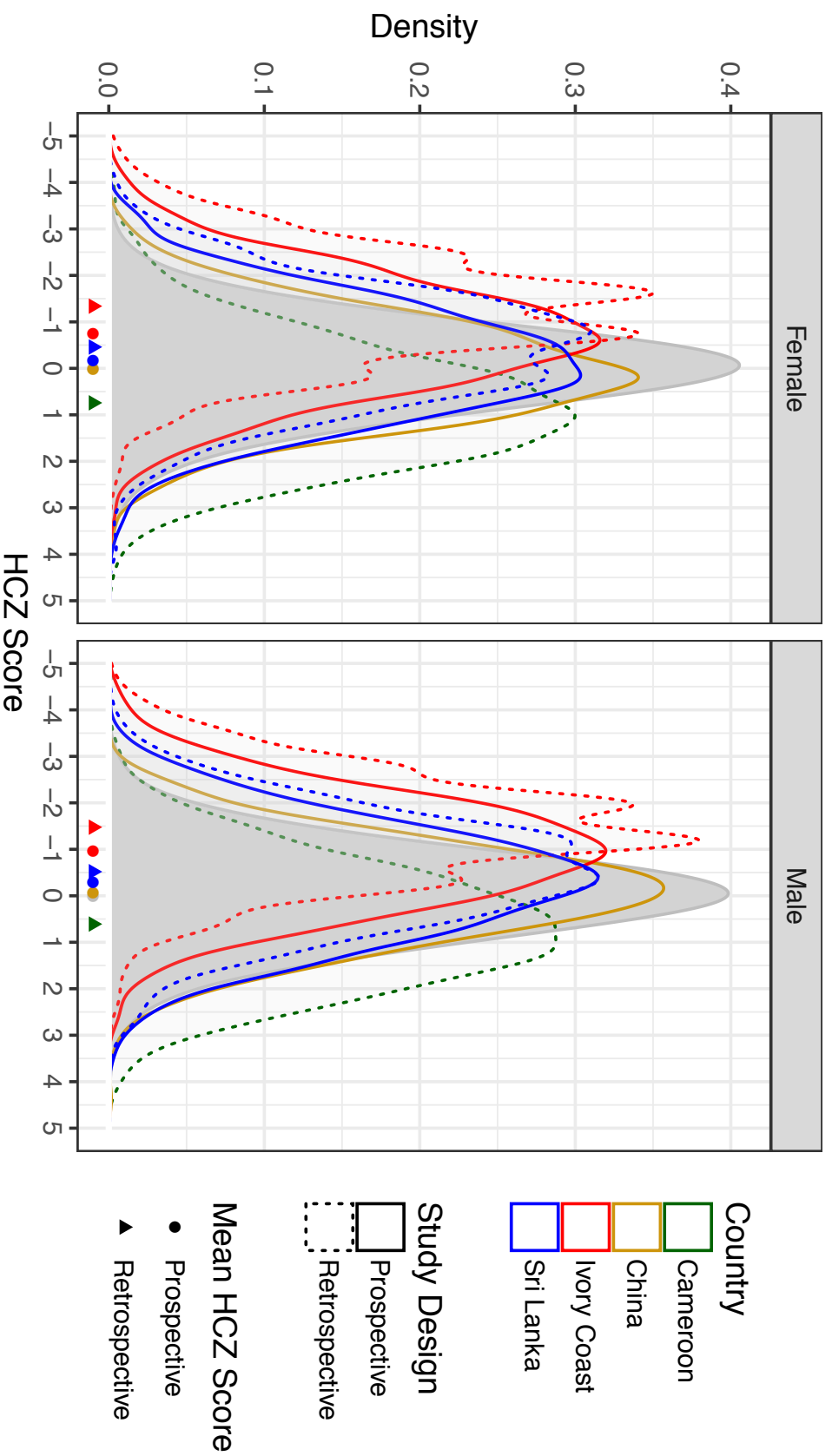


Figure 5: Distribution of Head Circumference Z-Scores (HCZ), by sex, according to the INTERGROWTH-21st standards (in grey), for retrospectively collected data (Cameroon, Ivory Coast, Sri Lanka) and prospectively collected data (Ivory Coast, Sri Lanka, China).

Table 12: Mean head circumference Z-score (HCZ) & microcephaly prevalence by study site, type of data collection, and sex.

		Mean HCZ ± SD	Total microcephaly (≤-2SD)	Moderate microcephaly ≤-2SD & >-3SD	Severe microcephaly ≤-3SD
RETROSPECTIVE DATA COLLECTION					
Cameroon	Total (n=4427)	0.67 ± 1.33	129 (2.9%)	105 (2.4%)	24 (0.5%)
	Male (n=2242)	0.61 ± 1.32 ^a	62 (2.8%)	49 (2.2%)	13 (0.6%)
	Female (n=2185)	0.74 ± 1.33 ^a	67 (3.1%)	56 (2.6%)	11 (0.5%)
Ivory Coast	Total (n= 2957)	-1.41 ± 1.20	1029 (34.8%)	762 (25.8%)	267 (9.0%)
	Male (n=1542)	-1.48 ± 1.16 ^b	632 (41.0%)	506 (32.8%)	126 (8.2%)
	Female (n=1415)	-1.33 ± 1.25 ^b	397 (28.1%)	256 (18.1%)	141 (10.0%)
Sri Lanka	Total (n=3059)	-0.49 ± 1.27	335 (11.0%)	280 (9.2%)	55 (1.8%)
	Male (n=1592)	-0.52 ± 1.28	193 (12.1%)	165 (10.4%)	28 (1.8%)
	Female (n=1467)	-0.46 ± 1.27	132 (9.0%)	115 (7.8%)	27 (1.8%)
PROSPECTIVE DATA COLLECTION					
Ivory Coast	Total (n=3307)	-0.85 ± 1.24	616 (18.6%)	482 (14.6%)	134 (4.1%)
	Male (n=1698)	-0.96 ± 1.20 ^a	341 (20.1%)	272 (16.0%)	69 (4.1%)
	Female (n=1609)	-0.74 ± 1.28 ^a	275 (17.1%)	210 (13.1%)	65 (4.0%)
Sri Lanka	Total (n=3142)	-0.22 ± 1.24	236 (7.5%)	203 (6.5%)	33 (1.1%)
	Male (n=1561)	-0.16 ± 1.25 ^c	126 (8.1%)	113 (7.2%)	13 (0.8%)
	Female (n=1581)	-0.28 ± 1.23 ^c	110 (7.0%)	90 (5.7%)	20 (1.3%)
China	Total (n= 3022)	-0.02 ± 1.13	115 (3.8%)	108 (3.6%)	7 (0.2%)
	Male (n=1621)	-0.06 ± 1.11	60 (3.7%)	59 (3.6%)	1 (0.1%)
	Female (n=1401)	0.02 ± 1.16	55 (3.9%)	49 (3.5%)	6 (0.4%)

*Denotes significant difference between male and female mean HCZ ^ap<0.001 ^bp=0.002 ^cp=0.007

- **Discussion**

Defining microcephaly strictly as less than -2SD according to the INTERGROWTH-21st standards resulted in a prevalence at birth at our study sites in sub-Saharan Africa and Asia that far exceeded estimates observed in birth defect registries of Europe, the United States, and Latin America (Cragan et al., 2016; Morris et al., 2016; Orioli et al., 2017). These findings were in part expected, since the approximation of the distributions underlying these standards suggests that 2.2% of the healthy newborns included in the creation of the INTERGROWTH-21st standards would be classified as having at least moderate microcephaly given this definition (Altman and Ohuma, 2013). This prevalence would already be between 25 and 125 times the proportion of microcephaly as estimated through recent reports from birth defects registries (Cragan et al., 2016; Morris et al., 2016; Orioli et al., 2017). Supporting this, the ECLAMC (Latin American Collaborative Study of Congenital Malformations) determined a pre-Zika hospital-based microcephaly baseline prevalence of 0.08% in Brazil (Orioli et al., 2017), whereas another study from Brazil that applied a standard definition (-2SD according to the INTERGROWTH-21st standards) to two hospital populations, without other criteria, found that more than 2.5% of infants were labelled as having microcephaly (Silva et al., 2018). These differences are not surprising considering that microcephaly cases in birth defects registries are likely classified as so by clinicians who have considered additional factors such as proportionality of the infant, dysmorphic features, and regional norms.

There are many limitations of determining the prevalence of microcephaly in real-life settings if using pooled international standards that reflect ideal growth under optimal conditions. These constraints may explain the variation in our own estimates, as well as some of the extreme estimates for some sites, such as Abidjan, that we obtained. Certain clinical practices and tools are needed in order for newborn data to best be compared with growth standards, some of which are not always feasible in real-life settings of low-income countries. First, accurate measurement of the infant head circumference is needed, requiring multiple measurements using a non-stretch measuring tape with correct positioning on the neonate's head. In the analysis we present, these methods were employed in a controlled fashion during the prospective surveillance in China, Sri Lanka and Ivory Coast, but not in the retrospective

data from birth registers. In both Sri Lanka and Ivory Coast, the prospectively collected data that followed reinforcement of head measurement techniques and introduction of standard non-stretch headbands demonstrated mean HCZ estimates closer to zero when compared to the retrospective data; this indicates that reduction of measurement error leads to increased, but not total, assimilation with the INTERGROWTH-21st standards. Furthermore, estimation of gestational age, which is best done with an ultrasound assessment in the first trimester, or otherwise using the date of last menstruation (Villar et al., 2013; Villar et al., 2014), is a measure that is complex to determine for a high proportion of women in our two participating countries in sub-Saharan Africa. In Abidjan, around 60% of the women presented for the first time at the hospital towards the end of their pregnancy, leading to uterine measurements being used to help estimate the gestational age. In Guangzhou, which is not a low-income setting, women typically undergo 4-8 ultrasounds per pregnancy, allowing for precise determination of gestational age and early detection and abortion of infants with any abnormalities. This level of care and availability of tools may partially explain the similarity of the Guangzhou prospectively collected data to that of INTERGROWTH-21st standards.

The differences observed between the populations in each of our study sites and the INTERGROWTH-21st standards likely also reflect regional differences in the head sizes of infants due to environmental and socioeconomic factors, such as poor nutrition and circulation of infectious agents. These factors are, by definition, limited as much as possible in the creation of prescriptive growth standards, but cannot be teased out when comparing real-life data to the standards on a large-scale for surveillance purposes. Some criteria employed in the creation of the INTERGROWTH-21st standards, such as that for maternal height and body mass index, led to exclusion of more than 10% of otherwise eligible women and disproportionately affected specific countries (Villar et al., 2014). In a real-life setting where factors influenced by environmental and socioeconomic factors (e.g. maternal height and weight) are not adjusted for, the distribution of infant head circumference Z scores may be shifted away the pooled standard, further exacerbating extreme microcephaly estimates when using a fixed cut-off. For example, within our two sub-Saharan Africa sites, the included hospitals in Ivory Coast, whose HCZ are shifted left compared to the INTERGROWTH-21st distribution (mean HCZ -1.39SD with retrospective collection, -0.85SD with prospective collection), are public with low-income catchment areas, while the hospital in Cameroon, with

data shifted right with a mean HCZ 0.75SD (retrospective collection), is a semi-private institution with most patients coming from a higher socioeconomic status, and therefore likely with different environmental exposures.

Variation in fetal growth has been noted recently across the 10 countries included in the creation of WHO fetal growth standards (Kiserud et al., 2017), as well as across the four ethnic groups included in the National Institute for Child Health and Human Development (NICHD) fetal growth study in the United States (Buck Louis et al., 2015). Even after strict inclusion criteria, the INTERGROWTH-21st study noted variance in the mean HCZ for each of their participating countries when compared to their eventual pooled standard, which they call the standardized site discrepancy (SSD). Their eventual range of SSD for head circumference at birth varied from as low as -0.55SD to as high as 0.42 SD depending on the study country (Villar et al., 2014), which was within their predetermined limits to justify a pooled growth standard.²⁰ It was demonstrated by Albert and Grantz (2014) that this allowed variance means that the probability of falling below the 5th percentile of the INTERGROWTH-21st percentiles would be as high as 12.6% in a setting with an SSD of -0.5 and as low as 1.6% in a setting with an SSD of 0.5. Applied to microcephaly surveillance, in the first case, such pooled standards could lead to over-diagnosis in healthy infants, and in the second, under-diagnosis of potential clinical cases. The mean HCZ (a measure similar to the INTERGROWTH-21st 'SSD') from our study sites had an even wider range, from -1.41 to 0.67SD, which may explain the very high proportion of microcephaly classifications that we observed in some countries, such as Ivory Coast. Indeed some recent studies corroborate this, noting that replacement of regionally specific growth charts with the INTERGROWTH-21st growth standards would reclassify a significant proportion of infants/foetuses as having either macrocephaly or microcephaly in Canada and China, respectively (Cheng et al., 2016; Liu et al., 2017). A systematic review evaluating the WHO pooled international growth standards also corroborates this finding, with individual country means matching particularly poorly to the head circumference standards and leading to misdiagnosis of micro and macrocephaly (Natale et al., 2014).

- **Conclusions**

Our study raises several issues regarding microcephaly surveillance. The use of a -2SD cut-off based on the INTERGROWTH-21st chart implies that by definition, around 2% of healthy newborns will be categorised as having microcephaly. As mentioned by Morris and colleagues (2016), this will result in a high proportion of babies labelled as microcephalic who will have no detectable neurological impairment, generating unnecessary additional diagnostic costs and anxiety. To this group will be added those who have underlying morbid or nutritional conditions associated with small body size, and who have been excluded from the elaboration of pooled prescriptive standards that describe ideal growth. These conditions may be particularly common in poor settings of low-income countries. Finally, in regions with high variation in head circumference size due to environmental and socioeconomic factors, the proportion of babies diagnosed with microcephaly may reach proportions so high that any surveillance or diagnostic work-up based on this definition would become very impractical. The Ivory Coast maternities sampled in our study, with 19% of babies diagnosed with microcephaly through reinforced prospective surveillance, may be one example of that situation.

As the ZIKV epidemic has ended (PAHO, 2017b), the focus of surveillance shifts towards increased specificity in identifying neurological birth defects. As a result, a cut-off of -3SD for microcephaly surveillance should be reconsidered, to conform with EUROCAT as well as guidelines and a systematic review predating the ZIKV epidemic (ECLAMC, 2015; EUROCAT, 2017; WHO, 2014; Woods and Parker, 2013). The predictive value of developmental disabilities for infants with an at-birth head circumference Z-score of less than -3SD compared to the norm, as opposed to -2SD, is also much higher (Dolk, 1991), and this should be further validated using data from cohorts of infants with ZIKV exposure during pregnancy. In addition to a more specific cut-off, consideration of the proportionality of newborn head circumference length and/or weight should be considered; inclusion of these criteria will mimic the procedures of birth defects registries more closely, and is echoed in updated recommendations from the WHO and CDC (CDC, 2018b; WHO, 2016g). Finally, there is a need to perform further country-specific and regional studies to develop local standards for foetal and newborn head circumference that can be used on a large-scale for

surveillance purposes. A growing body of research, including our own study, challenges the idea that foetal and newborn growth across the world can be assessed with a ‘one size fits all’ standard (Buck Louis et al., 2015; Gaillard and Jaddoe, 2014; Kiserud et al., 2017; Natale et al., 2014). If the international definitions for microcephaly adapt to be considerate of this evidence, our global epidemiological understanding of this condition will benefit.

2.4 Discussion: Estimates and definitions of ZIKV-related birth defects

In the largest prospective cohort of pregnancies with confirmed ZIKV exposure, we found a risk of 7.0% (95%CI: 5.0-9.5) birth defects possibly linked to ZIKV infection, which was reduced to approximately 1.6% (95%CI: 0.4-4.1) after consideration of a control group. The reason for the dramatic reduction in our estimate was the very high proportion of infants originally included who were labeled as having a ZIKV-related birth defect because of ‘metric microcephaly’; meaning microcephaly defined so based on head circumference measurements according to a normalized growth curve for gestational age and sex, rather than based on or supported by a clinician’s diagnosis (Orioli et al., 2017). Further supporting the non-specificity and inappropriateness of defining disease-related microcephaly through only ‘metrics’, were our findings from two sub-Saharan African and two Asian settings that demonstrated elevated and highly regionally-variable proportions of infants being labeled as having microcephaly when using such a definition. These results, and our attempts to compare them to other contemporary studies on the same topic, highlight the difficulties in generating accurate estimates of disease burden following emerging disease events. Below I will discuss how, in the case of ZIKV-related birth defects, these complications may be arising due to incomparability in epidemiological studies, and use of non-specific case definitions.

- **Drawing a consensus on the risk of ZIKV-related birth defects**

At present, three key studies have estimated the risk of birth defects following ZIKV infection during pregnancy; these studies have been performed each in different settings and with slightly differing methodologies. In a prospective cohort study in Brazil, 125 women with rash at any moment during pregnancy were enrolled and followed until either pregnancy loss or up until one month after birth for live born infants (Brasil et al., 2016). In our prospective cohort study in the French Territories in the Americas (FTA), 546 women with any symptom of ZIKV infection at any moment during pregnancy were enrolled and followed until either pregnancy loss or until hospital discharge following birth for live born infants (Hoen et al., 2018). In the United States territories, a registry based study compiled longitudinal information on the pregnancies of 1279 ZIKV symptomatic women until either pregnancy loss or hospital discharge for live born infants (Shapiro-Mendoza, 2017). In all of the figures

mentioned above and risk estimates further discussed below, only women with RT-PCR confirmed ZIKV infection are considered. Without adjustment of control groups (for now), the estimates from the FTA and the USZIPR are similar with 7.0% (95%CI: 4.0-9.5) and 4.9% (95%CI: 3.8-6.3), respectively, but are much lower than the 46.4% (95%CI: 37.4-55.5) observed in Brazil. The reason for the stark differences between these estimates is yet to be addressed formally, but may be due to differences in any of: study methodologies, the definitions of birth defects ‘possibly linked to ZIKV’ that were used, or biological mediating or interactive mechanisms that differ regionally.

Methods and bias in studies addressing the risk of ZIKV-related birth defects

The main methodological characteristics of the three prospective studies addressing the question of the risk of ZIKV-related birth defects following infection during pregnancy are summarized in Table 13. Enrollment was slightly different in each study. In Brazil and the FTA, only ZIKV symptomatic women who presented to hospital were included; in Brazil, the presence of rash was necessary for inclusion, while in the FTA any symptom of infection was allowed (e.g. fever, rash, conjunctivitis, etcetera). In both studies, inclusion of symptomatic women presenting to hospital is likely to select for pregnancies with more severe infection manifestations. In addition, it is possible that a specific selection for women presenting with rash may further increase the chance for severe symptomatic infection in the Brazilian study; although the presence of rash in 95% of our own participants makes this, as a reason for differences in estimates, seem unlikely (Brasil et al., 2016; Hoen et al., 2018). As mentioned by the authors of the study in the US Territories, inclusion through a registry-based study may either select for more symptomatic pregnant women and pregnancies with already detected abnormalities, or rather may lead to underreporting of birth defects in pregnancy losses (Reynolds et al., 2017); the former could lead to an overestimate and the latter an underestimate of birth defects possibly linked to ZIKV. Our own research shows that symptom severity may be linked to increased viral load (Pellerin et al., unpublished data); however, higher viral load has not necessarily been linked to an increase in Zika-related birth defects (Halai et al., 2017; Pellerin et al., unpublished data).

Still focusing on methodological differences, an alternative, more plausible, explanation for stark differences in study estimates between the FTA and US Territories, when compared to Brazil, may be in the imaging procedures and length of follow-up for each study. In Brazil, transfontenellar ultrasound was performed in all live born infants, with subsequent computed tomography (CT) and MRI examinations when any abnormality was detected (Brasil et al., 2016). In contrast, in the FTA only prenatal imaging was performed, with subsequent MRI if abnormalities were detected (Hoen et al., 2018). In the US Territories postnatal imaging was performed in only 52% of live born infants (Honein et al., 2017; Reynolds et al., 2017; Shapiro-Mendoza et al., 2017). Another important methodological difference is an increased length of follow-up in live born infants from Brazil when compared to those in the FTA and US Territories; this was up to one month of age for Brazilian infants, whereas follow-up was only until hospital discharge in the FTA and United States Territories. Increased CT and MRI imaging may lead to the identification of subtle brain abnormalities not picked up by pre and postnatal ultrasound examination that could indicate future ZIKV-related developmental abnormalities, and a longer follow-up period allows for further evaluation of clinical signs of CNS dysfunction (e.g. tonicities, reflexes, irritability, swallowing ability) that may not easily be observed in the first few days of life. Supporting this, the proportion of microcephaly at the time of birth was similar in Brazil and the FTA, with 3.8% and 5.4%, respectively, whereas findings related *only* to postnatal imaging and clinical signs of CNS dysfunction, with no abnormalities seen by prenatal ultrasound, physical examination at birth, or eye examinations, were 18.4% and 0.4%, respectively. On the other hand, the proportion of infants being admitted to intensive care was much higher in Brazil when compared to the FTA (21% vs. 1.3%), which does indicate that the infants in Brazil were in a worse overall health condition, irrespective of what detailed imaging was employed (Brasil et al., 2016; Hoen et al., 2018). In the data published from the US Territories, it is not possible to break down these estimates (Shapiro-Mendoza et al., 2017).

Table 13: Characteristics of studies following pregnancies with RT-PCR confirmed ZIKV exposure in symptomatic pregnant women

Location	Study design and inclusion	Sample Size*	Imaging	Clinical exam after pregnancy outcome	Control Group
Brazil <i>Brasil et al., 2016</i>	Prospective cohort study, enrolling pregnant women presenting with rash	125	Prenatal ultrasound (45%). Postnatal transfontanelar ultrasound offered to all ZIKV+ pregnancies, then recommended CT/MRI if abnormality detected.	Examinations up until 1 month of life by multidisciplinary team (neonatologists, neurologists, infectious disease specialists, geneticists, ophthalmologists, physical therapists)	Yes Postnatal imaging not performed in control group. Clinical examinations in control group not reported. Clinicians not blinded to ZIKV status of infants
FTA <i>Hoehn et al., 2018</i>	Prospective cohort study, enrolling pregnant women presenting with ZIKV symptoms	546	Prenatal ultrasound (80%), then recommended CT/MRI if abnormality detected. Postnatal ultrasound only for those enrolled in infant cohort (not reported).	Examinations by pediatrician at time of birth for all live born infants. Hearing and visual testing only for those enrolled in infant cohort (not reported).	No ZIKV negative control group identified later. Prenatal imaging less common (52%) but post-birth clinical exam similar in control group. Clinicians not blinded to ZIKV status of infants.
United States Territories (USZIPR) <i>Shapiro-Mendoza et al., 2017</i>	Registry-based compilation of prospective longitudinal data on ZIKV exposed pregnancies*	1279	Proportion of infants with prenatal imaging results not reported. Postnatal imaging performed in 52%.	Clinical examination performed at time of birth in all infants. Hearing examinations in 79% of infants.	No

*Symptom data collected but not an inclusion criterion

** Numbers reflect pregnant women with confirmed ZIKV infection by RT-PCR and symptomatic

Differences in defining and reporting on birth defects possibly linked to ZIKV

Although more intensive brain imaging and clinical examinations could be the reason for a higher proportion of infant abnormalities linked to ZIKV in Brazil when compared to the USZIPR or FTA, the differences in estimates may be further exacerbated by differences in what is reported as ‘adverse pregnancy outcome’, with the implication of being potentially linked to ZIKV, in different studies. Of the 58 (46%) adverse pregnancy outcomes notified in Brazil, 9 (7%) were fetal losses without any indication of brain abnormalities by prenatal ultrasound or autopsy, 4 (3%) were infants either small for gestational age or with macrosomia due to maternal gestational diabetes with other linked or non-specific findings, and 13 (10%) are solitary non-specific MRI and other post-natal imaging findings that would typically require further follow-up to determine if an abnormality is present (Brasil et al., 2016). The remaining 32 (26%, 95%CI: 18.2-34.2) defects, significantly different than the reported 46%, may more closely represent conditions that would be considered as abnormalities in the studies performed in the FTA and United States Territories. This adapted risk estimate is still much higher than those of the latter two studies, but, if combined with the increased length of follow-up into the first month of life for observation of CNS dysfunction, the results of the three studies become more easily reconcilable.

Regional differences that may lead to mediation or interaction

Finally, there may be a biological mediating or interacting mechanism that comes between ZIKV infection during pregnancy and adverse fetal outcomes. If such a mechanism exists, it may differ regionally due to environmental exposures or genetic differences. Poor socioeconomic conditions have been linked to increased microcephaly cases in Brazil, though this may simply indicate an area with higher birth rates and slightly increased incidence of ZIKV infection due to vector control deficiencies (Souza et al., 2018). Previous dengue virus exposure has been indicated to enhance ZIKV infection in human serum (Castanha et al., 2017), and increase adverse fetal outcomes in ZIKV infected pregnancies in mice (Rathore et al., 2018). In the Brazilian ZIKV infected pregnant women, 88% had previous exposure to dengue virus (Brasil et al., 2016); however, presence of DENV IgG was not associated with adverse pregnancy outcomes in their study (Halai et al., 2017). Although in very recent years

there has been minimal circulation of DENV in the FTA, especially in Guadeloupe and Martinique where most of the data from our work on ZIKV-related birth defects originates (INVS, 2015; 2016; 2017), there have been significant outbreaks in these regions in the past (L’Azou et al., 2014). Further work to evaluate the comparative proportion of pregnant women with past exposure to DENV in the FTA would be interesting for comparison to that in Brazil; in the participants in the USZIPR study, there may be very little previous DENV exposure. As with other congenital infections, maternal-foetal transmission does not occur in all ZIKV infected pregnancies, and the reasons for why it does happen in some cases, but not in others, deserves further study.

- **Defining ZIKV disease-related conditions**

Metric microcephaly

In the three studies discussed above, as well as in other key studies that focus on defining ZIKV-related birth defects and their risk, a standardized definition of microcephaly with a set head circumference cut-off (less than either -2SD or the 3rd percentile) according to an international pooled growth chart for gestational age and sex has been used (Brasil et al., 2016; de Araújo et al., 2016; Hoen et al., 2018; Honein et al., 2016; Reynolds et al., 2017; Shapiro-Mendoza et al., 2017). Standards used include the INTERGROWTH-21st Newborn Size at Birth Growth Charts, the WHO Child Growth Standards, and the revised Fenton growth chart (de Onis et al., 2004; Fenton and Kim, 2013; Villar et al., 2014); the former two are recommended by the CDC and WHO (CDC, 2016; WHO, 2016f). When this definition is used on its own to define a case, without needing clinician opinion or agreement that the infant is abnormal, it can be termed ‘metric’ microcephaly, as has been discussed by Orioli and colleagues (2017). Labeling an infant as having microcephaly based on measurement only does not reflect the real-life practice of diagnosis for this condition, outside of research and ZIKV-induced surveillance (Orioli et al., 2017). For example, an infant with a head circumference of -2.5 SD and length of -2.2 SD, according to a standardized growth chart, may not be flagged as possibly abnormal by a pediatrician, especially the infant has no dysmorphic features and the mother is 150cm tall. Alternatively, an infant with head

circumference of -1.9 SD with partially collapsed skull, excess scalp and a length of 0.2 SD according to a standardized growth chart, may be noted as having microcephaly with recommended further imaging. In both examples, the growth standards may be useful at an individual level to determine whether the infant needs a closer examination, but an expert clinical opinion decides the next steps in diagnosis and care.

The use of one definition for a clinical condition in real-life and another for research purposes may lead to the perception of a highly distorted flux in cases if a focus on the manifestation suddenly increases due to an emerging disease event. In the case of microcephaly, this is obvious when we compare the reported pre-ZIKV prevalence of the condition, reported through birth defects registries, against the observed proportions when a ‘metric microcephaly’ definition is applied across a population of infants either pre or post-ZIKV. Table 14 demonstrates this, along with a further comparison to the proportion of microcephaly in studies in ZIKV exposed neonates, and the magnitude of increase that may then be perceived and reported. A key factor underlying this large difference is that, by definition, use of a cut-off at less than -2SD (or 3rd percentile) against a normally distributed standardized growth chart, *should* automatically implicate 2-3% of *healthy* infants as having microcephaly. Although this may seem obvious, high proportions of microcephaly seen in studies applying metric microcephaly definitions to hospital-based or other study populations have been interpreted as clinically relevant in the wake of the ZIKV epidemic. In Brazil and Guatemala, when high baseline microcephaly was observed using pre-ZIKV era data, the interpretations were ‘microcephaly was endemic... before circulation of the Zika virus’ and presence of ‘high background congenital microcephaly’, respectively, with recommendations to improve screening techniques (Rick et al., 2017; Silva et al., 2018). In one of the Brazilian hospitals studied by Silva and colleagues, the 2.5% prevalence of microcephaly seen should could alternatively be interpreted as “similar to the expected amount of infants with head circumference Z-scores less than -2SD according to the growth standard used”; the INTERGROWTH-21st newborn standards, used in that study, prescribe that 2.2% of healthy infants will have microcephaly (Villar et al., 2014).

Table 14: Microcephaly prevalence in live births when applying “real-life” clinically relevant definitions of the condition vs. a standardized metric definition

Region	Pre-ZIKV or non-ZIKV baseline estimates		ZIKV-exposed		Perceived magnitude of increase	
	Birth Defects Registry Prevalence(s) – year(s)	Metric definition Prevalence(s) – year(s)	Metric definition Prevalence(s) – year(s)	Real-life vs Metric $ZIKV/Pre$ (or non)-ZIKV	Metric vs Metric $ZIKV/Pre$ (or non)-ZIKV	
South America	0.04%, 2005-14 <i>Orioli et al., 2017</i>	Not available	Not available	-	-	
Brazil	0.08%, 2005-14 <i>Orioli et al., 2017</i>	3.5% & 2.5%, 2010* <i>Silva et al., 2018</i>	3.8%, 2016 <i>Brasil et al., 2016</i>	48 times	1.1 to 1.5 times	
Guatemala (rural)	Not available	4.4%, 2014-16** <i>Riek et al. 2017</i>	Not available	-	-	
Europe	0.02%, 2003-12 <i>Morris et al., 2016</i>	Not available	Not available	-	-	
France	0.03%, 2003-12 <i>Morris et al., 2016</i>	8.4%, 2016-17 <i>Funk et al., unpublished***</i>	5.8%, 2016-17 <i>Hoehn et al., 2018</i>	193 times	0.7 times	
North America	Not available	Not available	Not available	-	-	
United States	0.09% [#] , 2009-13 <i>Craggan et al., 2016</i>	Not available	4.1%, 2016-17 <i>Honein et al., 2016</i>	46 times	-	
Canada (Quebec)	0.04%, 1989-2012 <i>Auger et al., 2018</i>	Not available	Not available	-	-	

Note: All estimates are hospital-based unless marked by [#] which indicates a population-based estimate

* Study performed in hospitals in two different cities

** Varied metric microcephaly case definitions applied to data from a pregnancy registry; 4.4% fit the most specific metric microcephaly definition.

*** See second full text article presented in the Zika-related birth defects chapter in this thesis manuscript

Regional differences in growth

Misleading baseline proportions of microcephaly may be further exacerbated when regional growth norms do not match well with the recommended international pooled growth standards used to define metric microcephaly. This has been previously reported through a systematic review for the WHO child growth standards (Natale et al., 2014). This was also clearly demonstrated through our own findings in four settings in sub-Saharan Africa and Asia, with as low as 3% and as high as 35% live born infants being labeled as having microcephaly using a -2SD cut-off according to the INTERGROWTH-21st newborn growth standards. Such variation should equally be considered when interpreting research on ZIKV-exposed populations in different settings.

The INTERGROWTH-21st Newborn Size at Birth growth standards were created through a large population based study which included cities in eight countries: Pelotas (Brazil), Turin (Italy), Muscat (Oman), Oxford (UK), Seattle (USA); Shunyi County, Beijing (China); the central area of Nagpur (India); and the Parklands suburb of Nairobi (Kenya). The study enrolled pregnant women in each region who had optimum health, nutrition, education and socioeconomic status, who lacked any significant gynaecological or obstetric history, and who met other pre-specified study inclusion criteria (Villar et al., 2013). In the case that the mother developed any severe condition during pregnancy, or in the event of a stillbirth, miscarriage, medical abortion, or live birth with congenital abnormalities, the participant was excluded. The INTERGROWTH-21st study group found that while skeletal indicators (including crown-rump length for early linear foetal size, and head circumference for foetal growth after 14 weeks gestation) differed across the eight countries, even after application of their strict inclusion criteria, the estimates were within a pre-specified degree high enough in order to justify pooling the findings (Villar et al., 2014; Altman and Ohuma, 2013). This allowed range of difference was 0.5SD; meaning that for a key indicator, any one country's growth distribution could differ by up to 0.5SD from the pooled standard. As mentioned in our paper, this allowed variance was criticized by some, who demonstrated that 13% of healthy infants could be labelled as abnormally small if the mean SD of a specific country sat at -0.5SD on the pooled standard (Albert and Grantz, 2014).

Although it is controversial to suggest that ethnic variation and genetics may play a role in fetal growth, abstaining from entering into that conversation does not exclude the realization that we should expect (sometimes extreme) variance in head circumference and other growth Z score distributions when applying pooled standards to an entire newborn population in a given region of the world. In a real-life newborn population for which the proportion of microcephaly is of interest, there cannot be any selection based on criteria such as maternal height, nutrition and environmental conditions. This can explain, non-controversially, the variation seen in proportion of infants at baseline with microcephaly, or other conditions, in different regions. For example, in India, a criterion that mothers needed to be >153 cm tall, led to the exclusion of many otherwise eligible women (i.e. good nutritional and socioeconomic status, etcetera) in the INTERGROWTH-21st study (Villar et al., 2014). As the average female height in India has been estimated at around 152 cm, creation of a growth chart using this criterion is not nationally representative, and would no doubt lead to extreme results if it was applied nationwide for surveillance (Mamidi et al., 2011; Steer, 2014). Persons on either side of the debate for or against regionally specific growth standards are unlikely to argue that microcephaly surveillance of all newborns in a village in South Kivu may produce a very different estimate when compared to a private hospital in Manhattan, if using a pooled growth standard. In a setting with nutritional or socioeconomic deficiencies or excesses, the artificially high or low proportions of baseline microcephaly automatically implied when using a ‘metric microcephaly’ definition and international pooled growth charts will become very problematic when trying to identify a real change in disease-related cases should an epidemic of ZIKV occur.

- **Improving accuracy and comparability of ZIKV-related birth defects findings**

Avoidance of many of the issues I have raised above can be achieved by comparison with an appropriate ZIKV non-exposed control group that has had, as much as possible, the same investigations as a ZIKV exposed fetal/newborn population of interest. In our own case, comparison with a control group left us to deduce that only 1.6% of foetuses/infants born to women infected during pregnancy in Guadeloupe during the epidemic period had severe abnormalities linked to ZIKV at the time of birth. Even though in our original prospective cohort study we used a metric definition of microcephaly, even this likely inappropriate diagnostic for

the condition was teased out through comparison with a control group who had the same clinical examination at birth. Control groups may also help to speed up conclusions on non-specific imaging and clinical findings in the early neonatal stages; in this case, it would be necessary that clinicians are blinded as to ZIKV exposure and that the same intensity of imaging is done in control groups. This was not the case for the control group used in the Brazilian study examining the risk of ZIKV-related birth defects (Brasil et al., 2016). In the case of inability to identify a control group, shifting away from a measurement-based definition of some conditions, such as microcephaly, and towards more clinically relevant pathology-based definitions is needed.

Transparency and clear communication between study authors can be a quick and easy way to deduce differences in study case definitions. In our own study on ZIKV-related birth defects, as well as that of Brasil and colleagues in Brazil, publishing of a supplementary appendix that clearly stated all the imaging and clinical findings of each infant diagnosed as ‘abnormal’ allows any person to compare what each research group has considered as a disease-related condition (Brasil et al., 2016; Hoen et al., 2018). Research groups addressing the topic of the risk of ZIKV-related birth defects in the future will ideally use the same approach, which, all together, will facilitate eventual meta-analyses.

Determination of the most accurate possible results regarding the risk of ZIKV-related birth defects following infection during pregnancy has important policy implications, for example, for the eventual vaccination of women of childbearing age. Without a vaccine yet available, there is an more pressing importance in deciphering the true risk of ZIKV-related birth defects, and that is a clear and accurate communication with women who are positive for ZIKV during pregnancy. This will limit anxiety and guide follow-up and family planning decisions in countries where these services are available. In many South American countries where access to safe abortion is restricted, requests for pregnancy termination through an online telemedicine alternative increased significantly during the ZIKV epidemic period; these magnitudes may be underestimated as they do not take into account those who sought unsafe alternatives (Aiken et al., 2016). These women, on whom the social and economic burden of having an infant with a severe disability will largely fall, deserve our best collaborative efforts in estimating the risk of ZIKV-related birth defects.

Part 3: Introduction of direct acting antivirals to treat HCV in Egypt

In this section, I will focus on research related to the introduction of new highly effective direct acting antivirals (DAA) to treat Hepatitis C (HCV) in Egypt – this was the original topic of my thesis from October 2014 until mid-2016. During these two years, I made multiple visits to Cairo to prepare a cohort study following 7500 persons treated with these drugs through the National Treatment Program, and worked on moving the protocol through various regulatory bodies in France. Although the full cohort study was halted (the reason for my thesis topic switch), and has only just re-begun in early 2018, valuable collaborations with smaller associated research groups during this time led to my significant involvement in four published and two ongoing pieces of linked research over the past four years.

I will start with some background information on HCV, with a specific focus on the evolution of available treatment options as well as the Egyptian context. The latter will include a brief description of a research article I worked on related to the prevalence of HCV in Egypt. I will then present two research articles that highlight the challenges in bringing together initial findings on potential adverse events of a new treatment after administration in real-life populations. A third article, a research letter, addresses a challenge of wide-scale introduction of new therapies for HCV – assuring retention of patients until the moment of cure to enable follow-up of non-responders with potential resistance mutations as well thorough program evaluation. I will then briefly discuss further implications of our findings, potential explanations and solutions for addressing the challenges of interpretation of results following an event such as effective treatment introduction, and propose important next steps.

3.1 Background

3.1.1 Discovery and Diagnosis of HCV

In 1975, researchers realized that a high proportion of transfusion related hepatitis was caused by an elusive *other* agent, rather than the already identified hepatitis A and hepatitis B viruses (Alter et al., 1975; Alter, 1999; Fienstone et al., 1975). ‘Non-A, non-B hepatitis’ (NANB) was shown to lead to liver disease in a high proportion of patients, however, without being able to isolate the agent behind it, prevention efforts and specific blood screening tests were not possible (Hoofnagle & Alter, 1985). In the 1980s, there were many unsuccessful attempts to isolate the pathogen causing NANB in vitro using traditional methods on blood from patients. Then, more than 10 years after the discovery of NANB, a research group headed by Michael Houghton from Chiron Corporation laboratories, with the support from the Centers for Disease Control and Prevention, were able to isolate a cDNA clone of derived from a virus after screening large amounts of blood from experimentally infected chimpanzees (Alter, 1999; Houghton, 2009). It was a flavivirus, a member of the family *Flaviviridae*, and was thereafter called the “Hepatitis C virus” (Choo et al, 1989). Rapid work that followed saw the identification of several HCV genotypes (up to seven), development of assays for detection of HCV antibodies and HCV RNA, and an epidemiological understanding that this virus had been the main cause of blood-transmitted NANB hepatitis (Alter, 1999; Kuo et al, 1989). In the United States, it quickly became standard procedure to test donated blood for antibodies against the pathogen, and with further improved enzyme immunoassays (EIA) in the early 1990s, transfusion-associated HCV was practically eliminated in high-income nations within a few years (Alter, 1999).

At present, HCV antibodies (anti-HCV) can be detected in oral fluid or blood within a few weeks of infection using the third generation enzyme immunoassays (EIA-3) in laboratory conditions, with sensitivities and specificities up to 98% and 100%, respectively. Rapid tests for anti-HCV are also available; for detection in blood with sensitivities and specificities reaching 98 and 100%, respectively, and for detection in oral fluid with sensitivities and specificities reaching 94 and 100%, respectively (Tang et al., 2017). Presence of anti-HCV indicates qualitatively whether or not a person has ever been infected, but is unable to distinguish between current (i.e.

active) and former infection. Nucleic acid tests are used to detect and quantify HCV RNA, expressed in ‘units international’ per ml (UI/ml), and are commonly carried out using PCR techniques (Chevaliez, 2011; Pawlotsky, 2003). Presence of HCV RNA indicates an active infection with ongoing viral replication. Rapid tests for detection of HCV RNA using venous blood are currently being evaluated, with demonstrated sensitivities and specificities of up to 100%, and the possibility of eventually using capillary blood (finger prick testing) (Grebely et al., 2017; Lamoury et al., 2018; Llibre et al., 2018).

3.1.2 Natural history of HCV infection

Following acute infection with HCV, between 15 and 40% of individuals clear the virus spontaneously with 6 months time (Westbrook & Dusheiko, 2014); this clearance is associated with various genetic factors, including presence of variants of the interleukin 28B (IL28B) gene, as well as the DQB1*0301 allele of the major histocompatibility complex class II (Alric et al., 1997; Ge et al., 2009; Thomas et al., 2009). While most persons with acute infection will be asymptomatic, a minority will have flu-like symptoms with more severe symptoms such as jaundice, abdominal pain, and lack of appetite (Westbrook & Dusheiko, 2014); this lack of symptoms makes studies on acute HCV infection complex to carry out. The most common complication of acute infection is progression to chronic infection and hepatitis, however, for those who do clear the virus spontaneously, long-term impact on the liver is very rare. It is important to note that clearance of HCV, either spontaneously or via treatment, does not preclude re-infection (Westbrook & Dusheiko, 2014).

For the majority of persons with acute infection, chronicity occurs, marked by continual hepatic inflammation. After 20 years and 30 years, chronic HCV infection has been seen to lead to cirrhosis in around 15% and 40% of persons, respectively (Thein et al., 2008; Westbrook & Dusheiko, 2014). Cirrhosis is caused by progressive fibrosis (scarring), of the liver, which is a result of chronic inflammation and perpetually elevated liver enzymes. Fibrosis is evaluated in stages, best diagnosed by liver biopsy, which relates to the level of scarring of the liver using the Metavir score: F0 indicates a lack of fibrosis, and F1-F2 mild-moderate fibrosis, F3 is moderate-severe fibrosis without cirrhosis, and F4 is cirrhosis. Cirrhosis indicates that scarring has

encompassed the liver to such a degree that it is unable to perform its usual functions. The timing by which each patient develops and then moves towards increased fibrosis levels differs and is not necessarily linear; it is influenced by the virus itself, the individual, and the environment (Datz et al., 1999; Westbrook & Dusheiko, 2014; Yi et al., 2004).

For those with cirrhosis, the yearly incidence risk for hepatocellular carcinoma (HCC) is between 1 and 4% (El-Serag et al., 2006; Goodgame et al., 2003). If presence of HCC lesions are caught at an early stage, then local ablation or surgical resection procedures can successfully be used as treatment, with up to 70% of patients having 5-year survival following this therapy (Lin et al., 2012). If detected at an intermediate stage, transarterial therapies, such as transarterial chemoembolization (TACE), are usually recommended to treat HCC, however, studies evaluating whether or not this intervention increases average survival time have varying conclusions (Oliveri et al., 2011). Surgery is another option for persons with intermediate stage HCC, but with 5-year survival estimated at less than 20% (Chen et al., 2006). At late or terminal stages of HCC, oral sorafenib can be given, sometimes in addition to transarterial therapy, with median survival being typically less than a year (Bruix & Sherman, 2011; Qu et al., 2012). Unless a person is at the very earliest stages of this cancer, liver transplantation is also an option for treatment. With liver transplantation, the chance of 5-year survival for persons with HCC increases up to 80% (Mazzaferro et al., 2009; Poon et al., 2007). This success rate is due to the fact that liver transplantation not only removes the cancer, but also eliminates all previous liver damage. Unfortunately, the feasibility of transplantation is low due to a shortage of donors and strict eligibility criteria (Lin et al., 2012). Influencing survival, the 5-year risk of HCC recurrence after successful treatment, of even some of the earliest stages, is up to 70% (Hanazaki et al., 2000; Poon et al., 2002; Portolani et al., 2006).

An alternative risk for persons with cirrhosis, occurring in 3-6% of persons annually, is decompensation - defined as the occurrence of variceal haemorrhages, ascites and encephalopathy (Westbrook & Dusheiko, 2014). One-fifth of persons having a decompensation event will not survive until the following year (Thein et al., 2008).

3.1.3 Treatments for HCV

- **The Interferon-Era (1990 – 2014)**

Interferons (IFN) are proteins that occur naturally in the human body and have a wide range of actions. Various types of IFN are commercially produced for use in treating disease. In the case of hepatitis C, both IFN- α 2a and IFN- α 2b, subtypes of IFN- α that differ only by one amino acid, are active. Use of IFN- α to treat HCV began shortly after discovery of the virus, in 1990 (Poordad & Dieterich, 2012). Original treatment regimens that prescribed injected doses of IFN- α for six months to one year led to a sustained virological response (SVR) in between 10 and 20% of patients (Carithers & Emerson, 1997; Haria & Benfield, 1995; Poynard et al, 1996; Saracco & Rizzetto, 1995). In the case of HCV, SVR indicate successful clearance of the virus, and is assessed 24 weeks (SVR24; used in the IFN-era) or 12 weeks (SVR12; used with more recent regimens) after the end of treatment. In the decade or so to follow, some improvements to treatment with IFN- α included addition of another anti-viral, ribavirin (RBV), as well as pegylation of IFN (i.e. peg-IFN), a process that allows for an increased half-life of, and tolerance to, the molecule. Combination therapy of parenteral peg-IFN- α and orally administered RBV could typically lead to SVR in around 40 and 50% of chronic hepatitis C patients with genotypes 1 or 4 (G1, G4) if given for 48 weeks, and between 70 and 80% for those with genotypes 2 or 3 (G2, G3) if given for 24 weeks (Brok et al, 2005; Fried et al, 2002; Hadziyannis et al, 2004; Kjaergard et al, 2001; Manns et al, 2001; Simin et al, 2007; Zayed et al, 2016).

IFN is not a well-tolerated treatment, and is contraindicated in persons with advanced liver disease. Although severe side effects were rare for persons undergoing HCV therapy with IFN- α , almost all patients experience some mild-moderate symptoms. A ‘flu-like’ syndrome (i.e. fatigue, nausea, etcetera) is seen in almost all persons taking this therapy- the severity of this side effect is reduced with increased time on treatment for most patients (Dusheiko, 1997). Psychiatric side effects, such as depressive episodes and cognitive disorders are seen frequently, in around a quarter of patients (Dusheiko 1997; Udina et al, 2012). Such side effects have a negative impact on quality of life early on in treatment, and have led to discontinuation and dosage changes in up to 30% of patients (Bonkovsky et al, 1999; Dusheiko, 1997; Hunt et al, 1997; Ware et al, 1999; Zeuzem et al, 2000).

- **Direct acting antivirals (2014 to present)**

Direct acting antivirals (DAAs) are agents that act on parts of the viral life cycle that are specific to HCV itself. Advances in the in-vitro cultivation of HCV in the last decade or so have allowed for identification of many potential targets for such treatments. The class names of the DAAs reflect the part of the viral genome that is suppressed by their action: NS3/4A protease inhibitors block the ability of the virus to protect itself and cause damage to the host liver cell, NS5B nucleoside and non-nucleoside inhibitors block the ability of HCV to replicate itself, NS5A inhibitors block viral replication and the ability of HCV to interfere with host-cell immune defenses (Poordad & Dieterich, 2012).

In 2011, the FDA approved the first DAAs against HCV (FDA, 2011a; FDA, 2011b). These two agents, called boceprevir and telaprevir, are protease inhibitors that had demonstrated achievement of SVR in around 75% of treatment-naïve patients, and 65% of treatment-experienced patients in clinical trials when given in combination with IFN and RBV (Park et al., 2014). However, this triple therapy was associated with higher discontinuation of treatment due to adverse events when compared to IFN + RBV therapy alone; more common side effects included anemia, rash and itching, nausea, and taste distortion (Cooper et al., 2012; Dang et al., 2011; Park et al., 2014). In addition, development of resistance was a common reason for patients to fail treatment (Macartney et al., 2014; Wyles, 2012).

In 2013, a NS5B inhibitor called sofosbuvir (SOF), manufactured by Gilead Sciences under the name Sovaldi®, was approved for treatment of G1-4 of HCV by the FDA (FDA, 2013). This second generation DAA led to SVR in 90% of treatment-naïve HCV patients, with any of the four main genotypes, when given in combination with peg-IFN & RBV for 12 weeks of treatment (Kowdley et al., 2013; Lawitz and Lalezari et al., 2013; Lawitz and Mangia et al., 2013). In addition, for historically ‘difficult to treat’ populations (e.g. treatment non-responders, cirrhotics), dual therapy of SOF plus RBV for 24 weeks demonstrated SVR in up to 70% of persons enrolled in clinical trials (Osinusi et al., 2013). Swiftly following the approval to treat persons with HCV with SOF using dual or triple therapy, other DAAs were released by Gilead Sciences, Bristol-Myers Squibb, AbbVie, Janssen Pharmaceuticals, and Merck (FDA, 2017).

For chronic HCV patients of all genotypes and regardless of treatment experience, there are now many all-oral treatment options available, many given as a single tablet containing multiple fixed dose DAAs. Recommended treatment durations are currently from 8-12 weeks for non-cirrhotic patients and 12-16 weeks for patients with compensated cirrhosis. In this case, treatment experience refers to therapy with peg-IFN plus RBV with or without SOF, or SOF plus RBV (EASL, 2018). Clinical trial and real-life evaluations of these treatment regimens show similar cure rates of > 95% in these populations with all-oral regimens (Afdhal and Reddy et al., 2014; Afdhal and Zeuzem et al., 2014; Asselah et al., 2018; Feld et al., 2015; Ferenci et al., 2014; Forns et al., 2017; Jacobson et al., 2017; Kohli et al., 2015; Kowdley et al., 2014; Krishnan et al., 2017; Landis et al., 2017; Naggie et al., 2015; Rockstroh et al., 2015; Toyoda et al., 2018; Tsai et al., 2017; Wei et al., 2016; Welzel et al., 2016; Zeuzem et al., 2015; Zeuzem et al., 2018). In patients with severe liver disease (i.e. decompensated cirrhosis) and those who have received liver transplantation, there now are also treatment options, however, DAAs that act as protease inhibitors are not recommended (EASL, 2018). For this population, real-world evaluations and clinical trials demonstrate cure rates of >85% for persons with moderate decompensation, and up to 80% in those with severe decompensation with HCV genotypes 1 and 4 (Charlton et al., 2015; Manns et al., 2016; Foster et al., 2016, EASL, 2018).

The adverse events experienced using second generation DAAs are considerably milder when compared to IFN containing regimens, and lead to treatment discontinuation in only around 1% of patients. Typical side effects include fatigue, headache, nausea, benign diarrhea, and for some regimens, dermatological manifestations such as itching and rash (Dufour et al., 2017; EASL, 2018; Jacobson et al., 2017). Rare severe adverse events have included episodes of severe pulmonary arterial hypertension and cardiac arrhythmias, the latter due to contraindication with other treatments (EASL, 2018; Renard et al., 2016; Renet et al., 2015). Persons with decompensated cirrhosis are more likely to experience severe adverse events to treatment (EASL, 2018). While the new DAAs have higher barriers to resistance than the first generation ones, resistant-mutations have been detected in persons not responding to treatment, and this confers a lower likelihood of achieving SVR (Pawlotsky, 2016). Further research and monitoring in the area of DAA resistance is needed.

- **Benefits of achieving an SVR**

Achievement of SVR for patients with mild to moderate liver damage is associated with reversal of liver damage, reduction in incidence of HCC, and overall increased survival (D'Ambrosio et al., 2012; George et al., 2009; Morgan et al., 2012; Poynard et al., 2002; van der Meer et al., 2012; Veldt et al., 2007;) In similar patients treated with all-oral DAAs, improvements in hepatic function and overall mortality have been seen (Backus et al., 2018; Mandorfer et al., 2016; van der Meer & Berenguer, 2016). However, more time will be needed to fully quantify long-term benefits and improvement in liver function (van der Meer & Berenguer, 2016). In addition, for one-third to one-half of persons with moderate and severe decompensation that are treated with DAAs, achievement of SVR has been associated with improvement in liver function scores in both clinical trials and real-world evaluations; longer-term studies will be needed to determine if overall liver function and survival is improved (EASL, 2018; van der Meer & Berenguer, 2016). Achieving SVR also has a positive impact on patients' health-related quality of life, regardless of type of regimen this is achieved by (Bernstein et al., 2002; Younossi et al., 2018).

- **The cost of the miracle drugs**

The release of Sovaldi[®] (i.e. SOF) by Gilead Sciences was not met with unbridled joy in the international community, even considering its huge perceived benefits according to published clinical trials. The cost of a 12-week supply of the drug was marketed originally at 84000 United States dollars (USD) - about 1000 USD a pill (Reuters, 2014a). The backlash to this included protests, such as a 'death-in' staged at an event hosted by Gilead Sciences, condemnation from various health organizations, and legal movements for patent opposition (Treatment Action Group, 2014; The New York Times, 2014; MSF, 2017).

As of 2018, some middle-income countries such as India, Egypt, and Brazil, have refused the patent or have successfully applied for exceptions with Gilead Sciences and other pharmaceutical companies in order to produce generic versions of the drug (Gilead Sciences Inc, 2015; The New York Times, 2015; Pharmaceutical-Technology News, 2018; Douglass et al.,

2018). Further, many middle and high-income nations have successfully negotiated down the prices for DAA regimens with Gilead and other pharmaceutical companies. However, in some countries such as the United States, Denmark, and Poland, treatment costs remain very high and lead to patient eligibility selection (e.g. based on fibrosis level) that is not mandated by the treatments themselves (Douglass et al., 2018).

3.1.4 Global epidemiology of HCV

The adult global prevalence of anti-HCV was estimated to be 2.5% in 2016 using data from 138 countries collected between the years 2000 and 2015. Of this, it was estimated that approximately 67% of persons are viraemic, signifying around 120 million chronic HCV infections globally (Petruziello et al., 2016). The most predominant genotype is 1 (G1), estimated to have a global prevalence of 46-49%, followed by G3 (18-30%), G4 (13-17%), and G2 (11-13%) (Gower et al., 2014; Messina et al., 2015; Petruziello et al., 2016). By region, the proportion of persons with chronic infection, and the predominant genotype, vary greatly (see Table 15). Associated with the differences in prevalence per region are variations in the most common modes of transmission as well as locally available resources for prevention, testing, and treating. HCV is a blood-borne pathogen, and the most common methods of transmission include unsafe medical injections and surgical procedures, illicit injection drug use, and blood transfusion (Shepherd et al., 2005). In low-middle income countries, transmission has occurred mainly through exposure to unsafe medical procedures and a lack of injection safety (El-Ghitany et al., 2015; Eze et al., 2014; Malhotra et al., 2015; Rerambiah et al., 2014); however, it is estimated that the number of new healthcare associated cases has decreased by more than 80% in these settings since the year 2000 (Kane et al., 1999; Pepin et al., 2014). In high-income countries, injection drug use is the main risk factor for transmission; this accounts for up to 80% of new HCV cases in many European countries as well as in North America and Australia (Alter, 2002; Dore et al., 2003; Judd et al., 2005; Mele et al., 2000; Negro, 2014; Suryaprasad et al., 2014; Thorpe et al., 2002).

Table 15: Regional prevalence of viraemic (active) HCV infections globally (information taken from Petruzzello et al., 2016)

Name	Region			Most affected sub region		Least affected sub-region	
	% Population Viraemic	# Active infections	Predominant Genotype	Name (% Viraemic)	Predominant Genotype	Name (% Viraemic)	Predominant genotype
Africa	2.0	20 million	G4 (28%)	Central Sub-Saharan (4.1%)	G4 (83%)	Southern Sub-Saharan (0.6%)	G5 (36%)*
North Africa / Middle East**	1.9	9 million	G4 (65%)	N/A	N/A	N/A	N/A
Americas	1.0	9 million	G1 (75%)	Caribbean (1.1%)	G1 (83%)	North America <i>high income</i> (0.9%)	G1 (66%)
Asia	1.8	72 million	G1 (47%)	Central (2.8%)	G1 (70%)	Asia Pacific <i>high income</i> (0.8%)	G1 (59%)
Australasia***	1.3	0.4 million	G1 (55%)	N/A	N/A	N/A	N/A
Europe	1.3	10 million	G1 (64%)	Eastern Europe (2.2%)	G1 (68%)	Western Europe (0.6%)	G1 (55%)

*Only estimates on genotype came from surveys done in South Africa

** Data not available from many countries in this region (only includes: Algeria, Egypt, Iran, Iraq, Israel, Libya, Morocco, Qatar, Saudi Arabia, Tunisia, Turkey, Yemen), and therefore, region not broken down into sub-regions.

***This region incorporates New Zealand and Australia only and is not broken down into sub-region

Blood transfusion is considered to have been the biggest driver of HCV transmission, but dropped drastically as a contributor to incident infections in high-income countries with the implementation of ‘all-volunteer’ blood donation programs in the 1970s and 80s as well as screening of blood products in the early 1990s after discovery of the virus (Alter et al., 1972; Doman, 1995). Unfortunately, in many low-income settings, blood donor and blood product screening is not enforced universally and so transmission through this pathway is still a risk factor (Marwaha and Sachdev, 2014). Vertical transmission of HCV infection, from mother to child, is estimated to occur in 3-8% of infected pregnancies, with increased risk if the mother is co-infected with human immunodeficiency virus (Dal Molin et al., 2002; Ferrero et al., 2003; Thomas et al., 1998; Yeung et al., 2001). Sexual transmission of HCV is also possible, but is extremely rare in heterosexual single-partner relationships, and more common for multiple-partner and higher-risk sexual behaviours (e.g. anal sex); the latter is true especially if co-infected with HIV (Alter et al., 1989; McFaul et al., 2015; Terrault et al., 2013; Vanhomerig et al., 2015).

Recent research suggests that the worldwide prevalence of HCV is decreasing, from approximately 2.8% estimated from data collected up until 2005, down to 2-2.5% in data collected up until 2015 (Petruziello et al., 2016). However, these trends, and an accurate estimation of their magnitude, may be nuanced by changes in sensitivity and specificity of diagnostic tests, as well as differences in data availability, over the past 25 years.

3.1.5 HCV in Egypt

From ancient times until quite recently, schistosomiasis (or Bilharzia), caused by the parasites *S. haematobium* and *S. mansoni*, was a major public health problem and the main cause of liver disease in Egypt (Abdel-Wahab et al, 1980; Abdel-Wahab, 1982; Scott 1937) Although an effective oral treatment for schistosomiasis (i.e. praziquantel) is now available, for most of the last century the recommended therapy was repeated injections of tartar emetic (Strickland & Ramirez, 2000). To tackle the large national prevalence of schistosomiasis, the Egyptian Ministry of Health and Population (MoHP) employed this latter treatment in countrywide campaigns as early as the 1920s and with increasing intensity up until the early 1980s (Frank et al, 2000;

Strickland, 2006). At that time, the dangers of blood-transmitted pathogens were still underestimated and glass-syringes were used repeatedly with insufficient sterilization over large groups of persons receiving treatment.

HCV seroprevalence studies taking place in the Egypt in the early 1990s indicated that a very high proportion of the adult population had been exposed to the virus, with estimates of around 10-25% anti-HCV in blood donors from urban areas, and 15-40% in non-blood donor populations living in rural areas (Abdel-Wahab et al, 1994; Arthur et al, 1997; Darwish et al, 1993; Darwish et al, 1996; Darwish et al, 2001; El Gohary et al, 1995; Kamel et al, 1992; Kamel et al, 1994; Mahamoud et al, 2013; Quinti et al, 1995). In 1995-96, a representative survey of 8499 Egyptians between 10 and 50 years of age demonstrated a national seroprevalence of 21.9% (95% CI: 21.0-22.8), with analyses confirming an association between presence of anti-HCV and parenteral therapy for schistosomiasis (Frank et al., 2000). Later, in 2008, a representative demographic health survey in 11126 Egyptians aged 15-59 years old demonstrated an updated seroprevalence of 14.7% (95% CI: 13.9-15.5) (Guerra et al, 2012). It is estimated that more than 90% of the HCV infection in Egypt is due to G4, which is rare in other parts of the world (Gower et al., 2014; Ray et al., 2000).

Although oral praziquantel is now used for treatment of schistosomiasis in Egypt, a high incidence of infection persists through unsafe medical injections and other surgical procedures, facilitated by the large HCV reservoir (Arafa et al., 2005; Mohsen et al., 2015; Paez Jimenez et al., 2009; Paez-Jimenez et al., 2010;). The incidence was estimated at approximately 150 000 infections annually by a modeling study performed using data from cohort studies in 2012 (Brebán et al., 2013).

- **Original research (short description): Hepatitis C prevalence in Egypt, 2014-2015**

The Egyptian Demographic Health Survey (EDHS) took place again in 2014, this time under the name of Egyptian Health Issues Survey (EHIS). Both of the national surveys in 2008 and 2015 were cross-sectional household surveys, conducted by El-Zanaty and Associates with support from the United States Aid of International Development-sponsored DHS-7 project. In

these surveys sampling weights were used to provide estimates considered representative of the Egyptian population on the basis of a complex, three-stage probability sampling approach (El-Zenati & Way, 2008; Ministry of Health Egypt, 2015). The surveys provide estimates of HCV prevalence in Egypt for the country as a whole as well as broken down for the major administrative regions (Urban Governorates, Lower Egypt, Upper Egypt and the Frontier Governorates). In both EDHS 2008 and EHIS 2015, those aged 15– 59 years were invited to participate, however, in 2015, children aged 6 months to 14 years were also included. The preliminary analysis of the EHIS 2015 demonstrated a new seroprevalence of 10.0% (95%CI: 9.5–10.5). When compared to the 14.7% (95% CI: 13.9-15.5) seroprevalence seen in 2008, this significant decline raised questions of whether or not the nationwide prevention and treatment efforts could be credited. At this point, we began some collaborative work with the MoHP in order to compare and interpret the differences between the 2008 and 2015 surveys.

We found that, in the 15–59-year age groups, the prevalence of HCV antibody was found to be 10.0% (95% CI 9.5–10.5) and that of HCV RNA to be 7.0% (95% CI 6.6–7.4). In children, 1–14 years old, the prevalence of anti-HCV and HCV RNA were 0.4% (95% CI 0.3–0.5) and 0.2% (95% CI 0.1–0.3), respectively. For both antibodies and HCV RNA, the prevalence was increasing by age group. Extrapolated, this would mean that 3.7 million persons have chronic HCV infection in the age group 15–59 in 2015 in Egypt. For chronic infection (presence of HCV RNA), when the 2015 results were compared to the 9.9% (95%CI: 9.3-10.5) of persons with HCV RNA in the EDHS of 2008, a 29% reduction was indicated. We then deduced that this apparent decrease was mostly due to the ‘cohort effect’ rather than prevention or treatment efforts; age groups with very high prevalence of infection were growing older and shifting out of the age range captured by the survey (Figure 6).

To estimate whether or not the effect of widely employed prevention efforts, such as infection control and needle safety programs, could be seen through the national surveys, we looked to the younger age groups, where seroprevalence reflects the cumulative incidence of the past years in the absence of mortality. In 2008 and 2015, the prevalence of HCV antibodies in those aged 15– 19 years was 4.1 and 1.0%, respectively; the percentage of relative risk reduction was 75% (95% CI 64–85), implying a very substantial reduction in HCV incidence in the past 20 years in this age group. However, we were not yet able to visualize any effect of the nationwide

HCV treatment program (to be described further below), in the reduction of the proportion of persons with HCV RNA compared to the proportion of persons with evidence of ever having been infected (anti-HCV).

In order to better estimate the prevalence of HCV in Egypt, and to further visualize both the cohort effect and the impact of HCV treatment, the next EHIS survey, likely to take place around 2020, will need to adapt to include older age groups. The PDF of this article can be found in Appendix 5.

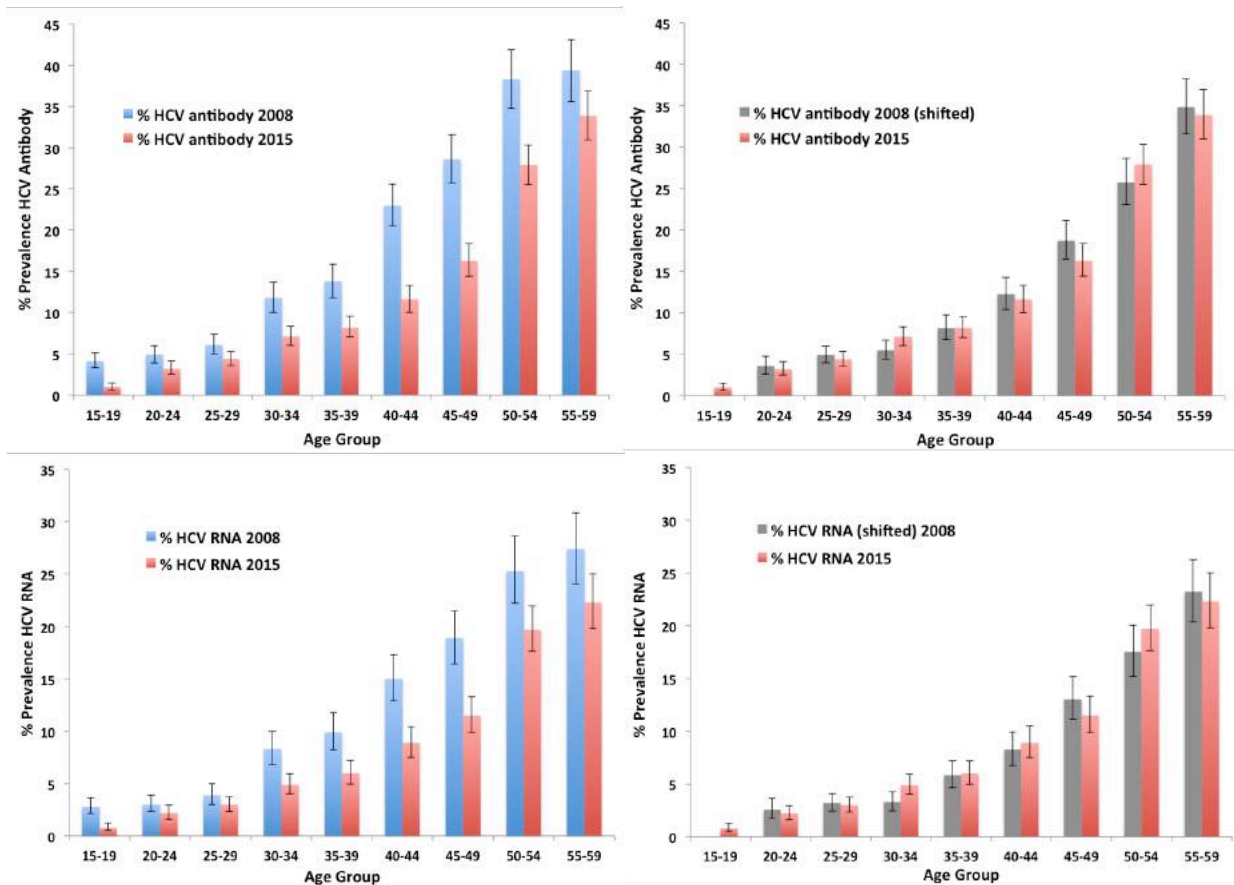


Figure 6. (A) Age-specific prevalence of hepatitis C virus (HCV) antibody-positive persons in 2008 and 2015 (left), then shifted (by 7 years) 2008 and 2015 (right). (B) Age-specific prevalence of HCV RNA-positive persons in 2008 and 2015 (left), then shifted (by 7 years) 2008 and 2015 (right).

- **National Treatment Program for HCV in Egypt**

In response to the high national HCV prevalence and associated disease burden, the Egyptian MoHP launched the National Committee for the Control of Viral Hepatitis (NCCVH) in 2006. This committee is composed of expert Egyptian hepatologists, an MoHP representative, and a few ad-hoc international experts. The key initial priority of the NCCVH was to promote access to care and treatment for persons infected with HCV, and they therefore quickly established an Egyptian National Treatment Program (NTP). In 2007, the first national HCV treatment centers were founded, distributed equally throughout the country based on population density as well as controlling for a feasible maximum distance of travel (i.e. < 50 kilometers) for patients seeking treatment. By the time that DAAs were introduced in late 2014, the National Treatment Program comprised 26 centers, and now, in 2018, it has expanded to include 74 centers nationwide (see Figure 7) (El Akel et al, 2017). From 2007- 2014, the regimen provided by the National Treatment Program was combined peg-IFN- α 2a or 2b with ribavirin over a period of 48 weeks. More than 350000 persons were treated with these regimens in Egypt between 2007 and 2014, with cure rates of approximately 50% (El Raziky et al, 2013; Waked et al, 2014). In 2014, an agreement was signed between the NCCVH and Gilead Sciences for the purchase of SOF at the cost of 900 USD for the full 12 weeks treatment course – a 99% reduction from the 84000 USD price for the same treatment in the United States. This deal fit within Gilead’s tiered ‘global pricing programme’ that considers the national income of each country as well as negotiations based on other country-specific conditions, such as the very high prevalence of HCV in Egypt (El Akel et al., 2017; Reuters, 2014b).

In October 2014, the first DAA-containing regimen used in Egypt was SOF in combination with peg-IFN and RBV for 12 weeks; this regimen could be given to so-called ‘easy to treat’ patients, without a prior history of treatment or current comorbidities. This was quickly followed by introduction of 24 weeks of SOF and RBV for those ineligible for IFN-containing regimens. Due to high demand for treatment, DAA regimens were initially given in priority to patients with bridging fibrosis (F3) and compensated cirrhosis (F4). Since 2014, many other branded oral IFN-free regimens have been introduced into the country, with equally low prices, primed by the climate of the original deal with Gilead for SOF. In addition, generic versions of many of these molecules are now being produced locally in Egypt, further lowering the prices for

each patient’s regimen down to 74 USD for a 12-week SOF-daclatasvir combination (El Akel et al, 2017). As of September 2018, regimens that are being used for ‘easy-to-treat’ patients (i.e. treatment naïve, low level of liver disease) include SOF with daclatasvir or paritaprevir-r/ombitasvir with RBV, each for 12 weeks. The regimen given to ‘difficult-to-treat’ patients (i.e. treatment experienced and/or moderate levels of liver disease) is SOF with daclatasvir and RBV. Special populations, such as those with failure to DAA regimens, advanced liver disease, co-infection with hepatitis B virus (HBV), chronic kidney disease, and post-transplant, can also be treated through the program through a variety of other available DAA regimens – these patients are often referred to specialized centers for follow-up (NCCVH, 2016).

It is estimated that now more than 1.5 million persons have received DAAs in Egypt through the National Treatment Program (El Kassas, Pers Comm), with real-life efficacy rates above 90% for easy to treat and above 80% in difficult to treat patients (Eletreby et al, 2017; Elsharkaway et al, 2017; Nagaty and Abd El-Wahab, 2017; El Kassas, Alborai et al., 2018).



Figure 7: Location of the 74 National Treatment Centers for HCV in Egypt, 2018

- **The Egyptian “model of care” for treating HCV with DAAs**

The flow of each HCV patient through the National Treatment Program has been thoroughly described by our colleagues from the NCCVH in their 2017 publication on the same topic, from which I borrow the name for this small section (El-Akel et al, 2017). I summarize it here briefly, based on my discussions with them as well as their publication.

In anticipation of the many persons with HCV wishing to be treated with DAAs, in 2014, in addition to opening up further treatment centers, the NCCVH also commissioned an online portal with which persons from anywhere in the country could register for a treatment assessment. After entering in some basic information online, including information on address, each person would be referred to a treatment center in close proximity to him or her with a specific appointment date. Using this online system, more than 300000 persons registered within the first week. The patient then attends their first appointment, usually bringing along some pre-specified laboratory results. If sufficient clinical and laboratory data are available during this first visit, hepatologists at the center will make a treatment decision for the patient, which should be confirmed by the NCCVH, or alternatively, refer the patient for further laboratory testing. In either situation, a follow-up appointment for the patient is determined. When ready, the patient starts their assigned treatment, returning every 2-4 weeks for monitoring visits until the end of their 12 or 24 week regimen. Clinical indicators, laboratory results, and adverse events are evaluated and recorded at each patient monitoring visit, and specific efforts are made to ensure that the patient sees the same hepatologist throughout their treatment. Following completion of treatment, the patient is requested to return to the center 12 weeks later for evaluation of SVR. Initially, almost 40% of patients did not return for this final visit, with negative implications for patient referral and program monitoring; the National Treatment Program then started issuing ‘cure certificates’. Due to the stigma surrounding HCV infection, and the fact that confirmation of HCV negativity is sometimes required for employment and marriage contracts, this intervention greatly improved patient retention until SVR. Patient data from the baseline, monitoring, and SVR visits are entered into a standardized online data management system that is centralized in Cairo (El Akel et al, 2017).

3.2 Collaborative epidemiological investigations within the National Treatment Program for HCV in Egypt

- **The ANRS 12332 ‘HepNile’ Cohort Study**

This cohort study, entitled “Evaluation of the ‘real-life’ efficacy and safety of antiviral treatments including new Direct Acting Antiviral agents among patients treated for chronic hepatitis C in three National Treatment Centres in Cairo”, was first funded by the French Agence nationale de recherche sur le Sida et les hépatites virales (ANRS) in early 2014, and was set to begin in early 2015. This is a prospective, non-interventional cohort study to monitor and further describe the “real life” efficacy and tolerance of DAA regimens among 7500 HCV patients at three National Treatment Centres in Cairo, over the period of 3 years. The only deviation of this cohort study from the standard care received through the National Treatment Program is establishment of a biobank for resistance studies - blood will be taken at baseline and end of treatment for all patients, and SVR12 visits for patients failing treatment.

The experience of setting up and running this cohort study, then analysing and interpreting the data collected through it, was originally meant to comprise my thesis project. However, between 2014 and 2015, the project experienced many regulatory delays. Despite this, we made many field visits to Cairo and made small steps forward with the protocol and study set-up procedures. Finally, on the brink of enrolling our first patients in January 2016, the project was halted indefinitely due to internal-political reasons outside of our control. About two years later, in November 2017, the NCCVH and the ANRS re-evaluated the interest of starting the project, and in January 2018, the cohort began enrolling its first participants. As of September 2018, more than 1500 patients have been enrolled into the cohort.

- **Research Collaboration with New Cairo Treatment Centre**

In one of the three collaborating centres within the HepNile cohort, New Cairo Hospital, the clinicians have formed a research group for conducting observational studies using data on

special populations and/or topics within the National Treatment Program. In 2015, we began collaborating with them on various subjects, including:

1. Evaluation of the validity of an earlier time point four weeks following the end of treatment rather than 12, for assessment of HCV cure (i.e. SVR)
2. Patient outcomes, and specifically, risk of recurrence, for persons with a history of hepatocellular carcinoma
3. Risk of reactivation for persons receiving direct acting antivirals for HCV who are co-infected with Hepatitis B virus
4. Safety, efficacy, and quality of life outcomes in elderly patients (>65 years old) being treated with direct acting antivirals
5. Validity of the PROQOL-HCV questionnaire for use in Egypt in Arabic.
6. Efficacy and safety of direct acting antivirals in liver transplant patients

The first three subjects listed above are completed and published, and are those that I will present in this section. The fourth and fifth topics, related to quality of life of persons taking DAAs, are on-going, and some preliminary results and implications will be mentioned in the discussion of this chapter. The final topic, regarding liver transplantation, is currently on hold.

3.2.1 Original research (full article): HCC recurrence after DAAs

In early 2016, a research group in Spain published a report signaling a ‘higher than expected’ incidence of hepatocellular carcinoma (HCC) recurrence for persons being treated with DAAs. Persons infected with HCV and having had successful ablation of HCC tumors (or lesions) in the past, comprise a special population that was, in the IFN-era of treatment, largely excluded from therapy. Linked to this, it is also a population who were little or not-at-all included in clinical trials testing the DAAs. Therefore, this report from Spain generated considerable concern and rapid attempts by clinical research groups to evaluate the same topic in their settings.

With our colleagues from the New Cairo Hospital HCV Treatment Centre in Egypt, we decided to weigh in on this question. The coordinator of this centre, our collaborator Pr. Mohamed El Kassas, also ran a HCC treatment centre in Cairo, dealing exclusively with patients having early stage tumors where simple ablation and surgical techniques are practiced. Following successful tumor removal, patients are continually followed up at this centre every three to six months and some will eventually commence treatment for HCV with DAAs. Due to the unique context of a centralized National Treatment Program for HCV and the involvement of our collaborator in it, we were also able to obtain longitudinal information about each person’s DAA treatment. Therefore, we could perform a survival analysis, using as our base population all the patients treated at one HCC centre, then followed from the time of successful HCC ablation, factoring in the time of DAA administration in some of those patients while not in others, and finally observing the difference in their outcome – either recurrence, or censoring, within a two year period. In addition, considering that the reason for international concern on this topic was the possibility of causality (i.e. that it was the indeed the DAAs causing increased recurrence), we took care to implement specific inclusion and exclusion criteria for who could be included in the analysis; we tried our best to imagine the ideal patients included in and analysis conducted on a randomized controlled trial, according to the principals of causal inference research using observational data. At the time we performed this analysis, only one survival analysis that also treated DAAs as a time-varying exposure had been published, but this study was not able to follow patients from the time of original HCC ablation. In addition, most results that had been reported were percentages of recurrence in persons with former HCC treated with DAAs without

ability to compare with those not-treated with DAAs; in this case, it is indeed complicated to find an appropriate control group and often, historical figures are used for comparison instead.

In the paper that follows, you will see a more detailed description of the population included, analysis performed, results obtained, and finally a discussion that shows the difficulties we faced in interpreting our findings in relation with the others that had been already published.

The PDF of this article can be found in Appendix 5.

Increased recurrence rates of hepatocellular carcinoma after DAA therapy in a hepatitis C infected Egyptian cohort: a comparative analysis

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- **Abstract**

Background: In Egypt, hepatocellular carcinoma (HCC) is the most common form of cancer and direct acting antivirals (DAAs) are administered on a large scale. In this unique setting, we aimed to determine the association of DAA exposure with early phase HCC recurrence in patients with a history of hepatitis C (HCV)-related liver cancer. *Methods:* A prospective cohort study of an HCV infected population from one Egyptian specialized HCC management center starting from the time of successful HCC intervention. The incidence rates of HCC recurrence between DAA exposed and non-exposed patients were compared, starting from date of HCC complete radiological response and censoring after two years. DAA exposure was treated as time-varying. Two Poisson regressions models were used to control for potential differences in the exposed and non-exposed group; multivariable adjustment and balancing using inverse probability of treatment weighting (IPTW). *Results:* We included 116 patients: 53 treated with DAAs and 63 not treated with DAAs. There was 37.7% and 25.4% recurrence in each group after a median of 16.0 and 23.0 months follow up, respectively. Poisson regression using IPTW demonstrated an association between DAAs and HCC recurrence with an incidence rate ratio of 3.83 (95% CI: 2.02-7.25), which was similar in the multivariable adjusted model and various sensitivity analyses. *Conclusion:* These results add important evidence towards the probable role of DAAs in HCC recurrence and stress the need for further mechanistic studies and clinical trials to accurately confirm this role and to identify patient characteristics that may be associated with this disconsolate event.

- **Introduction**

Several studies and meta-analyses have concluded that eradication of HCV with antiviral therapy would reduce the risk of HCC in patients with chronic HCV, independent of their fibrosis stage (Morgan et al., 2013). During the era of interferon-based therapy, patients with a sustained virological response (SVR) including those on combination therapy with pegylated-interferon (peg-IFN) and ribavirin (RBV), showed both histological improvement through HCV eradication as well as a decrease in the risk of HCC development (Ikeda et al, 1999). Other research concluded that patients achieving SVR through interferon-based therapies who had previously received curative HCC treatment, including local ablation therapy and hepatic resection, had favorable outcomes compared with non-SVR patients. The introduction of the new wave of DAAs in 2014, with increased tolerance and effectiveness, was seen as a continuing step forward in the treatment of persons with a history of HCC and improvement in their overall prognosis.

The new, highly effective DAAs were expected to dramatically decrease HCV related liver disease progression to end-stage liver disease and HCC; however, these optimistic expectations were questioned by an initial report from Spain in 2016. Reig and colleagues (2016) reported a ‘more than expected’ early recurrence rate (27.6%) in patients with HCC who received DAA treatment after an initial good response to HCC treatment. This report represented a red flag and opened the door for a debate about the relationship between DAA treatment and HCC recurrence. Reports from Italy and the United States, both demonstrating and refuting any increase in recurrence following DAAs in varying groups were released soon after (Conti et al., 2017; Torres et al., 2016; Yang et al., 2016; Zavaglia et al., 2017). These initial reports lacked any form of comparative survival analysis between DAA exposed and non-exposed groups. Subsequently, a comparison of DAA exposed and non-exposed groups from French cohort, reported by Pol and colleagues (2016), treated DAA exposure as time varying and found no increased risk of recurrence in those exposed to DAAs (HR: 1.21, 95%CI 0.62–2.34). However, this cohort started following eventually DAA-exposed patients from a median 23 months after the original HCC diagnosis and was criticized for possible underreporting of HCC recurrence (Reig, Boix, and Bruix, 2017). A recent review by Reig, Boix, Marino and colleagues (2017) presents an overview of the conflicting evidence that has been presented so far in this debate; the types of

studies conducted, heterogeneity in the populations included, and variability in the analytical methods used, means that no firm conclusions have yet been drawn on this topic.

HCC is the most common cancer in Egypt (Ibrahim et al., 2014), largely due to the country having the highest global prevalence of HCV (Kandeel et al., 2016). Due to the availability of low-cost branded and generic DAAs, the Egyptian Ministry of Health and the National Committee for the Control of Viral Hepatitis have treated approximately one million Egyptian patients since 2014, with cure rates over 90% using various DAA combinations (El-Akel et al., 2017). Therefore, we examined HCC recurrence within two years of initial HCC complete radiological response in an HCV infected Egyptian cohort, for those who either were or were not given DAAs through use of comparative time-dependent analysis and propensity scoring.

- **Materials And Methods**

Study design and participants

This study was carried out at one HCC treatment center in Cairo, Egypt. Patients of all Barcelona Clinic Liver Cancer (BCLC) stages and prognoses were consulted at this center through clinical and imaging examinations and those with BCLC stages 0 and A were treated through local ablation procedures; these patients had a maximum of three cancerous lesions, with the largest lesion being <5cm in diameter. Local ablative procedures available at this center included: radiofrequency ablation (RFA), microwave ablation (MWA), percutaneous ethanol injection (PEI), surgical resection, and transarterial chemoembolization (TACE). Following the ablative procedure, patients were followed up after one and three months with dynamic computerized tomography (CT) and ultrasound imaging to assess tumor response. After complete radiological response according to modified response evaluation criteria for solid tumors (RECIST) (hereafter referred to as ‘complete radiological response’) had been confirmed by a senior radiologist using the mentioned imaging techniques at both month one and months three visits, the patients returned for follow-up imaging every three to six months, for continued confirmation of complete radiological response until death or loss-to-follow-up. HCC follow-up

imaging at these time intervals was the same for patients both eventually treated and not treated with DAAs.

Following HCC complete radiological response, patients were referred back to local hepatologists at varying National HCV Treatment Centers where they were examined for DAA treatment eligibility. If the patient's clinical indicators fit within the national HCV treatment guidelines, they were given, free of charge, either a three or six-month regimen of DAA. These patients' viral loads were assessed at the end of their DAA treatment period (EOT) as well as 12 weeks post-treatment in order to establish whether or not there has been a sustained virological response (SVR12). The choice of whether or not a patient with a history of HCC will receive DAAs was dependent on the patient decision to seek treatment and the decision of the consulting hepatologist at the HCV treatment center. Patient eligibility, in the Egyptian context, refers to the patient having no contraindication to DAAs, a good prognosis, and limited liver damage; during the first wave of DAA introduction this meant that patients with Child-Pugh scores greater than 6 were not eligible for DAA treatment, nor those greater than 65 years of age. The decisions of a consulting hepatologist to either treat immediately, wait to treat, or never treat, a patient with a history of HCC was either based on treatment ineligibility or their own personal practice methods; some hepatologists recommended waiting during the first two years post HCC complete radiological response as this is a period when recurrence is common.

According to the current Egyptian national treatment guidelines, all-oral DAAs can be given as soon as one month following the HCC ablative maneuver, as long as the patient was seen to have a complete radiological response through dynamic CT and ultrasound during this visit. Patients with a history of HCC being examined for DAA treatment eligibility need to have had a dynamic CT scan and ultrasound confirming lack of HCC recurrence in the 3 months prior to the DAA start date; any patient who has not had this imaging performed within this time frame through their regular visits at the HCC Treatment Center, was required to return to the center to do so.

Procedures

For the presented analysis, we applied various inclusion and exclusion criteria to the total population of BCLC A and 0 patients who visited the HCC treatment center for local ablation between January 2013 and March 2016 (Figure 8).

We consecutively included patients who achieved HCC complete radiological response according to modified RECIST criteria between 2013 and 2016. All patients needed to be HCV positive and not co-infected with HBV or HIV. HCV genotype was not specified for inclusion and indeed genotyping was not done on all patients, however, most HCV infection in Egypt is with genotype 4 (Abdel-Hamid et al., 2007). All included patients were treated for their HCC with local ablative procedures including: RFA, MWA, PEI, and surgical resection.

We excluded patients who were treated using TACE, as this maneuver can be considered palliative. For those who were treated with DAAs during the two-year analysis window, we excluded any patients who had received interferon-containing DAA regimens (i.e. Sofosbuvir/Interferon/RBV). We also excluded any patients with early recurrence within three months of the date of complete radiological response, in order to further ensure that newly detected tumors represented true recurrence, and were not residual but previously undetected (Torres et al., 2016).

This study obtained ethical approval by the Institutional Review Board for Human Subject Research at the National Hepatology and Tropical Medicine Research Institute in Cairo, Egypt, which is organized and operated according to the Declaration of Helsinki for Human Subject Research (2013). All patients provided written informed consent to have their data included in this analysis.

Statistical analysis

After applying the stated inclusion and exclusion criteria to our original population, persons eventually treated or not-treated with DAAs within the two-year follow-up period were compared for their baseline characteristics (i.e. at the time of HCC ablative maneuver) using the

Student's t-test for continuous variables and chi-square or Fisher's exact test for categorical variables. For time-dependent analysis, Poisson regression models were preferred to Cox models because they allow the estimation of incidence rates according to DAA exposure (or non-exposure). Censoring was done when a patient died, was lost to follow-up, or was at the end of their two-year follow-up period. For the main analysis, DAA treatment was treated as time varying, with exposure starting from the date of DAA start and ending at the endpoint/censoring (thus reflecting a current or past exposure to DAA).

Incidence rate ratio for DAA treatment exposure was estimated using univariable and multivariable Poisson regression models. Covariates included in the multivariable model were: time since entry into the cohort (<8 months, 8-16 months, 16-24 months), sex, age (categorized as < or \geq 65 years old), baseline Child-Pugh score (categorized as \leq or >6) and whether or not the patient had ever had gastroesophageal varices (a possible indicator of portal hypertension). Furthermore, in order to minimize the effect of confounding by treatment indication, we used inverse probability of treatment weighting (IPTW) using propensity scores (Austin, 2014). The probability of receiving DAA treatment was modelled based on time since entry into the cohort, sex, age, baseline Child-Pugh score, and history of gastroesophageal varices, and stabilized weights were calculated using the R package "ipw" (van der Wal & Geskus, 2011) The average treatment effect was estimated using a robust variance estimator in order to account for the weighted nature of the sample. IPTW diagnostics were conducted as recommended by Austin and Stuart (2015).

A sensitivity analysis removed all patients with Child-Pugh score 7 and over the age of 65, as these were DAA treatment ineligibility criteria during the first phase of DAA introduction in Egypt. For a sub-analysis, DAA-treatment exposure, still treated as time varying, was broken down into three periods: no treatment, the first six months after start of DAA treatment, and six months after the start of DAA treatment. A further sub-analysis examined the difference between exposure to three and six-month DAA treatment regimens. All statistical analyses were conducted using R.

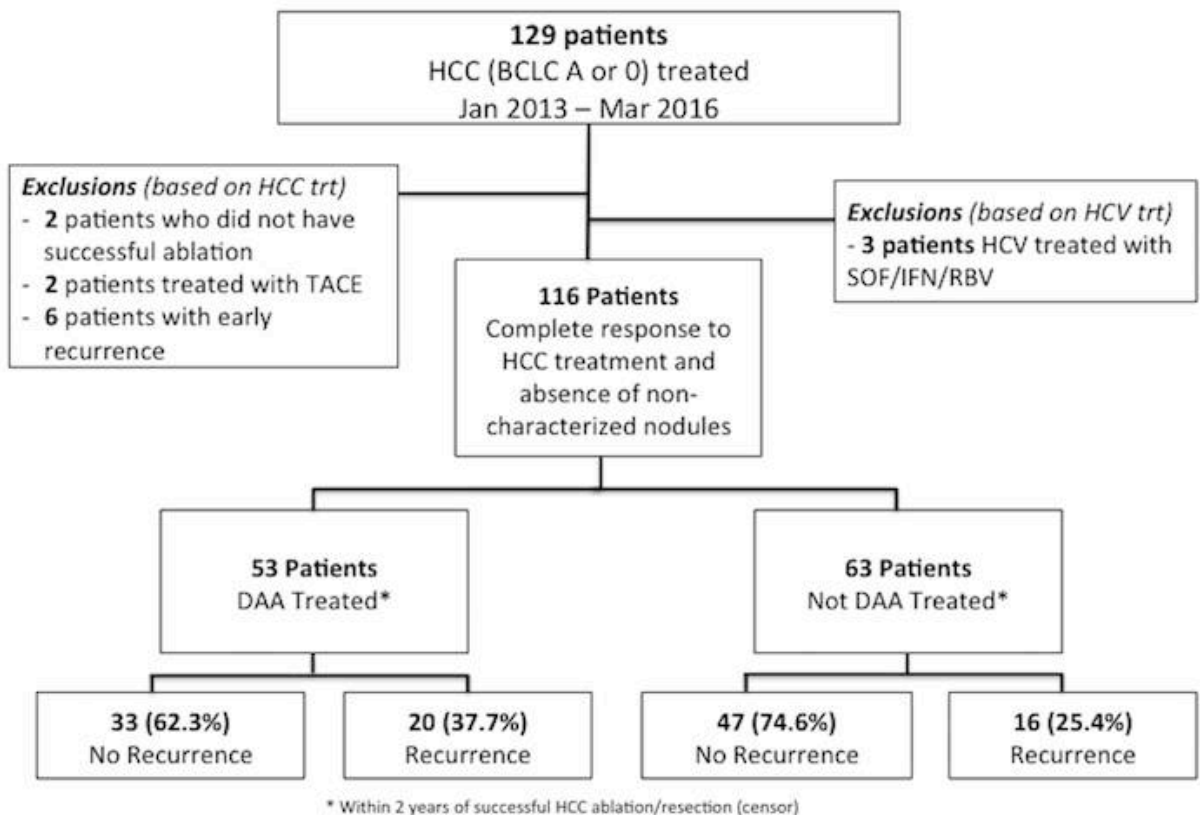


Figure 8: Application of inclusion and exclusion criteria to study population with basic recurrence proportions

- **Results**

Between January 2013 and May 2016, 129 HCV-infected HCC patients, all BCLC stage A or 0, were consulted and treated with local ablation procedures at the HCC Treatment Center in Cairo, Egypt. Of these, we excluded: two patients who were treated with TACE; two patients who never achieved a complete radiological response; six patients who had early recurrence of HCC (two of these were already taking DAAs); three patients who were eventually treated with DAA regimens containing Interferon. This left us with a total of 116 patients, 53 of whom eventually received DAAs during the two year analysis follow-up period and 63 who did not (Figure 8, links 2 & 3 in Appendix 4).

Table 16: Baseline characteristics, at the time of HCC complete radiological response, for patients eventually treated and not treated with DAAs

Baseline Characteristic	DAA Treated (N=53)	Not DAA Treated (N=63)	p-value
Male, # (%)	35 (66.0)	41 (65.1)	0.91
Age, mean (P25-P75)	56.7 (52-62)	57.3 (51-62)	0.70
Diabetes, # (%)	17 (32.1)	16 (25.4)	0.43
Child-Pugh score			0.006*
5	26 (49.1)	30 (47.6)	
6	26 (49.1)	22 (34.9)	
7	1 (1.9)	11 (17.5)	
# Hepatic focal lesions [HFL]			0.06
1	50 (94.3)	57 (90.5)	
2	1 (1.9)	6 (9.5)	
3	2 (3.8)	0	
History of gastroesophageal varices	30 (56.6%)	42 (66.7%)	0.34
Largest HFL in cm, median (P25-P75)	2.3 (2.0-3.0)	2.5 (2.0-3.0)	0.50
HCC Treatment Maneuver, # (%)			0.30
RFA	31 (58.5)	48 (76.2)	
PEI or PEI/RFA	13 (24.5)	7 (11.1)	
MWA	7 (13.2)	6 (9.5)	
Surgical Resection	2 (3.8)	2 (3.2)	

* Child-Pugh score 5 and 6 against Child-Pugh score

The DAA exposed and non-exposed groups were similar in terms of sex, age, and diabetes as well as their number of hepatic focal lesions and size of their largest lesion at baseline (Table 16). No patients in our cohort were alcoholic or had a history of alcoholism.

The Child-Pugh score was lower in those treated with DAAs; 1.9% of the DAA-exposed group had a score of 7 whilst the DAA non-exposed had 17.5% (p=0.006). The ablative maneuvers performed on patients in both groups were similar; the majority of patients in both

groups underwent radiofrequency ablation. The DAA exposed group received varying all-oral regimens for either three or six-month periods. SVR12 was confirmed in 77.4% of the DAA treated patients overall, and in 89.2% of patients when excluding those treated with Sofosbuvir/RBV (Table 17).

Table 17: DAA treatment regimens, end of treatment and SVR12 proportions in the DAA exposed group (N=53)

DAA Treatment Regimen	Duration of Treatment (months)	# Treated	% EOT	% SVR12
SOF/RBV	6	16 (30.2)	75.0	50.0
SIM/SOF	3	9 (17.0)	100	88.9
SOF/DCV/RBV	3	8 (15.1)	100	100
	6	1 (1.9)	100	100
SOF/DCV	3	11 (20.8)	90.9	81.8
	6	4 (7.5)	100	75.0
SOF/LDV/RBV	6	2 (3.8)	100	100
SOF/LDV	6	2 (3.8)	100	100
Total	-	53 (100)	90.6	77.4

Note: EOT= undetectable viremia at end of DAA treatment, SVR12 = sustained virological response 12 weeks after the end of DAA treatment

Among the 53 patients treated with DAAs, we observed 37.7% recurrence after a median of 16.0 months follow up. Among the 63 patients not treated with DAAs we observed a 25.4% recurrence after a median 23.0 months follow up. The association between DAA exposure and recurrence can be seen in Table 18. The unadjusted rate, per 100 person-months, of recurrence was 1.00 (95% Confidence Interval, CI: 0.51-1.49) in the DAA non-exposed group versus 4.06 (95% CI: 2.30-5.85) in the DAA exposed group, representing a crude Incidence Rate Ratio (IRR) of 4.08 (95% CI: 2.14-7.76) associated with DAA treatment. After controlling for time since HCC complete radiological response (i.e. time in cohort) as well as sex, age, Child-Pugh score,

and history of gastroesophageal varices using IPTW, exposure to DAA treatment was associated with a significantly increased risk of recurrence of 3.82 (95% CI: 2.00-7.30), consistently with results found in the multivariate analysis (Table 18). The results of IPTW diagnostics can be found by following link 4 in Appendix 4.

For patients with recurrence, the location of cancerous lesions was found in a new site for 15 (93.8%) of those not treated with DAAs and 19 (95.0%) of those treated with DAAs. In examination of whether or not patients treated with DAAs had a more aggressive recurrence compared to their non-DAA exposed counterparts, we observed that six (30.0%) of the recurring patients with DAA exposure had greater than three hepatic focal lesions, but we did not have enough statistical power to show whether or not this was higher than the two (18.0%) patients with this characteristic in the DAA non-exposed recurring population. Among the DAA treated group, the incidence of recurrence was not different among those who did achieve SRV12 as compared to those who did not (unadjusted IRR = 1.64, 95% IC: 0.64 – 4.20). Although three (2.6%) patients were censored due to being lost to follow-up, there was no censorship due to death within the 2 years following inclusion in the study for patients without recurrence. No patients underwent liver transplantation.

When excluding patients who did not achieve SVR12 (n=12), the recurrence IRR was 4.18 (95% CI=1.64-10.69). The results were consistent when considering only patients treated with six-month DAA regimens (IRR=3.39; 95% CI=1.56-7.37) or when considering only patients treated with three-month regimens (IRR=3.66; 95% CI=1.73-7.76). A sensitivity analysis using IPTW and excluding all patients with Child-Pugh score of 7 and aged >65 demonstrated a recurrence IRR of 5.62 (95% CI= 2.52-12.18) for DAA exposed vs non-exposed patients. A second sensitivity analysis split DAA exposure into two periods: the first six months after the start of DAAs and the time following six months after start of DAAs. Using multivariable Poisson regression (non-dichotomous nature of this exposure variable prevented the use of IPTW), we found an adjusted recurrence IRR of 3.24 (95% CI=1.50-7.01) in the first six months of DAA exposure and 4.17 (95% CI=1.73-10.05) in the post six-month DAA exposed groups respectively.

Table 18: Recurrence rates and rate-ratios comparing DAA exposed and non-exposed patients

DAA exposure	Recurrence Rates			Univariable Poisson Regression			Multivariable Poisson Regression			Poisson Regression using IPTW			
	Events	PM Rate*	95% CI	IRR	95% CI	P	IRR	95% CI	p	IRR	95% CI	p	
DAA exposure	No DAAs	16	1.00	0.51-1.49	1	ref	<0.001	1	ref	<0.001	1	ref	<0.001
	DAAs	20	4.06	2.30-5.85	4.08	2.14-7.76	0.03	3.61	1.84-7.11	0.07	3.82	2.00-7.30	<0.001
Time since cohort entry	0-8 months	8	906	0.67	0.08-1.26	1	ref	1	ref	0.16	-	-	-
	9-16 months	19	728	2.39	1.20-3.59	2.96	1.31-6.69	2.55	1.11-5.86	0.26	-	-	-
	17-24 months	9	463	1.69	0.44-2.93	2.22	0.86-5.65	1.96	0.79-4.86	0.01	-	-	-
Sex	Female	6	802	0.749	0.01-1.14	1	ref	1	ref	0.002	-	-	-
	Male	30	1296	2.32	1.20-2.95	3.09	1.29-7.41	3.45	1.44-8.25	0.16	-	-	-
Age	<65	33	1780	1.85	0.69-4.61	1	ref	1	ref	0.16	-	-	-
	≥65	3	318	0.94	0.00-3.49	0.51	0.16-1.66	1.47	0.53-4.10	0.16	-	-	-
	Child-Pugh Score	5/6	32	1875	1.71	1.10-2.30	1	ref	1	ref	0.50	-	-
Gastroesophageal varices	Never	13	822	1.58	0.72-2.44	1	ref	1	ref	0.44	-	-	-
	Ever	23	1275	1.80	1.10-2.54	1.14	0.58-2.24	1.31	0.67-2.56	0.70	-	-	-

Note: PM=person-months; 95% CI=95% Confidence Interval; IRR=Incidence Rate Ratio; IPTW= Inverse Probability of Treatment Weighting; *Rate is per 100 person-months; the probability of receiving treatment was modelled using the covariates presented in the table

- **Discussion**

Our data point to a highly (i.e. almost 4 times) increased rate of recurrence after DAA treatment for patients with a history of successfully treated HCC, when compared to similar patients who were not given DAAs. This significantly higher rate of recurrence in the DAA-exposed group remained after adjustment for baseline factors and time since HCC complete radiological response through inverse probability weighting, as well as across the sensitivity analyses performed. As far as we know, this is the first propensity scored comparative time-dependent analysis for DAA exposed and non-exposed patients followed from the moment of HCC complete radiological response.

It has been suggested that rapid changes to the immune surveillance system and/or anti-tumor response following DAA treatment could be the reason for the apparent increase in HCC recurrence (Nault & Colombo, 2016). A recent observational study by Villani et al (2016), supported this idea through demonstration that during treatment with DAAs, an angiogenesis inducer called vascular endothelial growth factor, which supports tumor development, increases significantly and can remain high until 3 months after DAA treatment. However, our own results showed a similar association between DAAs and HCC recurrence for those with longer (6 month) vs. shorter (3 month) DAA exposures (IRR 3.39 vs. 3.66, respectively).

The necessary assumption of this comparative analysis is that DAA-exposed and non-exposed patients are similar. However, it can be argued that in an era of highly expensive DAA treatments for HCV, it is possible that clinicians systematically choose to treat patients with certain clinical indicators over others; for example, in Egypt, treating patients with better prognoses has been estimated as more cost-effective based on quality-adjusted life expectancy outcomes (Obach et al., 2014). Our inclusion criteria, especially treatment initiation from 2013 onwards and rapid complete radiological response in response to initial HCC treatment (i.e. seen already at one month post-ablative maneuver), were specifically chosen to improve comparability between treated and untreated patients. As a result, baseline characteristics of the DAA exposed and DAA non-exposed groups in the study presented here appeared to be balanced, except for baseline Child-Pugh score.

Moreover, in the analysis, we used IPTW in order to further balance measured covariates between treated and untreated patients, including possible time-varying confounders such as time since inclusion in the cohort. Our sensitivity analysis, which further controlled the groups through exclusion of all patients with a Child-Pugh score of 7, showed the same magnitudes of effect and significance level as our primary analysis.

Table 19: Previous studies evaluating the association between DAA and frequency of recurrence in patients who achieved complete radiological response following HCC treatment

Author	Country	# DAA exposed	Median time (months) HCC treatment to DAA exposure	Percent recurrence in DAA exposed; (median # months*)	Rate Ratio; (p-value)
A cohort of DAA-exposed patients without a control group (descriptive study)					
Reig et al., 2016	Spain	58	11.2	27.6 (5.7 months)	-
Conti et al., 2016	Italy	59	12.4	28.8 (not available)	-
Torres et al., 2016	USA	8	7.5	0 (12 months)	-
Zavaglia et al., 2017	Italy	31	19.3	3.2 (8 months)	-
Time-dependent analysis DAA-exposed and non-exposed patients (analytical study)					
Pol et al., 2016	France	189	>22.8**	12.7 (20.2 months)	1.2 (p=0.58)
El Kassas et al	Egypt	53	8.0	37.7 (16.0 months)	3.8 (p<0.001)

*DAA initiation until HCC recurrence in studies of type 1, and median months total follow-up in studies of type 2 **Median 22.8 months from HCC diagnosis to cohort inclusion, and an additional median 1.4 months until DAA administration in those eventually exposed to DAAs

It is possible that there are other confounding factors that were not collected as part of this study. Indeed, although baseline characteristics are similar for all patients in our study, we do not have updated records of liver enzyme changes at each imaging follow-up visit. We argue, though, that we can assume that any selection bias in DAA administration would trend towards the treatment of those with better prognosis (e.g. younger age, limited liver damage), at least in the case of patients with a history of HCC in Egypt. Within the Egyptian National Treatment Program, a patient with a history of HCC would not be given DAAs immediately following HCC complete radiological response because of either ineligibility for treatment according to the Egyptian national treatment guidelines, or due to the personal opinion of the attending hepatologist regarding the need to wait to make sure the cancer is truly gone first. Ineligibility for treatment in this case relates mainly to when a patient had a worse prognosis (e.g. older age, more deteriorated liver function); this was the case for 11 of our patients who had Child-Pugh score 7, and two patients who were too old (>65 years), who did not receive DAAs according to the applied treatment protocol at that time. For the remaining 50 patients who did not receive DAAs within our two-year analysis period, 14 eventually received DAAs afterwards, 15 had recurrence within this time and were therefore not eligible and for 21 the attending hepatologist decided not to recommend treatment at all within the viewed follow-up period. Although our results cannot approximate a clinical trial in terms of random treatment assignment, we do assess that any underlying differing factors between the groups would likely be negative confounders; we would expect patients with a better prognosis to be treated with DAAs.

One of the biggest strengths of this study was our ability to follow, and analyze recurrence depending on eventual DAA exposure in an entire cohort of HCV positive patients from the moment of HCC complete radiological response. This has not yet been done in any other robust study; the survival analysis performed by Pol et al followed patients from time of ‘cohort inclusion’; this was a median 1.9 and 1.6 years after HCC diagnosis for DAA exposed and unexposed groups respectively (Pol et al., 2016). All other currently published papers on this subject report recurrence in DAA exposed groups after varying median number of months following their HCC complete radiological response. A demonstration of these differences, using some of the first studies published

within this debate, can be seen in Table 19. The issue with a delayed observation window in these populations is two-fold: 1. The biological mechanisms behind early (within two years) and late (> two years) phase recurrence have been reported to differ (Imamura et al., 2003), and 2. All patients with recurrence or death prior to the observation window are inherently excluded from the analysis and final recurrence rates. The former indicates that studies observing recurrence in the first two years since HCC complete radiological response, such as the study we present here or that of Reig (2016) or Conti (2016) are not comparable to those of Pol (2016) or Zavaglia (2017). The latter means that some studies, such as that of Pol (2016) and Zavaglia (2017), who have reported ‘no apparent effect of DAAs’, have done so in survivor populations. Recurrence in the first two years since HCC complete radiological response has been linked to microscopic vascular invasion, high serum AFP levels and having had non-anatomical resection, whereas after two years since HCC complete radiological response, tumors can be considered *de novo* and are linked to the grade of hepatitis activity, tumor nodule multiplicity and gross tumor classification (Imamura et al., 2003).

In order to draw any interim guidance from the currently published data on this subject, the findings that have been put forth to date should be considered and grouped according to the population included, analysis methods used and the time-window of observation for the included patients. Perhaps DAA treatment of surviving patients without any recurrence two years after HCC complete radiological response poses no additional risk, whereas administration of DAAs to patients in the first two years after HCC complete radiological response should be avoided until clinical trials provide more concrete evidence of their benefit.

3.2.2 Original research (short description): HBV reactivation following DAAs

Following the introduction of DAAs, several published case reports signalled a risk of HBV reactivation and HBV-related hepatitis during the course of treatment in patients with chronic HCV infection co-infected with chronic HBV (presence of HBV surface antigen - HBsAg) as well as those with past or resolved HBV infection (presence of hepatitis B core antigen anti-HBc) (Collins et al., 2015; Ende et al., 2015; Takayama et al., 2016). This led to guidelines by the American (AASLD) and European (EASL) Associations for the Study of Liver Disease for the monitoring and treatment of patients showing signs of reactivation (AASLD, 2017; EASL, 2017a; EASL, 2017b); however, large discrepancies in these recommendations highlighted the lack of robust evidence on this topic. Few cohort studies had been performed evaluating the magnitude of the risk of reactivation, and these studies were limited due to heterogeneity in study participants (inclusion of patients under HBV antiviral treatment), varying criteria for HBV reactivation, and small sample sizes in estimates for concurrent HBV infection (Gane et al., 2016; Liu et al., 2017; Londono et al., 2017; Wang et al., 2017; Yeh et al., 2017).

Therefore, in Egypt, we prospectively followed HBsAg-positive persons undergoing interferon-free DAAs through the National Treatment Program, to estimate the risk of HBV reactivation and HBV-related hepatitis. In addition, we conducted a meta-analysis to estimate the reactivation risk using published data obtained from a systematic review of PubMed/Embase, in addition to our Egyptian data. We applied a standard definition of HBV reactivation and hepatitis proposed by two international associations for the study of the liver, the AASLD and the Asian Pacific Association (APASL) (AASLD, 2017; Sarin et al., 2017)

Of 4471 patients with chronic HCV being treated in the National Treatment Program in Egypt, 35 HBsAg-positive patients started interferon-free DAAs without HBV nucleos(t)ide analogues. Ten experienced HBV reactivation (28.6%), of whom 1 developed hepatitis (10.0%). The systematic review we did identified 18 eligible studies. The meta-analysis then showed that the pooled reactivation risk in HBsAg-positive patients was 18.2% (95% CI: 7.9%-30.7%) without HBV therapy (see Figure 9) and

0.0% (95% CI: 0.0%-0.0%) with HBV nucleos(t)ide analogues. The pooled risk of hepatitis in those with HBV reactivation was 12.6% (95% CI: 0.0%-34.7%). The pooled reactivation risk in HBsAg-negative, antibody to HBV core antigen-positive (anti-HBc-positive) patients was negligible (0.1%, 95% CI: 0.0%-0.3%), irrespective of the presence of antibody to HBsAg (anti-HBs).

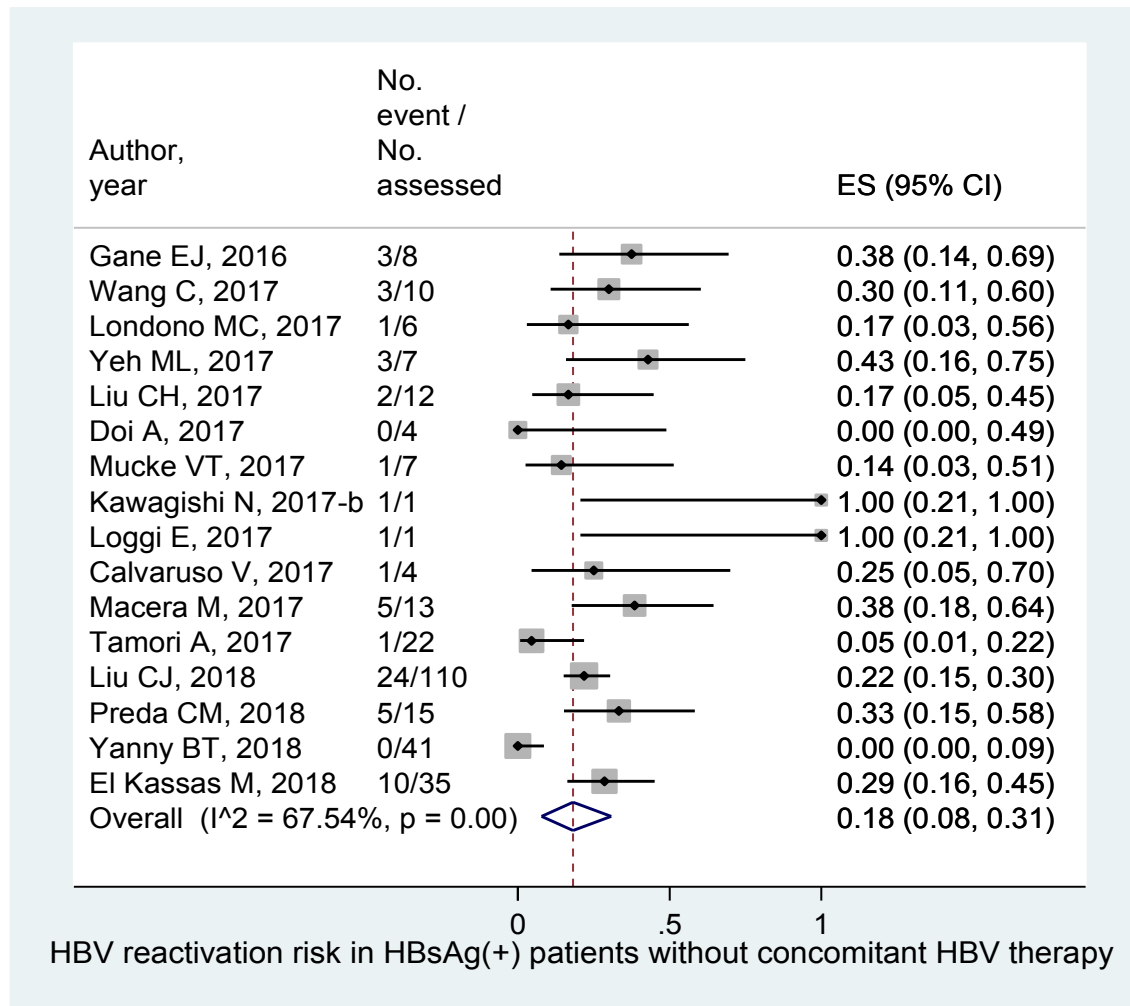


Figure 9: Pooled risk of HBV reactivation in HBsAg-positive patients without concomitant anti-HBV nucleos(t)ide analogue therapy

Our study confirmed the importance of screening for HBsAg and anti-HBc in patients undergoing DAA therapy. For those negative for HBsAg but positive for anti-HBc, the European (EASL) recommendation of monitoring and testing for HBV DNA in case of ALT elevation seems reasonable as HBV reactivation and particularly HBV-related hepatitis are rare in this group (EASL, 2017a; EASL, 2017b). For those positive for HBsAg, we concluded that further studies are needed to determine the best strategy between systematic nucleos(t)ide analogue prophylaxis or, rather, “on-demand” HBV therapy implemented only when HBV DNA levels become elevated during the course of DAA treatment.

The PDF of this article can be found in Appendix 5.

3.2.3 Original research (full article): Validity of earlier SVR testing after DAAs

With the introduction of DAAs, the duration of a person's HCV treatment went from almost one year, to only 2-3 months, in a very short period of time. However, the wait time to find out if the person had achieved SVR remained recommended at 3 months following treatment. This long gap between the last visit at which a patient receives treatment and the visit where they understand whether or not a cure was achieved, is problematic in the context of the large, centralized National Treatment Program in Egypt. Many patients are lost to follow-up between the end of treatment and the SVR12 visit and this leads to a lack of proper referral and resistance testing for patients failing treatment, as well as a high proportion of missing data inhibiting 'real-world' efficacy and program evaluations.

Using data from a cohort of patients treated at New Cairo Hospital Treatment Centre, we attempted to see whether or not an earlier time point for assessment of SVR, four weeks after the end of treatment rather than 12, would provide adequate sensitivity for observing all true treatment failures. This is biologically plausible considering the use of new highly potent treatments. Regardless of the result, this would inform the policy of the Egyptian National Treatment Program, and, if positive, would possibly allow for an alleviation of the logistical hurdle faced when attempting to retaining the very large volume of patients applying to be treated with the newly introduced DAAs.

The PDF of this article can be found in Appendix 5.

Letter: Concordance of SVR4 and SVR12 following direct acting antiviral treatment in Egypt.

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In your recently published paper by Omar and colleagues (2018), we are presented with the results of 18,378 patients treated for chronic hepatitis C (HCV) with direct acting antivirals (DAAs) through the Egyptian National Treatment Program. During the same time period within the program, retention of patients until the assessment of sustained virological response (SVR12) posed a significant logistical hurdle, and led to 29-40% of patients being lost-to-follow-up (El-Akel et al., 2017; Elsharkawy et al., 2017). Use of a more prompt endpoint, 4 weeks after the end of treatment (SVR4), could potentially lower these proportions and subsequently support evaluations of Egyptian and other national treatment programs administering DAAs; however, SVR4 has previously shown to be inadequate (Burgess et al., 2016).

To compare SVR4 to the conventional endpoint, SVR12, in a real-life setting, the program carried out a prospective, observational cohort study of patients receiving interferon-free DAAs at the New Cairo Hospital treatment centre in Cairo, Egypt, between September 2014 and December 2016. All patients eligible for treatment according to the Egyptian National Guidelines were invited to participate. Participants received two vouchers for free quantitative HCV RNA testing 4 and 12 weeks post-treatment and real-time PCR was done at the centre's laboratory or other selected national laboratories. Patients had clinical follow-up at the centre every four weeks until the end of treatment, then at 4 and 12 weeks post-treatment.

Of the 2899 patients treated at the centre who agreed to participate, 233 (8.0%) were treated with IFN containing DAA regimens, and 196 (6.8%) were lost to follow-up, leaving 2470 (85.2%) eligible for analysis. Patient characteristics are described in Table 20. The DAAs administered include: sofosbuvir and daclatasvir with or without ribavirin (n=1559, 63.1%), sofosbuvir and simeprevir (n=586, 23.7%), sofosbuvir with ribavirin (n=285, 11.5%), ombitasvir/paritaprevir with ribavirin (n=40, 1.6%). Five and 15 patients had detectable HCV RNA at four and 12 weeks post-treatment, leading to an estimation of SVR4 and SVR12 as 99.8% (95%CI: 99.5%-99.9%) and 99.4% (95%CI: 99.0%-99.7%), respectively. The sensitivity of viral load testing at 4 compared to 12 weeks post-treatment was 33.3% (5/15, 95%CI: 11.8%-61.6%) and the specificity was 100% (2455/2455; one-sided 97.5%CI: 99.9%-100%). The negative and positive predictive values were 99.6%, (2455/2465, 95%CI: 99.3%-99.8%) and 100% (5/5, one-sided 97.5%CI: 47.8%-100%), respectively.

In this study, viral load testing four weeks post-treatment misclassified a small number of individuals (i.e. 10/2470, 0.4%), but did not identify the majority (66.6%) of eventual treatment failures. Practically, within the Egyptian National Treatment Program, which aims to treat 350,000 persons annually, this would leave 1400 viremic persons returning to their communities as transmitters, with a higher risk of complications, and without a referral for further DAA treatment (Dieperink et al., 2014; van der Meer et al., 2012; Waked et al., 2014). Evaluations of other intermediate endpoints, such as SVR8, as well as studies examining patient factors contributing to relapse between the end of treatment and SVR12, are needed. In the meantime, various other measures have been employed in Egypt to ensure higher proportions of patients undertake viral load testing at SVR12, including text message and phone call reminders, as well as delivery of 'cure' certificates upon demonstration of SVR12 results (El Akel et al., 2017; Gomaa et al., 2017).

Table 20: Baseline characteristics and viral load dynamics for treatment failing discordant and concordant patients, with summary baseline characteristics for all study patients.

Baseline Evaluation						SVR4	SVR12
Sex	Age	Viral load log ₁₀ IU/ml	Past HCV treatment	DAA Regimen/ Duration in wks	Fib4 score	Viral load* log ₁₀ IU/ml	Viral load* log ₁₀ IU/ml
Discordant treatment failures (n=10), summary: male=7 (70.0); age=55.4 (11.4); viral load in log₁₀ IU/ml=13.1 (1.5); treatment experienced=0; fib4 score=6.6 (4.9)							
F	64	14.3	No	SOF/DCV/RBV; 12	5.7	Not detected	9.8
M	67	10.8	No	SOF/RBV; 24	14.4	Not detected	Detected;UA
M	61	14.7	No	SOF/DCV/RBV; 12	15.8	Not detected	10.7
M	47	12.5	No	SOF/SIM; 12	2.3	Not detected	11.2
M	29	11.5	No	SOF/DCV; 12	0.6	Not detected	11.0
F	57	13.1	No	SOF/DCV/RBV; 12	4.8	Not detected	11.4
M	58	12.9	No	SOF/RBV; 24	5.4	Not detected	Detected;UA
M	59	13.9	No	SOF/SIM; 12	8.8	Not detected	13.2
M	47	15.3	No	SOF/DCV/RBV; 12	4.8	Not detected	10.6
F	65	12.0	No	SOF/SIM; 12	3.9	Not detected	11.7
Concordant treatment failures (n=5), summary: male=2 (40.0); age=56.0 (7.8); viral load in log₁₀ IU/ml= 13.0 (0.9); treatment experienced=2 (20.0); fib4 score=7.6 (5.8)							
M	59	13.7	No	SOF/RBV; 24	4.9	Detected;UA	13.7
F	68	13.0	No	SOF/RBV; 24	17.7	14.0	Detected;UA
F	53	11.8	IFN/RBV	SOF/SIM; 12	7.3	6.9	6.9
M	52	12.2	IFN/RBV	SOF/RBV; 24	4.6	Detected;UA	Detected;UA
F	48	14.0	No	SOF/RBV; 24	3.7	Detected;UA	11.1
Concordant treatment successes (n=2455), summary: male=1045 (42.6 %); age=51.8 (11.3); viral load in log₁₀ IU/ml=13.0 (1.9); treatment experience=249 (10.1); fib4 score=2.4 (2.2)							
ALL PATIENTS (N=2470), summary: male=1054 (42.7); age=51.8 (11.3); viral load in log₁₀ IU/ml=13.0 (1.9); treatment experience=251 (10.2); fib4 score=2.5 (2.3)							

*UA indicates a detected viral load, but quantitative value unavailable. **Note:** summary statistics are presented as n (%) or mean (SD) for categorical and continuous variables, respectively.

3.3 Discussion: Evaluating the introduction of DAAs in Egypt

The introduction of DAAs is a game-changer for the control of HCV, and increased access to these treatments, through availability of generics and expansion of treatment programs, will enable drastic reductions in each nation's prevalence of chronic infections, possibly to the point of disease elimination in the near future (Anonymous, 2015; Ippolito et al., 2015; Polaris Observatory, 2017; WHO, 2016). Although the safety and efficacy of DAAs were evaluated in depth in clinical trials prior to approval, administration of the treatments to real-life populations in Egypt, and equally in other regions of the world, brought with it some unexpected questions and concerns. Using data from a very specific context – mass administration of the treatments through a centralized national treatment program in the country with the highest prevalence of HCV, we weighed in on some of the pressing questions that accompanied DAA introduction. Through this, we were also privy to the difficulties of bringing together and making conclusions from findings in the immediate time period following this emerging disease event; here, these challenges were due to lags in standardization of recommended study designs, analysis methods, and case definitions.

- **Monitoring adverse events in special populations after DAA introduction**

Two of the research topics we worked on related to the evaluation of adverse events in special populations of persons with HCV following treatment with DAAs; persons from these groups had not been included extensively in clinical trials. These adverse events were: recurrence of liver cancer, and reactivation of HBV. As neither of these side effects was explicitly expected based on experiences during the IFN-era, the first epidemiological studies describing them were conducted and reported in ways that did not necessarily facilitate comparability. Increased HCC recurrence, as an adverse event to HCV treatment, was unobservable prior to the advent of DAAs due to the fact that persons with advanced liver disease were contraindicated for treatment with IFN-based regimens. The causal effect of DAAs on an increase in recurrence is still under debate. Alternatively, reactivation of HBV infection after treatment for other diseases, such as

cancer, is well known (Hoofnagle, 2009), and it has also been previously reported in patients co-infected with HBV and HCV who were treated with IFN and RBV (Liu et al., 2012). Whether or not reactivation would occur with the new DAAs, and to what extent, was unknown prior to their introduction. Here I will discuss the implications that in this context, the conditions in which initial reports of these adverse events surfaced, have had on the types of studies conducted and reports produced surrounding them, and what that has meant for bringing them together to draw conclusions.

HCC recurrence

In epidemiology, we shy away from overtly stating, especially in writing, that we are trying our best to understand causal effects. However, after painstakingly planning out sampling strategies and study designs, fretting over loss-to-follow-up, carefully choosing our analysis model, and adjusting for confounders, we discredit ourselves by not admitting that this is indeed what we are attempting to approximate (Hernan, 2018a; Hernan, 2018b). For the many public health topics for which it will always be highly unfeasible, if not impossible, to conduct a randomized controlled trial, we can eventually consider accumulation of non-randomized observational studies, weighing reports with ‘stronger’ versus ‘weaker’ study designs differently, to make causal conclusions with policy implications. For example, the fact that there has been no randomized controlled trials observing the effect of smoking on lung cancer incidence has not prevented worldwide cigarette taxes and legislation on smoking in public spaces. In attempting to understand whether or not DAAs lead to increased recurrence of HCC, if we could, we would draw consensus from randomized trials. However, even if ethically and logistically it is eventually possible to carry out a randomized trial, it will be many years before we have mid-long term comparative survival data for exposed and non-exposed patients. In the meantime, what we can do is try our best to use the cohorts and the data we have to approximate the ideal trials we would conduct (Hernan, 2018a; Hernan, 2018b). Hypothetically, if we could, we would follow a group of patients from the time of their original HCC cure (i.e. the moment when recurrence risk starts), assign them randomly to

either receive DAA treatment at a certain time or not, and then perform frequent and regulated imaging to check for signs of recurring cancer. In the real-world, with the data we have from Egypt, the best way we found to mimic this was following an entire group of persons treated for HCC from the time of their successful ablation, use inverse probability weighting with measured confounders in our analysis to approximate randomized treatment assignment, and treat DAA initiation as a time-dependent exposure, censoring our analysis after two years. After doing this, we found a highly significant increase in the risk of recurrence for those treated with DAAs (IRR: 3.82, 95%CI: 2.00-7.30).

Our methods and reporting differed greatly from many of the other groups who had already published at that time. The original harbingers of increased HCC recurrence due to DAAs tended to report percentages of persons with recurrence, which were interpreted as either high or low compared to historical estimates in groups of persons treated with the new regimens (Conti et al., 2016; Reig et al., 2016; Zavaglia et al., 2016). While these reports were useful in starting debate and prompting other groups to signal similar or contrary experiences, such study designs and reported figures, without identifying a control group or employing survival analysis, did not provide us with risk-ratios for comparison and discussion against our own findings. One other survival analysis with a control group had been performed at the time we were working on ours; this French study concluded no effect of DAAs on recurrence (HR: 1.21, 95%CI 0.62–2.34) (Pol et al., 2016). However, as we mentioned in the discussion of our paper, our results are possibly not appropriately compared with those of this French cohort as the follow-up starting points and median time after original HCC cure at which DAA administration, differed. In the discussion of our paper we mentioned that this could lead to a different interpretation of each our own and the French study, rather than indicating conflicting results; the French study shows no increased risk of recurrence if persons who have survived for two years after original HCC cure and ours shows increased risk of recurrence if DAAs are administered within a few months of tumor ablation. In other words, the design and analysis of each of our studies emulated different trials, and therefore, the results of each should not be considered together, just as they wouldn't be had they actually been reported from randomized studies with different populations and

protocols. Alternatively, and very importantly, interpreting any estimates as demonstrating causality, even when it seems a trial has been well imitated, comes with the very important assumption that there are no significant unmeasured confounders (Hernan, 2018a; Hernan, 2018b). If eventually deemed ethical, a randomized trial comparing early versus late DAA treatment (e.g. within six months of cancer cure or after) for persons with a history of liver cancer would respond well to the question I have posed above.

In the time since our analysis has been carried out on recurrence of HCC, there have been many further studies, including some survival analyses with control groups, reported. There have also been reviews and meta-analyses attempting to bring these findings together; for many of these, the general conclusion is that there can still be no conclusion, and that further well designed studies are needed (Butt, Sharif, et al., 2018; Guarino et al., 2018; Saraiya et al., 2018; Waziry et al., 2017). The first red flags for this adverse event were reported just shortly after DAAs started to be used in this special population of persons with former liver cancer, and in that setting, consideration of all study designs and reported figures as possible evidence for or against this side effect may be beneficial. However, in late 2018, an increased emphasis by researchers, as well as the journals that accept their reports, should be put on how the data we have can be used to generate policy implications in the absence of a real, and maybe never to be realized, randomized controlled trial. This could include recommendations and ‘calls’ by liver associations for the types of analyses that can be performed using non-randomized observational data, and/or eventual differential weighting in meta-analyses based on study and analysis design.

As a follow-up to this work on HCC recurrence, we are currently undertaking an updated survival analysis comparing the risk of overall mortality over a further extended time period for those receiving and not receiving DAAs. Our preliminary results demonstrate an eventual increased risk of death for persons receiving DAAs, but which seems to be entirely mediated by whether or not there has been recurrence. If corroborated by others, this finding would indicate a lack of benefit in giving these treatments to some populations of persons with a history of liver cancer.

HBV reactivation

HBV reactivation can be triggered by health events such as onset or, alternatively, treatment of other diseases, such as cancer chemotherapy (Hoofnagle, 2009), and had previously been seen in co-infected patients treated with IFN and RBV (Liu et al., 2012). Therefore, after case reports surfaces indicated that this event had occurred in persons taking DAAs, causality of the two events was not debated. In order to inform policy on monitoring and treatment in this case, a cohort study following patients with evidence of current or past infection with HBV through their DAA treatment and until the moment of cure assessment, will address the question of the magnitude of risk and consequences of this adverse event. We found two challenges in bringing together the findings from our own cohort study on this topic with others through a meta-analysis; non-consensus on the definition of HBV reactivation used in studies, and risk of bias in reported studies. Timing may play a role in the former; either that research is conducted and published rapidly following initial reports of an adverse event leading to non-consideration of eventual comparability, or alternatively, studies are conducted in the lag time prior to official recommendation of case definitions by reference associations (e.g. the American or European associations for the study of liver disease). Another challenge that we observed, while trying to bring findings together with our own through a meta-analysis was a high risk of bias in some studies. For example, we excluded some studies in populations of veterans in the United States who stated high original sample sizes, but also had very high losses-to-follow-up; in these two studies the final reported risk of reactivation used the original denominator of patients included and patients were not stratified on whether or not they received concomitant therapy against HBV (Belperio et al., 2017; Butt, Yan, et al. 2018). Inclusion of such studies could inaccurately sway meta-analyses. Criteria for risk of bias, and exclusion of studies with such characteristics is therefore necessary and possibly requires increased attention in investigations that immediately follow emerging disease events.

- **Ameliorating logistical hurdles linked to high uptake of DAAs**

Some challenges linked to an introduction of a new, highly effective treatment need not be negative from all sides, such as the appearance of unexpected adverse events. In the case of Egypt, an initial high uptake of persons into the large centralized national treatment program to administer DAAs quickly led to difficulties in retaining patients until the moment of their cure evaluation, with up to 40% of patients not returning with their SVR12 results (El Akel et al., 2017). In response to this, we examined whether or not an earlier time point, four weeks following the end of treatment (rather than 12 weeks), could equally identify most treatment failures. This is biologically plausible considering that the new treatments are highly potent and lead to rapid responses in most patients (EASL, 2018). We found that only one-third of eventual treatment failures were caught using this earlier endpoint, and recommended to remain using SVR12 in order to ensure adequate referral of patients with possible resistance. Few studies have looked into this question but further evaluations would be interesting, especially considering that even newer and more potent DAAs are being released, with proportions of patients achieving SVR12 reaching up to 100% (EASL, 2018). In the case of Egypt, for now, other methods are being used to retain patients, such as delivery of ‘cure’ certificates, which has been found effective in a context where infection with HCV is highly stigmatizing and proof of being infection-free is sometimes needed for work and marriage (El Akel et al., 2017).

Another important challenge, that has not been addressed by any of my research, and that has directly arisen from introduction of the highly effective DAAs and scale-up of treatment programs, is eventual diminution of persons applying to be treated. As discussed extensively in this chapter, in Egypt and many other countries, DAAs are becoming more accessible through generic production, and more widely used in persons infected due to tolerability and access to treatment programs. However, in a country where it is estimated that almost 6 million adult persons are infected, it is also estimated that the majority are not knowledgeable of their infection status (El Kassas, Elbaz et al., 2018). As a result, after treating approximately 1.5 million persons over the past 4 years through the National Treatment Program in Egypt, the waiting lists at the 74 treatment

centres, and the numbers of persons being treated per month have dwindled. Although screening of university students and health workers is now taking place mass testing of high-risk groups in Egypt, such as those in rural areas, is needed to continue addressing the need (El Kassa, Elbaz, et al., 2018). Further facilitating mass screening efforts will be availability of a robust and field-friendly rapid point-of-care test for active infections (i.e. detecting HCV RNA). Such tests are currently being developed and tested (Grebely et al., 2017; Lamoury et al., 2018; Llibre et al., 2018). Without improving this part of the care cascade, both in Egypt and worldwide, HCV elimination in the near future will not be possible.

**Part 4: Approaching epidemiological investigations
following emerging disease events**

This section is meant to serve as a brief and general conclusion to my thesis manuscript. It includes a few strategies for the initial organization of epidemiological investigations following emerging disease events, which may aid in developing research priorities and eventually reconciling findings. This will not be an exhaustive description of such methods, but rather will draw on a few examples from my own experiences during the thesis period.

I will start by discussing the utility of research consortiums and protocol standardization. I will then present expert opinion elicitation as a method for focusing hypotheses, and providing rapid information for policy makers; here I will present, as an example of such methods, an article that describes my use of this technique to identify exposure pathways for Middle East respiratory syndrome coronavirus (MERS-CoV).

Finally, I will give my concluding thoughts on my thesis work and the implication these findings have for persons affected by emerging disease events.

4.1 Research consortiums

Establishment of diverse research partnerships for infectious disease topics has many benefits and is being increasingly recommended and utilized. These consortia are seen to lead to streamlined and robust answers to pressing emerging disease questions, reduction of duplicity in efforts, and generation of new hypotheses through stimulating inter-group discussions (Dockrell, 2010). In addition, when those included in the consortium are from varied settings and types of expertise, this can lead to cross-group capacity building, and development of new regional partnerships where the disease in question has an impact. Many such research partnerships are composed of scientists from both high-income and low-middle income, settings; an additional benefit in this case can be the eventual diminution of global health inequalities, as long as the consortia have carefully thought out, shared governance (Global Ministerial Forum on Research for Health, 2008; Pratt and Hyder, 2016).

Both of the research studies on ZIKV-related birth defects that I have been involved with (i.e. the ZIKA-DFA-FE cohort study, and the ZIKV-related microcephaly surveillance study) are part of the ZIKAlliance consortium supported through the European Union's Horizon 2020 grant. This research consortium is composed of 54 partners across 18 countries worldwide, and 9 work packages. Information on this consortium can be found here: <https://zikalliance.tghn.org>. Within the first work package of this research network, is the aim to demonstrate the impact of ZIKV infection on fetuses/infants when the mother is infected during pregnancy. In terms of clinical and epidemiological studies, this is being approached through cohort studies of women infected with ZIKV during pregnancy; both the Brazilian cohort study and our own cohort study in the FTA are part of this group, in addition to a further multi-country cohort study with a standardized protocol that takes place across South America.

The clear benefit of having these varied cohort studies all involved in the same consortium is that it allows for presentations by, and discussions between, investigators at the multi-annual group meetings, therefore permitting eventual understanding of where methodological differences may lie between study protocols. Although not yet being

done, it could also eventually allow for collaboration between investigators and pooling of raw data, creating higher powered studies that may showcase regional variation, but also highlight why these differences may have been found. In addition, as this consortium, as with many of the others that have been put together to tackle ZIKV-related research topics, follow a 'one health' approach (i.e. combining investigators across epidemiological, clinical, microbiological, animal/vector and social sciences fields), there is the possibility to easily share and have input on hypotheses on biological reasons for regional variation when methodological differences do not explain all.

4.2 Protocol standardization

When comparing observational research studies, we are often unsure of whether or not the differences in results are due to underlying true variation by populations or simply to dissimilarities in the methods used or the data collected. This has been discussed as a possible reason for the present difficulty in drawing a consensus from findings on the risk of ZIKV-related birth defects as well as on the association between direct acting antivirals for HCV and recurrence of liver cancer, in this thesis. Use of harmonized research methods for certain study types and following guidelines on reporting can be used to ensure that a consensus can be drawn, as quickly as possible, from the studies that have been performed on an important emerging disease topic. The need for such standardization was highlighted following recent novel influenza epidemics and pandemics, after which two important consortiums, the International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC) and the Consortium for the Standardization of Influenza Seroepidemiology (CONSISE) have been collaboratively developing and promoting standardized research protocols with the WHO (Van Kerkhove, 2013; Van Kerkhove, 2016).

In 2016, the WHO also endorsed the creation and dissemination of standardized protocols for various types of ZIKV studies, including case-control studies for the association between ZIKV with microcephaly and ZIKV with Guillain-Barre Syndrome; cohort studies of ZIKV infected pregnant women and infants with ZIKV exposure in-utero; persistence of ZIKV in body fluids; and ZIKV seroprevalence studies. Personally, I participated in the drafting of the protocol for follow-up of infants born to women infected with ZIKV during pregnancy; this was done through review and combination of existing protocols for similar studies as well as through input and editing from experts. In June 2016, a meeting was held in Mexico City with researchers from across South America and Europe to discuss drafted versions of these protocols and agree on aspects that should be standardized across countries (Van Kerkhove et al., 2016). These protocols can be found at: <http://www.who.int/reproductivehealth/zika/zika-virus-research-agenda/en/>. While not all scientists will choose to adopt these prefabricated protocols, they can be used as guidance by well-established research groups and as a

protocol model for smaller groups with limited research experience in regions affected by the emerging disease.

The CONSolidated Standards Of Reporting Trials (CONSORT) statement, was first conceptualized in 1993 when a diverse set of epidemiologists and methodologists came together to develop a scale for the assessment of clinical trials, and realized that many key indicators were poorly reported (CONSORT, 2010). This was a finding supported by contemporary literature (Altman and Dore, 1990; Pocock et al, 1987). Other meetings and gatherings to follow, led to the CONSORT 2001 and, eventually, the CONSORT 2010, checklists for reporting of clinical trial results (Moher et al., 2001; Schulz et al., 2010). Along the same lines, the STrengthening the Reporting of OBServational studies in Epidemiology (STROBE) checklists, which exist for all of case-control, cross-sectional, and cohort studies, were first published in 2007 (Vandenbrouke et al., 2007; von Elm et al., 2007). Increasingly, and importantly, peer-reviewed journals require authors to fill out and submit these checklists along with their manuscript prior to its review. This fairly recent advancement, and the further adoption of this requirement by other journals in the future, will surely lead to increased comparability of published research findings and allow for merging of findings in robust meta-analyses.

4.3 Expert opinion elicitation

Expert opinion elicitation (EOE) is a tool that gathers inputs, from persons with relevant experience, regarding event probabilities, magnitude predictions, and relative importance of risks where empirical evidence is lacking. This has been employed in many scientific domains, such as, but not limited to, technology development, climate change forecasts, environmental health exposure impact, and emerging infectious disease risks (Cox et al., 2012; Gale et al., 2010; Goutard et al., 2012; Horst et al., 1998; Morgan, 2014; Syed et al., 2010). Specifically with regards to emerging diseases, this method can be useful in generating rapid ‘best guess’ assessments that inform necessary policy actions, prior to the planning and conduct of appropriate research studies (Russell et al., 2017).

While EOE studies can be seen as a quick way of gathering information, it is still necessary to conduct them with a carefully thought out methodology, to avoid misleading results. The first consideration is the choice of relevant experts. This can be done either through informal methods, such as identification of potential participants by the study coordinators, who are normally also experts in the field, based on who they know to be knowledgeable and willing. To expand on this, ‘snowball’ recruitment can be done where an initial group of experts chosen by the study coordinators are asked to refer their colleagues who would be interested and appropriate. Alternatively, a less subjective method, such as a systematic search of articles on related subjects and identification and proposal of the study to the most frequently appearing authors, can be done (Morgan, 2014).

Prior to commencing the elicitation activity, it is recommended that experts be given a thorough summary of existing evidence on the subject of interest; this will limit the chance that the expert will be biased by ‘availability’ and make their judgment only based on information they have recently been confronted with (Tversky and Kahneman, 1974). This is typically done through provision of a recent and robust systematic review (Morgan, 2014). Following this, experts will respond to qualitative and quantitative questions on the likelihood and magnitude of certain risks associated with the emerging

disease topic at hand. Ideally, this is done through a well-designed questionnaire that is given to the expert in person through a face-to-face interview. However, use of technological aids, such as online software and questionnaires can also be used to deliver the questions to the participants. Eventually, for analysis of the results, it may be necessary to weight more heavily some expert opinions over others, as some persons will be more knowledgeable and/or certain in their answers than others. Here, calibration is an ideal method to understand the quality of each expert opinion; this includes asking a few ‘seed’ questions for which there actually is empirical evidence, and that the expert’s answers can be compared to (Cooke, 1991; Cooke and Goossens, 2008). In addition, and especially when little to no already quantified risk estimates exist on a subject, experts can be asked for their personally assessed level of certainty in their own answer, for example on a scale of 1 (not confident) to 5 (very confident) (Morgan, 2014).

Finally, the results from each expert needs to be combined, either with the goal of drawing a consensus across those participating, or rather to present the range of possible expert answers and opinions on a subject. One well-known consensus method is the ‘Delphi’ technique, which was first employed in decision making by the United States Air Force in the 1950s (Dalkey and Helmer, 1972). When applied to gathering expert opinions in scientific fields, the Delphi technique requires multiple rounds of the elicitation exercise. The first round proceeds as I have described above, followed by a presentation, to a given expert, of how their answers compared to the responses of their colleagues. The expert is then given the chance to revise their response, and typically a second round where experts can discuss and justify their results together, with a final group decision on a ‘consensus’ result, is produced (Dalkey, 1962; Linstone and Turoff, 1975). Alternative to the Delphi method, in certain fields such as climate science, investigators may want to understand the full range of risks and predictions that experts believe possible (Oppenheimer et al., 2007). This second approach, where consensus is not sought, is seen to be an advantage of EOE methods by many (Aspinall, 2010; Oppenheimer et al., 2007; US Environmental Protection Agency, 2011).

4.3.1 Original research (full article): MERS-CoV expert opinion elicitation

MERS is caused by a coronavirus that was discovered in 2012 in Saudi Arabia. Infection with the virus can lead to severe respiratory illness, including pneumonia, and an estimated fatality of up to 35% (WHO, 2018). However, since it was first identified six years ago, few disease parameters have been solidly quantified. For example, direct and indirect contact with camels is a clear risk factor for infection (Alraddadi et al., 2016), however, whether or not camels also play the role of the main reservoir for this virus, is not yet well understood.

To follow will be the description of an expert opinion elicitation study that I performed in collaboration with colleagues from the Institut Pasteur (and international network partners) and the International Centre for Cooperation in Agronomical Research for Development (CIRAD), on the topic of exposure pathways for MERS-CoV. Originally, senior scientists from these institutions aimed to perform a risk assessment, including known disease parameters and supplementing them with inputs from EOE. However, due to a lack of quantified risks, such a study was not possible. This led us to focus on the EOE alone, hoping that it would give guidance for further robust studies.

This article is being presented, not to focus on MERS-CoV as an emerging disease example (this is outside the breadth of this thesis), but rather to showcase the use and limitations of a method such as EOE in demonstrating the range of predicted risks for an emerging disease, and in narrowing hypotheses for recommended research.

The PDF of this article can be found in Appendix 5.

MERS-CoV at the animal–human interface: inputs on exposure pathways from an expert-opinion elicitation

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- **Abstract**

Nearly 4 years after the first report of the emergence of Middle-East respiratory syndrome Coronavirus (MERS-CoV) and nearly 1800 human cases later, the ecology of MERS-CoV, its epidemiology, and more than risk factors of MERS-CoV transmission between camels are poorly understood. Knowledge about the pathways and mechanisms of transmission from animals to humans is limited; as of yet, transmission risks have not been quantified. Moreover the divergent sanitary situations and exposures to animals among populations in the Arabian Peninsula, where human primary cases appear to dominate, vs. other regions in the Middle East and Africa, with no reported human clinical cases and where the virus has been detected only in dromedaries, represents huge scientific and health challenges. Here, we have used expert-opinion elicitation in order to obtain ideas on relative importance of MERS-CoV risk factors and estimates of transmission risks from various types of contact between humans and dromedaries. Fourteen experts with diverse and extensive experience in MERS-CoV relevant fields were enrolled and completed an online questionnaire that examined pathways based on several scenarios, e.g., camels–camels, camels–human, bats/other species to camels/humans, and the role of diverse biological substances (milk, urine, etc.) and potential fomites. Experts believed that dromedary camels play the largest role in MERS-CoV infection of other dromedaries; however, they also indicated a significant influence of the season (i.e. calving or weaning periods) on transmission risk. All experts thought that MERS-CoV-infected dromedaries and asymptomatic humans play the most important role in infection of humans, with bats and other species presenting a possible, but yet undefined, risk. Direct and indirect contact of humans with dromedary camels were

identified as the most risky types of contact, when compared to consumption of various camel products, with estimated “most likely” incidence risks of at least 22 and 13% for direct and indirect contact, respectively. The results of our study are consistent with available, yet very limited, published data regarding the potential pathways of transmission of MERS-CoV at the animal–human interface. These results identify key knowledge gaps and highlight the need for more comprehensive, yet focused research to be conducted to better understand transmission between dromedaries and humans.

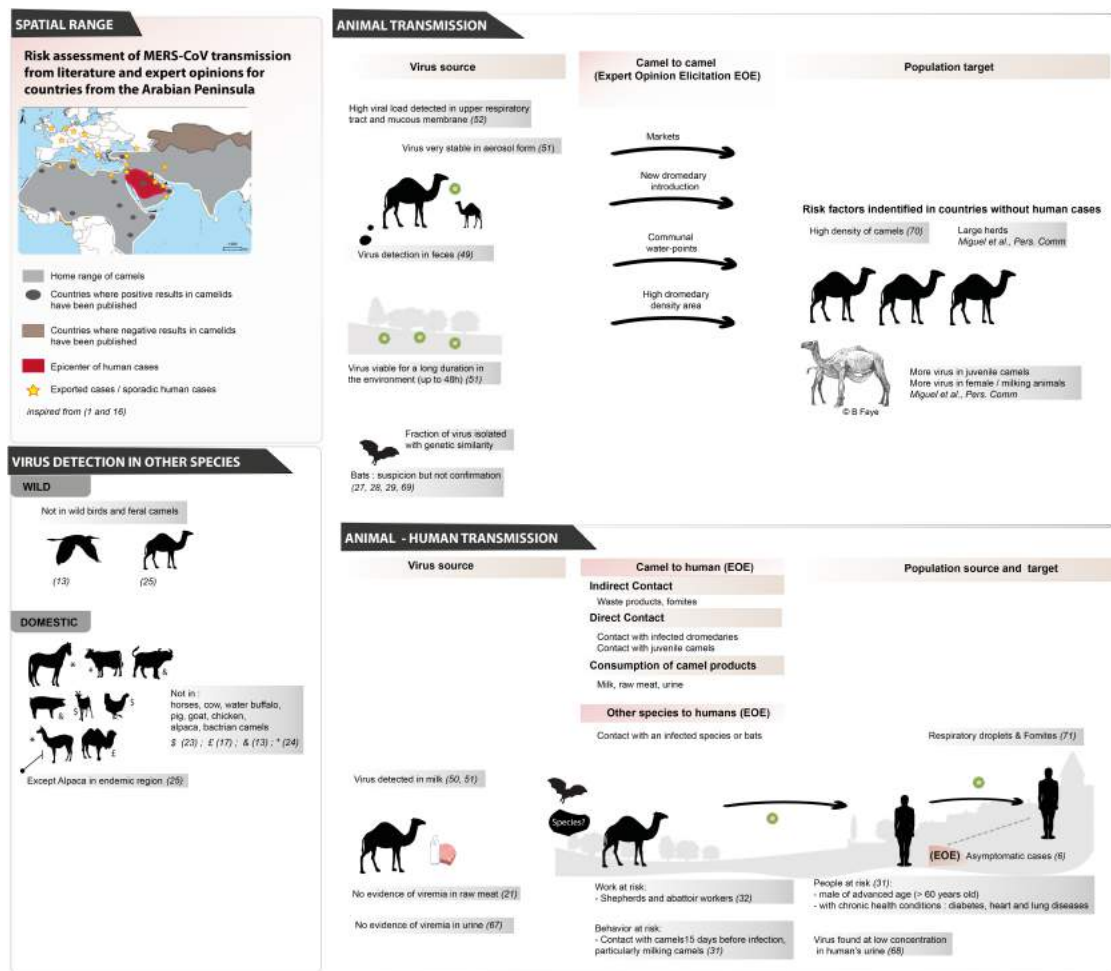
- **Introduction**

Nearly 4 years after the first report of the emergence of Middle- East respiratory syndrome Coronavirus (MERS-CoV) in humans and more than 1800 human cases later (WHO, 2016), mainly in Saudi Arabia (~75% of cases and almost all of the primary cases), the ecology of MERS-CoV and its epidemiology remain poorly understood (Embarek and Van Kerkhove, 2015). Human-to-human transmission of MERS-CoV accounts for approximately half of all the MERS-CoV cases reported to date (Embarek et al., 2015). Inter-human transmission has been well documented in health care-associated outbreaks in the Middle East and Korea (Assiri et al., 2013; Drosten et al., 2015; Ki et al., 2015), and there appears to be limited inter-human transmission in household settings (Drosten et al., 2014).

Many studies have now identified dromedary camels (*Camelus dromedarius*; dromedaries) as a natural host for MERS-CoV, and there appears to be ample evidence of widespread infection (either past or present) in dromedaries in the Middle East (Azhar et al., 2014; Hemida et al., 2015; Hemida, Perera et al., 2014; Nowotny and Kolodziejek, 2014), and in many parts of Africa (Deem et al., 2015; Müller et al., 2014; Perera et al., 2013; Reusken, Haagmans, et al., 2013; Reusken, Messadi et al., 2014). High levels of MERS-CoV specific seroprevalence have been observed in dromedaries, ranging from 0% in Central Asia to as much as 100% in Africa and the Arabian Peninsula (Azhar et al., 2014; Deem et al., 2015; Hemida et al., 2015; Hemida, Perera et al., 2014; Miguel, El Idrissi et al., 2016; Miguel, Perera et al., 2016; Müller et al., 2014; Nowotny and

Kolodziejek, 2014; Perera et al., 2013; Reusken, Haagmans, et al., 2013; Reusken, Messadi et al., 2014) (see Figure 10). MERS-CoV strains isolated from dromedaries are genetically and phenotypically very similar or identical to those infecting humans (Chan et al., 2014; Farag et al., 2015).

Figure 10: Review of MERS-CoV exposure pathways for animal-to-animal transmission and animal-to-human transmission based on literature evidence and the expert opinions elicited in this study



Note: As it is not possible to further adapt this figure, the references are as follows #1 is WHO, 2016; #6 is Drosten et al., 2014; #13 is Perera et al., 2013; #16 is Miguel, El Idrissi et al., 2016; #17 is Miguel, Perera et al., 2016; #21 is Gossner et al., 2014; #23 is Hemida et al., 2013; #24 is Reusken, Ababneh et al., 2013; #25 is Reusken et al., 2016; #27 is; #28 is Ithete et al., 2013; #29 is Memish et al., 2013; #31 is Alraddadi et al., 2016; #32 is Müller et al., 2015; #49 is Hemida, Chu et al., 2014; #51 is van Doremalen et al., 2013; #52 is Khalafalla et al., 2015; #67 is Adney et al., 2014; #68 is Drosten et al., 2013; #69 is Munster et al., 2016; #70 is Corman, Jores et al., 2014; #71 is Al-Tawfiq and Memish, 2014.

Since the beginning of the MERS-CoV outbreak, animals and specifically dromedaries, have been suspected of playing a role in transmission. The global camel population has more than doubled in the past 50 years, reaching ~30 million today, 95% of which are dromedaries. Approximately 60% of camels are found in East African countries, which are important exporters to the Arabian Peninsula and Egypt (Faye, 2013). Camels play a major role in socio-cultural traditions in Saudi Arabia; a place where the camel population has increased from 80,000 to 200,000 heads over the last 50 years; a number which some experts estimate is actually closer to 800,000 heads (Gossner et al., 2014). In parallel, a drastic decrease (from 10 to 1.5%) of nomadic camel populations has been observed over this time period in favor of permanent (or semi-permanent) settlements often at the borders of cities (Gossner et al., 2014). It is possible that the mentioned changes in global dromedary population dynamics have led to an increased spread and heightened detection of MERS-CoV in this species, both of which have made dromedaries the focus of most of the research conducted on MERS-CoV to date.

While coronaviruses are widespread in the animal kingdom (Woo et al., 2012), MERS-CoV seems to have a narrow host range. In the last few years, a large spectrum of domestic species have been negative after MERS-CoV serology tests, including horses, cattle, pig, water buffalo, chickens, goats, and Bactrian camels (Hemida et al., 2013; Miguel, Perera et al., 2016; Perera et al., 2013; Reusken, Ababneh et al., 2013; Reusken, Haagmans et al., 2013;). An exception was published recently when antibodies were detected in Alpaca (*Vicugna pacos*) in Qatar; this is notably in a specific region where MERS-CoV is already endemic in dromedary camels (Reusken et al., 2016) (Figure 10).

A number of studies on wild birds and swine in Hong Kong, feral camels in Australia and bats in several countries have not identified MERS-CoV in these species (Cramer et al., 2015; Perera et al., 2013) (Figure 10). Putative precursors of MERS-CoV have been detected in species of African bats (Ithete et al., 2013), and Corman, Ithete and colleagues (2014) raised hypotheses on the emergence of MERS-CoV from other animal species. They characterized the full genome of an African bat virus closely related to MERS-CoV and showed that human, camel, and bat viruses have phylogenetic

relationships although these bat viruses are not closely similar to MERS-CoV. They suggest that, according to available serologic data on camels and humans since 2012 and molecular investigations of known cases, MERS-CoV moved from bats to camels in sub-Saharan Africa. They also suggest that camelids could be “mixing vessels for MERS-CoV and other mammalian CoVs” and that the virus can be transmitted between humans and camels (Corman, Ithete et al., 2014). Up to now, MERS-CoV-like viruses have not been detected in any species other than camels, with the exception of an unconfirmed report of the detection of a very small fragment of MERS-CoV-like RNA in a specimen from a *Taphozous perforatus* bat collected in Saudi Arabia (Memish et al., 2013). *T. perforatus* and other bat species sampled in Egypt and Lebanon did not reveal MERS-CoV like viruses, although other coronaviruses were detected (Shehata et al., 2016).

However, after more than 1800 reported cases over the past 4 years from 27 countries, only one case-control study evaluating non-human risk factors for infection has been performed and published. This study, which included 30 primary cases and 116 age, sex, and neighborhood-matched controls, confirmed suspicions that direct and indirect exposure to dromedary camels in the 14 days prior to symptom onset are risk factors for infection. This study also found that advanced age (>60 years old), being male, and having certain underlying chronic health conditions, such as diabetes, heart conditions, and chronic lung disease, were independent risk factors for disease (Alraddadi et al., 2016). Several other seroprevalence studies evaluating the extent of MERS-CoV infection in occupationally exposed persons (e.g., farmers, herders, slaughterhouse workers) have identified that these populations have a higher levels of seroprevalence when compared to the general population (Müller et al., 2015; Reusken et al., 2015) (see Figure 10).

What is currently unclear is why all primary human MERS-CoV cases have been reported from the Arabian Peninsula (Embarek and Van Kerhove, 2015). Given that there is evidence of MERS-CoV circulation in dromedaries across large parts of Africa (Deem et al., 2015; Müller et al., 2014; Perera et al., 2013; Reusken, Haagmans et al., 2013; Reusken, Messadi et al., 2014), it is likely that cases of MERS-CoV in humans have been missed. There are several potential hypotheses to explain this. First, surveillance for

MERS-CoV in human populations focuses mostly on severe disease and on travelers returning from the Arabian Peninsula rather than on patients without a history of travel. Moreover, on-going surveillance in Saudi Arabia is now very intensive (Saudi Arabia Ministry of Health, 2013). Second, the prevalence of chronic underlying medical conditions in many countries in Africa is far lower than in the Middle East, with high rates of heart disease, diabetes, and obesity; third, it is likely that asymptomatic, mild or sub-clinical cases are missed with even the most robust surveillance systems. Fourth, the nature of contact with and the use of dromedary products differ between countries and cultures. Lastly, the viruses circulating in the Arabian Peninsula may be different. Although MERS-CoV in Africa are >99% identical at the nucleotide level with those in the Arabian Peninsula (Chu et al., 2015), it is conceivable that a few key amino- acid differences may make a major change in transmissibility and virulence.

In the case of MERS-CoV transmission, there is a large uncertainty about the various exposure pathways associated with new dromedary camel or human cases, and, although published research on MERS-CoV is actively increasing (Zyoud, 2016), few transmission risks have yet been quantified. There is an obvious need to collect more critical information from virological and eco-epidemiological studies, but also from social sciences (anthropology, sociology) studies about camel–human relationships, including behaviors at the interface. These studies can evaluate contact patterns, modes of transmission, viral shedding from animals, virus persistence in different environments, and biological samples. In view of all that remains to be done, we advocate a risk-ranking approach based on exposure pathways to guide allocation of resources for future data collection on the main sources of transmission of MERS-Cov. Risk assessment is a powerful modeling tool that enables decision-makers to determine the likelihood of disease occurrence and the magnitude of its consequences, which, in turn, allows identification of key steps and appropriate management measures to take. It is a structured and a systematic process that helps in the gathering of diverse and disparate information and data. However, when data are scarce and knowledge gaps are highly prevalent, such as with the recently identified MERS-CoV, too many transmission pathways would have been presented for the risk analysis. This is why we proposed, as a preliminary step, to call upon experts using expert- opinion elicitation (EOE), to explore

scenarios and hypotheses of transmission among animal(s), fomites, and humans. From the EOE outputs, a qualitative and/or quantitative risk assessment model could then be implemented. Expert-opinion elicitation has proven to be useful in other zoonotic disease risk assessments, especially in cases where little quantitative information for the disease is already known (Goutard et al., 2012; Horst et al., 1998). The aim of this work is to allow for a triage of highly likely and unlikely pathways, and highlight areas that deserve increased attention for field surveys and studies.

- **Materials and Methods**

In our study, experts were defined as being persons with relevant experience on the topic, including having extensive technical experience in epidemiological or virological research through MERS-CoV or related animal and/or human studies. Considering the recent emergence of the virus as a cause for human disease, extensive experience in MERS-CoV research itself was not an inclusion criteria; however, all included experts needed to have some experience working on MERS-CoV and/ or camel research topics within North Africa and the Arabian Peninsula, if not elsewhere. Furthermore, the experts' publications and professional affiliations should have been significant enough to reflect this expertise. Recruitment was done first through relevance screening, where the researchers chose persons based on their own judgment. Following this original recruitment, "snowball" recruitment was used; experts who chose to participate were asked to recommend other experts to fill out the questionnaire. We aimed to enroll at least 10 experts, with extensive experience in relevant fields, for the exercise and, therefore, started by emailing invitations to 13 persons. All persons recommended by the first group of experts were invited to participate if their expertise was judged relevant for our study. All experts gave an informed consent before starting their participation in the survey. Written consent was not necessary for this type of study; experts could withdraw themselves from the study at any time and all opinion "results" would be presented in an anonymous fashion.

Searching into MERS-CoV literature and meeting reports allowed us to identify potential pathways and risk factors needed for designing the EOE (FAO, 2016; Shapiro et al., 2016; Zyouud et al., 2016) (**Figure 10**).

The questionnaire was designed online using the tool Survey Monkey (www.surveymonkey.com). A pilot survey was sent to the team members in order to test the survey and optimize the consistency of the questions. A clear description of the study objectives and of what was expected for their participation was provided to experts in the invitation email. Following their acceptance to participate, the experts were emailed a link to the online survey. The beginning of the online survey included instructions, examples, and contact information of the administering researchers.

The questionnaire was designed to take about 30–40 min, and be filled in by experts individually using a link to online software (see link 5 in Appendix 4). It consisted of expertise questions, relative importance of risk factor questions, transmission risk estimations, and open-ended responses, in that order, all of which will be described in more detail below.

The analytical hierarchy process (AHP) is widely used in marketing research and has more recently been introduced as a tool in veterinary epidemiology (Horst et al., 1996; Horst et al., 1998; Saaty, 1980; Saaty, 1987; Wind and Saaty, 1980). AHP obtains opinions on the weight of the relative importance of one attribute of an object or event over another, through pair-wise comparisons. In our questionnaire, we used the technique to obtain experts opinions about the most relevant exposure pathways and their relative importance for five different animal–animal or animal–human transmission scenarios. Where appropriate, simple transmission diagrams were used to explain the potential exposure pathways in question. Experts were first asked to identify which exposure pathway they “believed in” out of a provided list, always with the opportunity to specify “other.” They were then asked to do pair-wise comparisons of each exposure pathway, comparing the risk factors of transmission, using the Saaty scale (Saaty, 1987) (Figure 11).

<i>More important</i>					<i>Less important</i>			
Extremely	Very strongly	Strongly	Moderately	Equivalent	Moderately	Strongly	Very strongly	Extremely
9	7	5	3	1	1/3	1/5	1/7	1/9

Figure 11: Simplified Saaty scale used for comparing risk factors in the analytical hierarchy process.

In order to obtain quantitative estimates on the transmission risks from dromedaries to humans, we asked experts for their 3-point estimation (minimum, most likely, and maximum) considering different types of exposure between 10 susceptible camel workers and dromedaries. Exposures included consumption of camel products (e.g., milk, urine, meat), direct and indirect contact; separate estimations were asked for different scenarios of younger (≤ 50 years) or older (> 50 years) camel workers and adult or juvenile dromedaries. Using the same method, experts were also asked their estimates for transmission between potentially asymptomatic camel workers and family contacts.

Finally, a few open-ended questions on factors that may increase or decrease transmission and were posed to experts. The survey was not anonymous in order to be able to come back to the experts in case of inconsistency in their answers. For every question, the experts were asked to respond not only expressing their opinion but also to assess their own confidence in their answers for each question, with a score from 1 to 5.

The analytical hierarchy process allowed us to weight each exposure pathway according to the level of importance given to it by each expert. Additional weight was attributed to each answer according to the level of confidence given by the expert. Then, for each pathway, a weighted aggregation of all expert answers was generated. An expert's data were excluded from the combined estimates in case of any of the following criteria: $< 30\%$ consistency ratio, obvious erroneous entry, missing data for part of or the entire question. In this case, a 30% consistency ratio cut-off, taking into consideration that the historically recommended 10% cut-off is shown to be too severe for comparison

matrices that have >3 variables and that the cut-off should increase with number of variables (Golden and Wang, 1989; Peláez, 2003); our questions introduced up to eight variables for each matrix. The overall level of agreement across experts in their ranking of the selected risk factors was calculated using the Kendall's W coefficient. The average weighted minimum, most likely and maximum transmission risk for each of the 3-point estimation questions was also generated using a similar weighting mechanism. An expert's data was excluded from the 3-point distribution combined estimates in case of consistently highly outlying estimates or missing data for part of or the entire distribution. Outliers were defined as estimated risks that were consistently greater than twice the estimates of all other experts. When possible, for missing or erroneous data, experts were re-contacted by email to clarify. The mean confidence level, across all included experts, was calculated for each pair-wise comparison and 3-point distribution question. Open-ended responses were summarized qualitatively.

- **Results**

Overall, 18 experts were contacted to take part in the questionnaire. Of these, 16 responded to the invitation, and 14 filled out the questionnaire in full, contributing data to this study. All respondents, except 1, had expertise in either MERS-CoV epidemiology and/or virology; the remaining expert had significant experience in camel production and husbandry and general epidemiology. Six and three respondents had experience in conducting studies of camels and bats, respectively. A detailed description of each participant's expertise can be found in Table 21.

MERS-CoV Infection of Dromedary Camels

On the topic of how dromedaries become infected with MERS-CoV, the following exposure pathways were presented to experts: infestation of infected bats in close proximity, daily close contact with infected camel workers (both ≤ 50 and > 50 years old), short-term contact with an infected dromedary herd, short-term contact with a non-

dromedary species infected with MERS-CoV, and infection occurring during dromedary calving season. All of the above risk factors were selected by at least 5/14 experts. However, the most highly selected and importantly weighted exposures were “short-term contact with an infected dromedary herd” and “timing coinciding with dromedary calving season” (Figure 12). Two experts selected the “other” option and specified the most risky season is dromedary-weaning season. Furthermore, one expert selected “other” and included the possible risk associated with contaminated camel feed.

Table 21. Included Expert Profiles

	Degree	Epidemiology		Virology		Camel Studies		Risk Analysis		Chiropterology (bats)	
		✓*	1-5 yrs	✓*	1-5 yrs	✓	10+ yrs	✓	1-5 yrs	✓	10+ yrs
1	MD	✓*	1-5 yrs	✓*	1-5 yrs						
2	DVM	✓*	10+ yrs	✓*	10+ yrs	✓	10+ yrs				
3	MD	✓*	10+ yrs	✓*	10+ yrs						
4	MPH	✓*	6-10 yrs			✓	1-5 yrs	✓	1-5 yrs		
5	PhD	✓*	10+ yrs	✓	1-5 yrs			✓	6-10 yrs		
6	DVM	✓*	10+ yrs	✓*	10+ yrs	✓	10+ yrs	✓	6-10 yrs	✓	10+ yrs
7	DVM	✓	10+ yrs	✓*	10+ yrs						
8	DVM	✓*	1-5 yrs								
9	PhD	✓*	1-5 yrs	✓*	6-10 yrs			✓	10+ yrs		
10	DVM	✓	10+ yrs			✓	10+ yrs				
11	DVM	✓*	10+ yrs					✓	6-10 yrs		
12	PhD	✓*	1-5 yrs			✓	1-5 yrs	✓	1-5 yrs		
13	MD	✓*	6-10 yrs	✓*	10+ yrs					✓	6-10 yrs
14	DVM	✓*	10+ yrs	✓*	10+ yrs	✓	1-5 yrs	✓	6-10 yrs	✓	1-5 yrs

* Including MERS-CoV specific

MERS-CoV Infection between Dromedary Herds

Risk factors that were presented to experts, when asking about the possibility of dromedaries from different herds infecting each other, were: nomadic dromedary herds, introduction of a new dromedary into the herd, high dromedary density area, dromedaries taken to racetracks, dromedaries entered into/taken to beauty contests, dromedaries brought to communal water- points, dromedaries brought to/sold at markets, dromedaries pass through border points. Each factor was considered risky by at least 5 (36%) of the 14

experts, and no additional risk factors from experts were provided. The most highly selected (i.e., >70% of experts) risk factors, in order of weighted importance, were: bringing dromedaries to markets, introduction of a new dromedary into the herd, high dromedary density area, and bringing dromedaries to communal water-points. The first three of these risk factors were given similar weights by experts, while the last (communal water-points) was thought to only be half as important as them. Experts had sufficient agreement on their ranking of risk factors (Kendalls $W = 0.25$, $p = 0.003$), and had a mean response certainty of 3.9 and 3.8 (out of 5) for choosing risk factors and the subsequent rankings, respectively.

MERS -CoV Primary Infection in Humans

Exposure pathways for human primary case occurrence included: infestation of MERS CoV infected bats in close proximity to human populations, contact with a MERS-CoV infected herd of dromedaries, contact with a non-dromedary MERS-CoV infected species, blood-biting pests (e.g., fleas, ticks) on an infected animal species or on humans, contact with another human who is asymptotically infected with MERS-CoV. All experts agreed that contact with infected dromedaries or asymptomatic humans were major risks for disease transmission, with the former being of higher risk (Figure 12). About a third of experts (29–36%) thought that contact with MERS-CoV infected species other than dromedaries or bats may also play a role in human infection. Only one expert considered the possibility of blood-biting pests transmitting infection between dromedaries or other species and humans. Experts suggested no “other” risk factors.

Table 22. Estimated Percentage Transmission Risk from Adult and Juvenile Dromedaries to Camel Workers (CW)

	Adult Dromedary				Juvenile Dromedary							
	≤50 years old CW		>50 years old CW		≤50 years old CW		>50 years old CW					
	Most likely	Min/Max	C*	Most likely	Min/Max	C*	Most likely	Min/Max	C*			
Milk	3	0/13	2.9	4	1/16	2.8	-	-	-	-		
Urine	3	0/9	3.2	3	0/12	2.9	5	0/12	3	4		
Raw Meat	4	0/15	2.8	3	0/13	2.9	1	1/6	2.9	5		
Direct Contact	25	4/45	2.9	29	5/55	3	22	7/39	2.9	33		
Indirect Contact	13	1/33	2.9	18	4/36	2.8	19	4/34	3.1	24		
											6/48	3.1

*C= Mean level of expert confidence for estimate with a scale of confidence between 1 (not confident) to 5 (very confident)

Transmission from Infected Dromedaries to Camel Workers

The following possible exposure pathways from dromedaries to camel workers were presented to the experts: direct contact (e.g., face-to-face, touching, kissing), indirect contact (e.g., cleaning camel environment, contact with dromedary waste), consumption of unpasteurized milk, consumption or use of dromedary urine, and consumption of raw dromedary meat. All pathways were thought to be possible by the experts (≥50% each) and direct contact with dromedaries was thought to be a transmission risk factor by all experts (Figure 13). When asked to quantify these risks, by estimating the likely incidence of human cases, separately for adult and juvenile camels and older (>50 years) and younger (≤50 years) camel workers, experts estimated direct and then indirect contact with the highest risk; generally there was a slightly higher risk estimated when contact was with juvenile camels, and a clear trend for higher estimated risk when older vs. younger camel workers were exposed (see Table 22). Specifically, the risk of transmission was thought to be low (≤5%) for camel workers consuming or using camel products, such as milk, urine, or raw meat. The estimation of

the incidence was quantified as being between 13 and 24% for indirect contact with an infected dromedary regardless of whether adult or juvenile, and between 22 and 33% with direct contact, varying by the age of both the camels and camel workers.

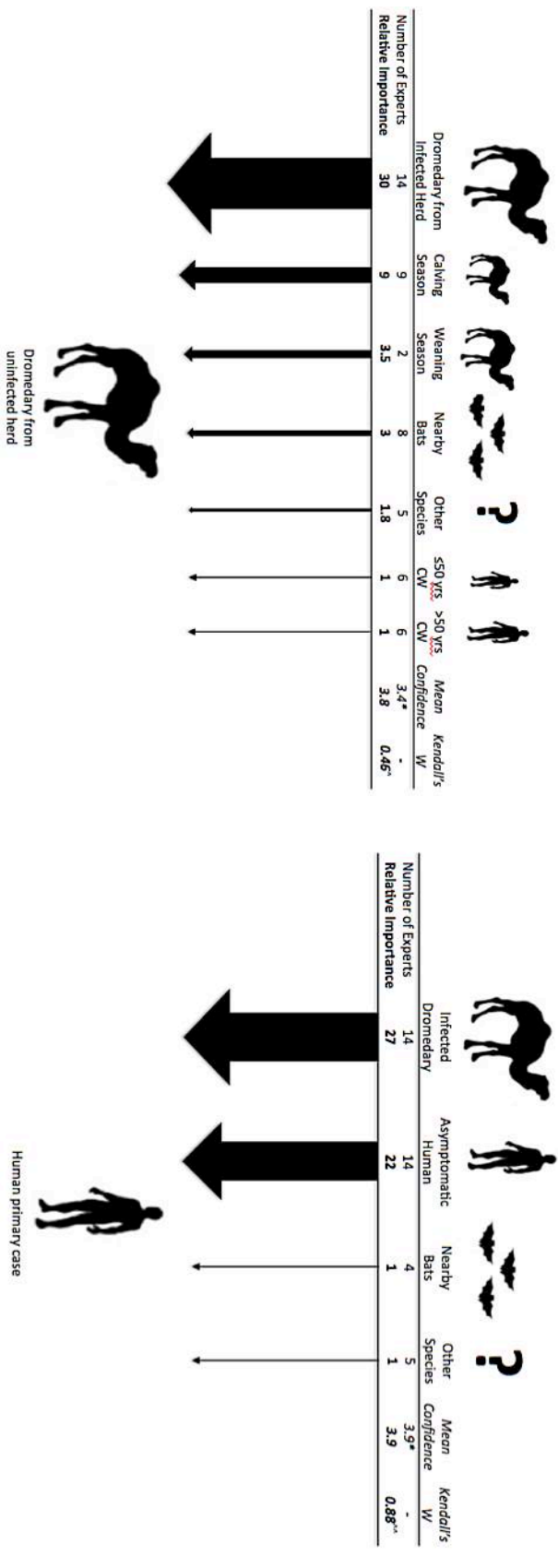


Figure 12: (A) (left) Exposure pathways and relative weights of risk factors for a dromedary camel from an uninfected herd to become infected with MERS-CoV. $\wedge p < 0.001$. (B) (right) Exposure pathways and relative weights of risk factors for a camel worker or other human to become infected with MERS-CoV. $\wedge \wedge p < 0.01$. *Mean confidence for overall choice of risk factors for this question with a scale of confidence between 1 (not confident) and 5 (very confident).

Transmission from Asymptomatic MERS-CoV Cases to Contacts

Experts were asked to estimate the risk of transmission from an asymptomatic infected individual to other individuals in close contact. The estimated “most likely” risk of transmission if the potentially asymptomatic camel workers were either ≤ 50 years or >50 years old was 9%. The experts had a mean confidence of 2.8 (out of 5) for their answers for both age groups.

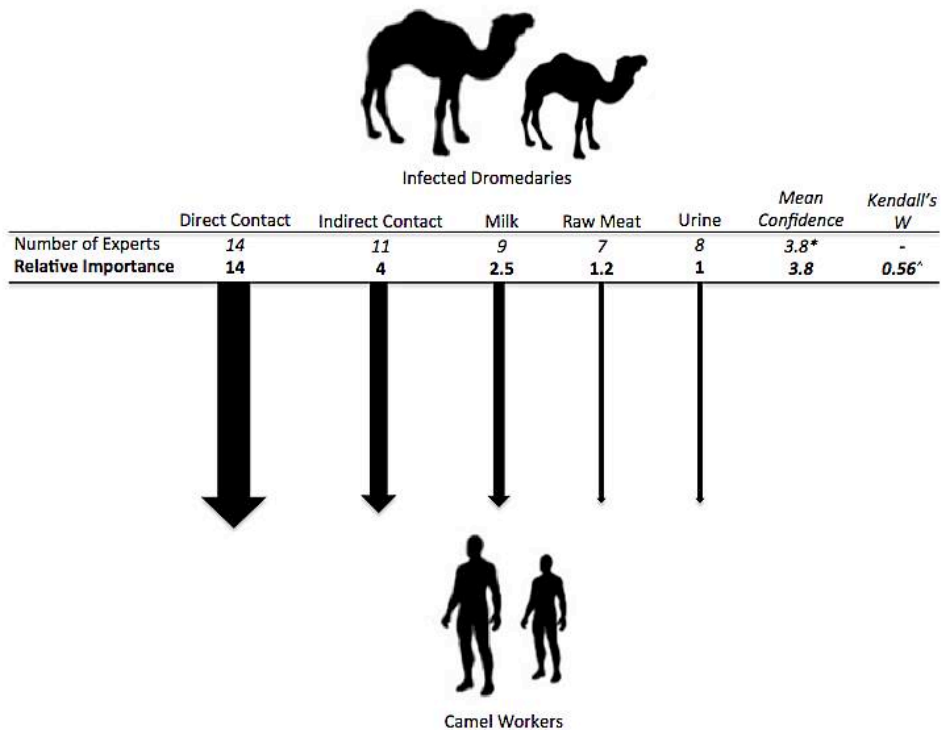


Figure 13: Exposure pathways and relative weights of risk factors for types of transmission between dromedaries and camel workers. $\wedge p < 0.01$. *Mean confidence for choice of risk factors for this question with a scale of confidence between 1 (not confident) and 5 (very confident)

Risk Factors for Symptomatic MERS-CoV Infection in Humans

At least 10 of the 14 experts agreed that older age (>50 years), being immunocompromised, and the amount of viral dose transmitted, increases the chances that infected camel workers or other persons will become symptomatic after MERS-CoV infection. Being immunocompromised was given the highest overall comparative weight as a risk factor, followed by amount of viral dose transmitted. Also, genetic susceptibility and recent occurrence of an epidemic period for another disease (e.g., Influenza) were identified as risky by three and four experts, respectively. Experts had a good level of agreement on their ranking of the selected risk factors (Kendalls $W = 0.61$, $p < 0.01$), and had a mean response certainty of 3.4 and 3.3 (out of 5) for choosing risk factors and the subsequent rankings, respectively.

Responses to Open-Ended Questions on Transmission Dynamics

Experts were asked which factors led to increase viral shedding in MERS-CoV infected dromedaries. The most highly suggested items included: juvenile dromedaries lacking antibody immunity ($n = 4$), immunosuppressive conditions and secondary disease ($n = 4$), animal density ($n = 2$) and stressful environments for the animals (e.g., at slaughterhouses, markets) ($n = 2$). Experts believed factors that may lead to increased or more efficient transmission between MERS-CoV infected dromedaries and humans include repeated close contact with dromedaries with the chance of contact with respiratory secretions ($n = 3$), host susceptibility or immune status ($n = 2$), increased virulence of the virus through genetic recombination or other ($n = 2$), and environmental contamination of camel-visited areas ($n = 2$). Experts were also asked whether or not they thought any other viruses might cross-immunize with MERS-CoV for either dromedaries or humans. Of the 12 experts who answered this question, 3 said “No,” while 6 were unsure or thought this was possible, and 3 experts believed that other coronaviruses might cross-immunize with MERS-CoV.

- **Discussion**

Our results use expert opinion to weigh the different transmission risks of MERS-CoV between animals and from animals to humans. Despite a lack of quantitative data, our results are supported by growing evidence from research published from MERS-CoV affected countries. Risk assessment is a tool that allows for the gathering of accessible data and information (e.g., expert opinion). The preliminary approach proposed in this paper synthesized available evidence regarding primary MERS-CoV transmission to humans. Our results highlight a general consensus in the order/rank of pathways, as well as for potential drivers and risk factors. According to the experts included in our study, dromedaries play a major role in transmission. However, the role of bats could not be ruled out and should be investigated further.

Despite the fact that new research reveals traces of antibodies against MERS-CoV in two livestock handlers in Kenya in 2013/14 (Liljander et al., 2016), it is surprising that no locally acquired primary human cases have been reported where humans and infected dromedary camels are present outside the Arabian Peninsula. Recent workshops on MERS-CoV (Doha in April 2015 and Cairo in May 2015), organized by WHO, FAO, and the World Organisation for Animal Health (OIE), have produced numerous recommendations to improve surveillance and suggest research in animal and human populations (FAO, 2015). One of these recommendations is to investigate whether and why MERS-CoV infections of humans appear not to occur in Africa despite the high levels of infection in dromedaries, and why the virus is apparently absent in camels in Central Asia (dromedary and Bactrian camels).

The exact role of dromedary camels as a potential reservoir for MERS-CoV is also still unclear, and further investigations should be carried out to identify the mechanism of virus transmission and quantify the stability of the virus in various conditions more clearly. MERS-CoV has been detected in the oropharyngeal tract, feces, milk, and meat of dromedaries (Gossner et al., 2014; Hemida, Chu et al., 2014; Reusken, Farag et al., 2014; van Doremalen et al., 2013). However, the modes of transmission are not clearly known. Our experts felt that the transmission risk from consumption of raw

camel products, including milk, meat, and urine is low. It is assumed that the infection from dromedary camels to humans occurs through droplets or contact as high viral loads have been detected in the upper respiratory tract and nasal mucous membrane of dromedaries (Khalafalla et al., 2015). However, milking activities and drinking unpasteurized milk, which is highly prevalent in Saudi Arabia (Faye et al., 2014), are considered as risky for the occurrence of primary cases in human populations. There is no evidence of MERS-CoV in camel meat, and it is known that cooking would kill the virus. One study from Qatar identified MERS-CoV in milk, but it was unclear whether the virus was excreted in the milk or if the milk had been contaminated by traditional milking techniques, which involves calves being used to initiate the milking process (Reusken, Farag et al., 2014).

The role of and the extent to which infected asymptomatic human cases play a role in transmission is unknown. WHO estimates that ~20% of reported MERS cases are asymptomatic (WHO, 2015a), but this estimate is likely underestimated given surveillance focuses on severe cases requiring hospitalization and evidence from serologic studies (WHO, 2015b; Müller et al., 2015). One study documented prolonged shedding of MERS-CoV in an asymptomatic health care worker (Al-Gethamy et al., 2014), which provides evidence that, if not properly isolated, asymptomatic cases in health care settings and in the community could lead to onward transmission. The experts included in this study believe that contact with asymptomatic cases is as important as that with infected dromedaries. Comprehensive testing of contacts of MERS-CoV patients, regardless of the presence of symptoms, is required to evaluate infection between known cases. The role of asymptomatic cases or carriers, if they are indeed infected, also needs careful consideration in the community setting. Not all reported primary cases can be traced back to contact with dromedaries, and it is likely, at least in some cases, that an asymptomatic or mildly symptomatic case may be an intermediary between dromedary contact and a symptomatic human case.

Table 23. Recommended MERS-CoV Studies at the Animal-Human Interface

Studies	Main outputs	Key Strengths	Shortcomings & Constraints
1. Experimental Studies			
1.1 Virology	Virus strains comparisons among animals and humans. Phylogeography	Deciphering of pathways between mammals species	Statistical power: require sufficient and representative strains to be analyzed
1.2 Experimental infections in bats and camels (and other livestock species)	Pathophysiology and clinical outcomes. Immunological response. Virus ecology, virus shedding in animals	Epidemiological parameters for modelling e.g. shedding, viral excretion	Bioethics. Biosecurity. Costly
2. (Empirical) Observational Studies			
2.1 Ecological studies on bats and camels	Roles as reservoirs and/or vectors of MERS-CoV	Identification of drivers of MERS-CoV ecology	Authorization to work on endangered bats. Need efficient non-invasive methods. Devices to follow livestock movements and bats migrations
2.2 Epidemiological studies	Prevalence and incidence in camels/humans. Serological test performance in humans/animals. At-risk behaviors and risk factors for MERS-disease in humans	Cross-sectional and ecological studies, which are relatively simple to be carried out	Costly for case-control and cohort studies
2.3 Sociology and anthropology studies	At-risk human behaviors at individual and community levels	Will feed epidemiological studies and models	Implementation of participatory approaches in pastoral and challenging territories (e.g. low-income countries, remote areas)
2.4 One Health surveillance systems	Follow-up of virus, antibodies, clinical signs in humans and animals	Detection of emergence in humans; Collection of viruses. Infection timeline.	Complex (need agreement among public health and vet services) and costly (need sustainability)
3. Modelling			
3.1 Probabilistic models (e.g. QRA)	At-risk pathways of transmission	Useful for disease management even if all mechanisms are not known	Long and iterative process for QRA. Data and information needed, including experiment data
3.2 Dynamic models (e.g. SIR, IBM, SNA)	Testing hypotheses (simulation) of MERS-CoV transmission. Drawing up the levels of vaccination needed	Deciphering of transmission ways between mammals species	Need data. Complex models required (SIR stratification animal/Human, joint models e.g. SIR and SNA, etc.)
3.3 MCDA	Decision process. Risk mapping for spatialised MCDA	Straightforward to be implemented (literature review and expert opinions)	Model validation (but could be done with Human cases in Arabian peninsula)

Legend: SIR = susceptible infectious recovered compartmental models; IBM = individual-based modelling or multi-agent systems; SNA = social network analysis or contact network analysis; MCDA = multi-criteria decision analysis; QRA = quantitative risk assessment

After 4 years, research on the role of camels and/or other sources of primary transmissions to human is inadequate. So far, most MERS-CoV studies have focused on virological or clinical aspects of the disease. No comprehensive analytical epidemiological studies have yet been carried out in MERS-CoV affected countries. With the exception of one case–control study (Alraddadi et al., 2016) and individual case studies following investigation into single cases, transmission between dromedary herds and between dromedaries and humans has not been well studied. Even these detailed investigations are limited in terms of deciphering the cause-effect relationship. As human cases of MERS are relatively sporadic/rare, case control studies, especially matched case–control studies, can be well adapted during epidemics or outbreaks investigations and must be performed. Cohort studies are the best option in order to compare incidence among exposed (e.g., camel workers, immunocompromised people, etc.) and non-exposed populations. However, conducting cohort studies for rare diseases may be difficult.

In regions without reported human clinical cases of MERS, cross-sectional surveys based on serological investigation in humans and identification and quantification of potential risk factors for infection (behaviors, husbandry, contacts with camels and camel products, etc.) will assist in the suggestion of hypotheses, if human infection is prevalent and statistically exploitable for inference at the population level. Outside of the Middle East, these studies need to be undertaken, especially outside of the Arabian Peninsula and in African countries where MERS-CoV has been detected and/or isolated in dromedaries. Outside of the Arabian Peninsula, a number of joint human/camel serological studies are currently underway in North Africa (Pasteur Institut, Pers Comm) and planned in sub-Saharan African countries (HKU and Cirad, Pers Comm). For instance, in Ethiopia where MERS-CoV strains have been detected in camels (Peiris et al., Pers Comm), studies in at-risk human communities (e.g., nomadic people in close contact with camels, abattoir workers) have to be implemented: both analytical epidemiological studies and surveys on acute febrile illness (Woyessa et al., 2014), including respiratory and other signs could lead to clues about MERS-CoV infection and/or MERS-disease in humans.

At a more global scale, understanding differences in exposures and behaviors of individuals with dromedaries across the Middle East and Africa is likely to explain some of the differences in potential infection risk. For that purpose, “ecological studies” could help to explore diverse drivers of transmission among different environments and societies. However, studies

based on aggregated data are prone to many biases (Lasserre et al., 2000) making it difficult to know if individuals have really been exposed to the risk factor in question. Furthermore, social sciences have to be enlisted in order to puzzle out the relationships between camels and humans. Outputs can serve for epidemiological studies and modeling (e.g., multi-agents systems, see hereunder). Additionally, improved surveillance systems in humans and animals in rural and nomadic areas are required for MERS-CoV considering possible changes of the public health situation due to virus evolution (e.g., toward more pathogenic strains or diffusion of strains from areas with human disease, etc.) over time, modification of camel husbandry, etc. For population-based studies, epidemiology and surveillance, we need to have species-adapted and validated serological tools. Indeed, performances of tests are often lacking and should be assessed using frequentist or Bayesian approaches.

In addition to epidemiological studies, additional data from viral ecology studies among camels and other species, including bats, are required; phylogeography studies of MERS-CoV, and ecological studies on bat species living in the proximity of camels and suspected to play a role in the circulation of the virus, including a better understanding of their home ranges, migration patterns, biology (especially reproduction), roosting sites, and mechanisms of contact with camels are needed. Studies of viral shedding in animals, of virus persistence in different biological specimens of humans and animals, and in the environment under different conditions would help to quantify, or at least help to characterize, potential transmission risks.

The effect of MERS-CoV on camel health is not well documented; is the camel an asymptomatic carrier (reservoir/vector) or can MERS-CoV infection induce mild symptoms and/or pave the way for secondary infections? To address this question, camel studies should focus not only on MERS but also on the diverse etiologies of respiratory syndromes (Wako et al., 2016). This could be significant because, first, if MERS is recognized as a camel disease, more research resources could be allocated, second, super-infections could play a role in MERS-CoV transmission traits. Finally, multi-pathogens studies and multi-disease surveillance in camel populations can improve, through an economy of scale, MERS-CoV detection and the collection of data and metadata. Similarly, health conditions and infectious and parasitic diseases of camels may have an impact on MERS-CoV ecology and/or MERS epidemiological features. Indeed,

immunosuppressive effects of several origins (husbandry and farming conditions, under-nutrition, deficiencies, parasites, co-infections, etc.) could enhance the infectivity of the MERS-CoV.

Considering the recent emergence of MERS-CoV as a zoonotic threat, and the lack of information already quantified on it, we appropriately included a small number of experts in this EOE exercise, but those who had diverse and extensive experience in relevant fields. The questions included in the exercise were feasible for persons who are not accustomed to formal prioritization methods; AHP is known to be adapted for complex information situations, to be intuitively understandable and to allow scientists to score the attributes with minimal confusion. Our study has several limitations. First, it is commonly considered best practice to give a training exercise on EOE methods as well as provide a multi-page literature review on the topic in question to participating experts, prior to administering the questionnaire, however, this was not done here. In this case, experts were provided with a detailed document describing how to fill out the questionnaire, with examples, and were invited to contact the authors if they had any questions or confusion (see link 5 in Appendix 4). It is possible to “calibrate” experts, by including some items in the questionnaire for which a general scientific consensus or quantification already exists; the expert response to these questions can then be matched to the real answer in order to see how close that expert arrives. There was no calibration done in this study, largely due to the fact that there are almost no solidly quantified risks associated with MERS-CoV at present. Experts were weighted instead only on their confidence level for each question answered; however, it is always possible that experts are overconfident, giving scores that do not reflect their real uncertainty on their knowledge of a variable. Linguistic uncertainty in the questionnaire could have led to some bias; experts with varied origins and experience can interpret questions and imagine contexts differently, and this can be exacerbated by ambiguity or lack of specificity in questions. For the aggregation of our results, we used a mathematical approach by combining the weighted estimates of all experts. Another option would have been to use a more inclusive and participatory behavioral approach that would allow experts to revise their answers after seeing those of others and eventually come to a consensus together. However, empirical results have suggested that mathematical methods can outperform behavioral techniques in certain circumstances (Lawrence et al., 1986; Seaver, 1978), and it is also possible that group dynamics could bias estimations of risks toward a more extreme consensus (Plous, 1993). Overall, the experts were more certain in answering the AHP questions, which involved

choosing and ranking risk factors. For all animal–animal or animal–human AHP questions, the experts consistently had mean confidence levels of close to 4 (out of 5), however, when asked to rank risk factors for asymptomatic human–human transmission, the mean confidence level was lower (closer to 3). When estimating minimum, most likely, and maximum transmission risks based on scenarios, the experts had lower overall mean confidence in their answers, with scores of between 2.8 and 3.2 for all estimations. This lower certainty is likely related to the fact that so few transmission risks for MERS have yet been quantified, whereas in choosing and ranking risk factors, there are already strong trends as presented in the literature.

Apart from virological, ecological, and epidemiological approaches, simulation models will allow for the testing of different scenarios of transmission, and this can be compared with reported cases. However, the scarcity of the data at the animal–human interface impedes the use of data-driven models like the stratified (animal–human) susceptible-exposed-infectious-recovered (SEIR) models, contact networks models, etc. Moreover, in order to analyze and simulate the complete pattern of the disease, there is also a need to capture the behaviors of animals and people (Funk et al., 2015). The individual-based model built on multi-agents systems is a computerized system combining multiple interacting agents (e.g., humans, animals) within a given environment (Bradhurst et al., 2016). Such a model could be built in close interaction with stakeholders (farmers, camel workers, etc.) and could drive toward more precise hypotheses about initial transmissions to humans (Amouroux et al., 2008; Macal, 2016).

This EOE study has several limitations but it is a preliminary step for implementing a more comprehensive risk assessment. Risk assessment is a time-consuming and iterative process that needs to be fed by several sources of data, lab experiments and field observations (see Table 23 for a summary of recommended studies). Risk communication, which is part of the risk analysis and closely linked to the risk assessment, is essential, especially considering that MERS is a major public health issue and could have indirect economic and social impacts on the “dromedary world.” The questionable responsibility of dromedaries regarding human MERS-CoV cases could indeed spur inappropriate and overdone control measures. More broadly, this EOE can help in identifying gaps and needs in terms of experimental, field and modeling studies that will give a better understanding of the zoonotic transmission pathways of MERS.

4.3.2 Discussion: EOE for supporting response to emerging disease events

Expert opinion elicitation can be a useful tool in emerging disease outbreaks, specifically to provide rapid guidance for policy action (Russell et al., 2017). In the case of the example provided above on MERS-CoV, the EOE study showed that experts enforced already widely proposed hypotheses on the importance of camels in transmission of the virus, but also demonstrated that some animals and exposures not typically prioritized in recent research deserved further investigations. Whether or not these conclusions will lead to research studies on typically neglected exposure pathways, that would not have been performed otherwise, remains to be seen.

A review by Morgan (2014), describes well how EOE can be either “used or abused”, and describes that when performed well, these studies can make useful inputs into important research questions, but, done poorly they can “lead to useless or even misleading results that lead decision makers astray, alienate experts, and wrongly discredit the entire approach”. The author goes on to describe how the development of questionnaires, interview questions, and other elicitation activities need to be well planned, along with an appropriate choice of experts and method for combining answers or drawing consensus. Although the Delphi method is popular for drawing consensus from expert advice, this technique has also been criticized as being less efficient at actually generating accurate predictions but highly effective at pressuring groups into conformity (Woudenberg, 1991). In Egypt, Cousien and colleagues (2014), used EOE along with the Delphi method to estimate the risks of transition between different stages of HCV-related cirrhosis. They found that the chosen experts had great difficulty in estimating quantitative probabilities, and were not able to reach a consensus. Indeed, for some topics, and some experts, use of EOE techniques and the need for individuals to generate risks, may not be natural or feasible (Morgan, 2014).

In the case of ZIKV-related birth defects, there are many disease parameters that are not yet well understood, some of which may be difficult to address through highly powered studies in a timely manner due to decreasing transmission of the virus. Baud and colleagues (2017) have summarized the key knowledge gaps in a recent review. Some of these, such as “What is the percentage of transmission from infected pregnant women to fetuses?” and “Does Zika virus

infection confer lifelong immunity?”, for which eventual studies may be complex and time consuming, it could be interesting to get initial inputs on the likelihoods of these parameters from persons with related experience. For example, with regards to the first question, although ZIKV as a vertically transmitted agent is novel, other infectious diseases that are transmitted mother-to-child have well-established experts from whom insights can be sought. In terms of the immunity bestowed from an infection with ZIKV, a consensus opinion from experts with extensive experience with other arboviral infections, such as yellow fever, could be solicited. Another interesting question, posed by Baud and colleagues (2017), is likelihood of a re-emergence of ZIKV in Africa and Asia. In this case, an EOE exercise including experts from a range of fields, such as epidemiology, genetics, and vector dynamics, may lead to published and focused recommendations for research in these regions. These can then be used to justify further funding opportunities for these regions where there is a definite risk of neglecting the impact of ZIKV (Meda et al., 2016).

4.4 Concluding remarks

In this thesis manuscript, I have described how, historically, the first epidemiological studies on emerging diseases have led to initial mis-estimations and misinterpretations of important disease parameters. This phenomenon was then supported by my own findings through research done on ZIKV-related birth defects and on the effects of new, highly effective treatments for HCV in Egyptian populations. Finally, I described how some strategies, such as involvement in diverse research networks, protocol standardization, and EOE, might help in focusing hypotheses and generating results that can lead to more rapid consensus estimates. While formal standardization and best practices for specific emerging disease investigations may be subject to delays, transparent and collaborative research will still be able to inform preventive actions in the meantime.

The time that it takes us to come to an agreement on risks related to emerging diseases and events linked to them, is the same time by which exposed populations are highly vulnerable. In the case of ZIKV, exposed women presenting with positive test results during pregnancy may not be provided with an accurate risk of their infant eventually having severe neurological defects; if currently overestimated, women in some countries may seek out unnecessary and unsafe abortions. For persons in remission from liver cancer waiting to be treated to clear their HCV infection, recommending or not the highly effective direct acting antivirals could lead to earlier recurrence with a related increased mortality risk. It is not for ourselves, aiming to publish in high impact journals, to whom we owe the production of collaborative and robust research, but rather to the persons who put their trust in us to inform them on the best probable course of action for their own health.

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3. Publication list

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- **Accepted**

Ali-Eldin Z, Shimakawa Y, Wifi MN, **Funk A**, Zaky S, El-Raey F, Esmat G, Fontanet A, El Kassas M. Risk of hepatitis B reactivation in chronic hepatitis C patients treated with direct-acting antivirals: a cohort study from Egypt and meta-analysis of the published data. *Liver Int.* 2018 May 8. doi: 10.1111/liv.13874

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4. Supplementary material links

1. All supplementary material for article Hoen et al., 2018
https://www.nejm.org/doi/suppl/10.1056/NEJMoa1709481/suppl_file/nejmoa1709481_appendix.pdf
2. Supplementary Figure 1A for article El Kassas et al., 2018
<https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fjvh.12854&file=jvh12854-sup-0001-FigS1a.tif>
3. Supplementary Figure 1B for article El Kassas et al., 2018
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4. Supplement 2 for article El Kassas et al., 2018
<https://onlinelibrary.wiley.com/doi/abs/10.1111/jvh.12854>
5. Questionnaire used in MERS EOE article Funk et al., 2016
<https://www.frontiersin.org/articles/10.3389/fvets.2016.00088/full>
(see 'Open Supplemental Data' button on right hand side of the page)

5. PDFs of published and submitted articles

Articles appear in the order of their appearance in the manuscript, as below:

1. Hoen et al., 2018. Pregnancy outcomes after Zika infection in the French Territories of America. *N Engl J Med*; 378:985-994.
2. Funk A et al., submitted to *Pediatrics* in October 2018. Microcephaly surveillance in Africa and Asia using a -2SD cut-off and international growth charts
3. Funk A et al., submitted to *BMJ* in September 2018. Comparison of the risk of birth defects in live births of pregnant women infected and not infected by Zika virus in Guadeloupe, 2016-2017.
4. Kandeel et al., 2017. The prevalence of hepatitis C virus infection in Egypt 2015: implications for future policy on prevention and treatment. *Liver Int*; 37(1):45-53.
5. El Kassas and Funk A et al., 2018. Increased recurrence rates of hepatocellular carcinoma after DAA therapy in a hepatitis C-infected Egyptian cohort: A comparative analysis. *J Viral Hepat.*;25(6):623-630
6. Ali-Eldin Z et al., 2018. Risk of hepatitis B reactivation in chronic hepatitis C patients treated with direct-acting antivirals: a cohort study from Egypt and meta-analysis of the published data. *Liver Int*; doi: 10.1111/liv.13874
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8. Funk et al., 2016. MERS-CoV at the Animal-Human interface: inputs on exposure pathways from an Expert-Opinion elicitation. *Front Vet Sci.*;3:88. eCollection 2016.

Pregnancy Outcomes after ZIKV Infection in French Territories in the Americas

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ABSTRACT

BACKGROUND

The risk of congenital neurologic defects related to Zika virus (ZIKV) infection has ranged from 6 to 42% in various reports. The aim of this study was to estimate this risk among pregnant women with symptomatic ZIKV infection in French territories in the Americas.

METHODS

From March 2016 through November 2016, we enrolled in this prospective cohort study pregnant women with symptomatic ZIKV infection that was confirmed by polymerase-chain-reaction (PCR) assay. The analysis included all data collected up to April 27, 2017, the date of the last delivery in the cohort.

RESULTS

Among the 555 fetuses and infants in the 546 pregnancies included in the analysis, 28 (5.0%) were not carried to term or were stillborn, and 527 were born alive. Neurologic and ocular defects possibly associated with ZIKV infection were seen in 39 fetuses and infants (7.0%; 95% confidence interval, 5.0 to 9.5); of these, 10 were not carried to term because of termination of pregnancy for medical reasons, 1 was stillborn, and 28 were live-born. Microcephaly (defined as head circumference more than 2 SD below the mean for sex and gestational age) was detected in 32 fetuses and infants (5.8%), of whom 9 (1.6%) had severe microcephaly (more than 3 SD below the mean). Neurologic and ocular defects were more common when ZIKV infection occurred during the first trimester (24 of 189 fetuses and infants [12.7%]) than when it occurred during the second trimester (9 of 252 [3.6%]) or third trimester (6 of 114 [5.3%]) ($P=0.001$).

CONCLUSIONS

Among pregnant women with symptomatic, PCR-confirmed ZIKV infection, birth defects possibly associated with ZIKV infection were present in 7% of fetuses and infants. Defects occurred more frequently in fetuses and infants whose mothers had been infected early in pregnancy. Longer-term follow-up of infants is required to assess any manifestations not detected at birth. (Funded by the French Ministry of Health and others; ClinicalTrials.gov number, NCT02916732.)

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A Quick Take
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IT HAS BEEN RECOGNIZED RECENTLY THAT Zika virus (ZIKV) infection during pregnancy can cause severe birth defects,¹ including microcephaly,² other brain defects,³ and the congenital Zika syndrome.⁴ However, the magnitude of this risk is not clearly defined. It was estimated to be higher than 40% in a prospective observational study in Brazil involving women who had symptomatic ZIKV infection during pregnancy.³ In the U.S. Zika Pregnancy Registry, the estimate was 6% overall and 11% when ZIKV exposure occurred during the first trimester.⁵ The latter estimate has been updated recently to 15%.⁶

The ZIKV epidemic in French territories in the Americas began in early 2016 and presented another opportunity to assess the risk of ZIKV-related congenital neurologic defects in a population of pregnant women living in a region in which a ZIKV outbreak occurred. The centralized antenatal and maternal care facilities enabled enhanced surveillance of all pregnancies during the ZIKV epidemic. We present here the pregnancy outcomes in a cohort of women living in French territories in the Americas (French Guiana, Guadeloupe, and Martinique) who had symptomatic, laboratory-confirmed ZIKV infection during pregnancy.

METHODS

STUDY OVERVIEW

ZIKA-DFA-FE was a cohort study that used four different recruitment methods in an attempt to capture all women whose pregnancies overlapped with the period of the ZIKV epidemic in French territories in the Americas (details are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org). The study received ethics approval from Comité de Protection des Personnes Sud-Ouest et Outre Mer III. All participants provided written informed consent. Full details of the study design can be found in the protocol, available at NEJM.org. The authors vouch for the completeness and accuracy of the data and analyses and for the fidelity of the study to the protocol. The study protocol was prepared with the help of the INSERM Research and Action Targeting Emerging Infectious Disease (REACTing) network.

A key component of the ZIKA-DFA-FE study was the prospective follow-up, until the end of

pregnancy, of women who had clinical symptoms of ZIKV infection during pregnancy. In accordance with the guidelines of the French High Council for Public Health⁷ and the French National College of Gynecologists and Obstetricians⁸ that were released on July 28, 2015, and February 5, 2016, respectively, whenever a pregnant woman presented to the outpatient clinic or emergency department of a participating center with symptoms consistent with acute ZIKV infection, she underwent a clinical examination, and blood and urine specimens were obtained for confirmation of recent ZIKV infection.

ENROLLMENT CRITERIA

Pregnant women with suspected ZIKV infection were referred to the prenatal diagnosis center in each territory, where they underwent testing for ZIKV infection and were invited to participate in the study. Women were included in this analysis if they met all of the following criteria: ongoing pregnancy at any gestational stage; clinical symptoms consistent with acute ZIKV infection, with at least one symptom of pruritic skin rash, fever, conjunctival hyperemia, arthralgia, or myalgia; and laboratory confirmation of recent ZIKV infection, on the basis of a positive result on a reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay performed on a specimen of blood, urine, or both. The date of ZIKV infection was considered to be the date of the first ZIKV-related symptom onset.

COHORT FOLLOW-UP AND END POINTS

Once women were enrolled, they underwent monthly clinical and ultrasonographic examinations until they had a pregnancy outcome. During these follow-up visits, a clinician also inquired about events that may have occurred (e.g., pregnancy complications or treatments received) since the previous visit. If a fetal abnormality was identified during a follow-up ultrasonographic examination, magnetic resonance imaging (MRI) of the fetus was performed, and the woman was followed up monthly with laboratory testing and ultrasonography, as reported elsewhere.⁹⁻¹¹ The end point for each woman enrolled in the study was the pregnancy outcome: delivery of a live-born infant with or without birth defects, miscarriage, termination of pregnancy for medical reasons, or stillbirth.

DATA AND SAMPLE COLLECTION IN MOTHERS

During the enrollment visit, sociodemographic data were collected for each woman. These data included age, ethnic group, residence, level of education, professional activity, and lifestyle factors. Clinical information — including the number of previous pregnancies and live births, history of adverse pregnancy outcomes, pertinent medical history, body-mass index, symptoms of ZIKV infection, gestational age of fetuses, and any clinically relevant medical event during pregnancy — was recorded during this baseline visit, and blood and urine specimens were obtained.

Laboratory tests included RT-PCR for the detection of ZIKV (RealStar Zika Virus RT-PCR Kit 1.0, Altona Diagnostics) in blood, urine, or both at baseline in all women and at the end of pregnancy in the case of fetal death, termination of pregnancy, or stillbirth. In addition, results of TORCH serologic testing^{12,13} (toxoplasmosis, other [syphilis, varicella, parvovirus infection, human immunodeficiency virus infection], rubella, cytomegalovirus infection, and herpes simplex virus infection), which is routinely performed during pregnancy in French territories in the Americas, were recorded. Serologic testing for cytomegalovirus was performed only on an elective basis, when fetal abnormalities were detected.

DATA COLLECTION IN INFANTS

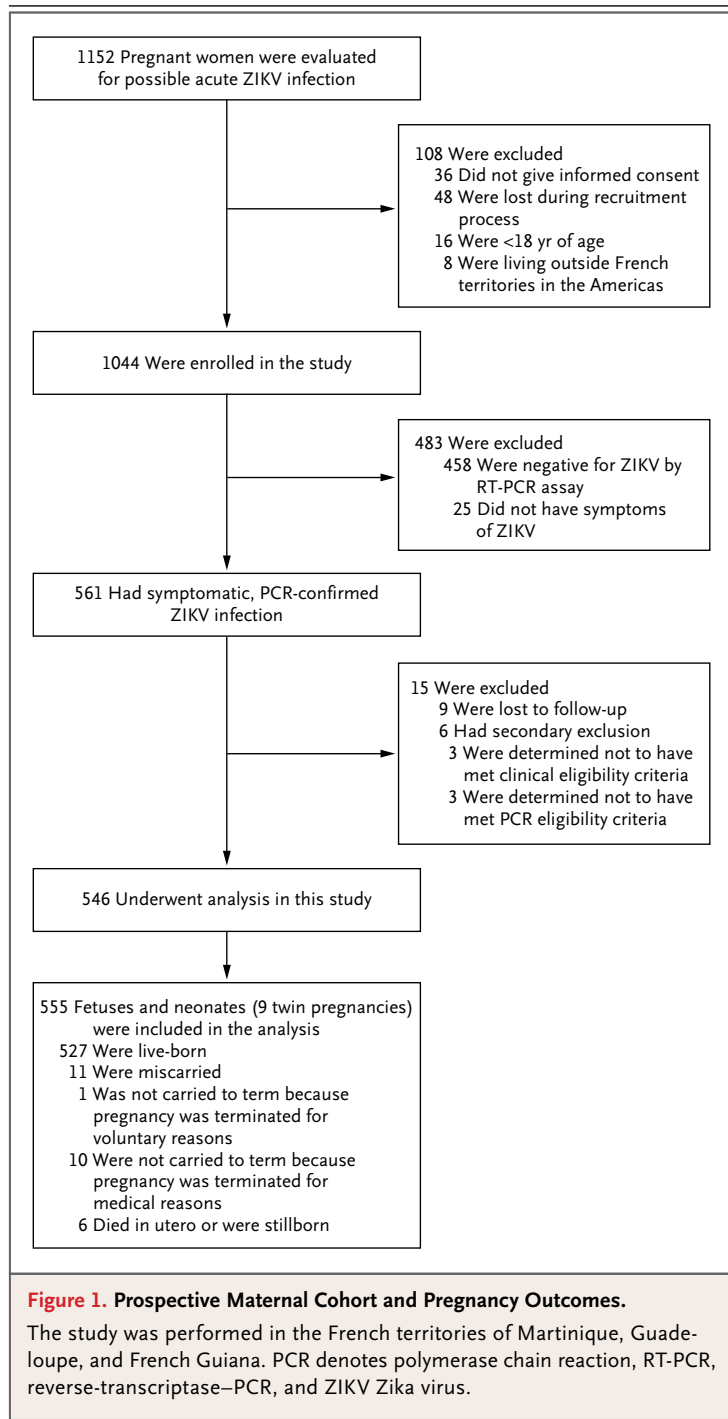
For live-born infants, maternal and cord-blood specimens were obtained on the day of delivery, and serum specimens were frozen. The following information on these infants was also recorded on the day of birth: gestational age, length, weight, head circumference, Apgar score at 5 minutes of life, and the results of a standardized clinical examination.

PREGNANCY OUTCOMES

Pregnancy outcomes included delivery of a live-born child (with or without abnormalities) or pregnancy loss through miscarriage, termination of pregnancy, or stillbirth. For the purpose of comparison with other studies,⁵ miscarriage was defined as intrauterine fetal death that occurred before a gestational age of 20 weeks. Stillbirth was defined as intrauterine fetal death that occurred at or after a gestational age of 20 weeks or intrapartum death during delivery.

Likewise, to allow for comparison with other studies, we summarized our data in two mutually exclusive categories: birth defects possibly associated with ZIKV infection⁵ (brain abnormalities with or without microcephaly regardless of the presence of additional birth defects); and neural-tube defects and other early brain malformations (e.g., anencephaly, acrania, encephalocele, holoprosencephaly, or arhinencephaly), eye abnormalities, and other consequences of central nervous system dysfunction among fetuses and infants who had neither evident brain abnormalities nor microcephaly. Consequences of central nervous system dysfunction included conditions such as arthrogryposis, clubfoot, congenital hip dysplasia, and congenital deafness. In the case of live birth, microcephaly was defined as moderate when the head circumference was between 3 SD and 2 SD below the mean and severe when the head circumference was more than 3 SD below the mean, on the basis of INTERGROWTH-21st standards (<http://intergrowth21.ndog.ox.ac.uk/>) for sex and gestational age. Moderate microcephaly was further defined as proportionate or disproportionate — proportionate if the neonate was small for gestational age and disproportionate if the neonate was not small for gestational age.¹⁴ Small for gestational age was defined as a weight more than 1.28 SD below the mean according to the INTERGROWTH-21st standards for sex and gestational age. In the case of pregnancy loss or termination of pregnancy for medical reasons, autopsy measurements when available and findings from the last ultrasonographic examination were used to assess for microcephaly. When ultrasonographic findings were used instead of autopsy data, microcephaly was defined as a head circumference more than 3 SD below the mean.

In addition, we specified the number of fetuses and infants who had any of the severe neurologic birth defects that are included in the currently proposed definition of the congenital Zika syndrome: severe microcephaly (head circumference more than 3 SD below the mean), brain abnormalities with a specific pattern of damage (e.g., calcifications, ventriculomegaly, or cortical malformations), damage to the back of the eye, joints with limited range of motion (e.g., clubfoot), or hypertonía that restricts body movement (e.g., arthrogryposis).⁴



STATISTICAL ANALYSIS

The analysis included all data collected up to April 27, 2017, the date of the last delivery in the cohort. The percentage of fetuses and infants with birth defects possibly associated with ZIKV infection was estimated according to the trimester in which pregnant women were infected, and we compared these values across the three groups

using Fisher's exact test. Data were analyzed with the use of Stata software, version 13 (StataCorp).

RESULTS

PATIENTS

From March 2, 2016, to November 24, 2016, a total of 1152 pregnant women were evaluated at prenatal diagnosis centers for possible acute ZIKV infection. Of these, 108 were not enrolled in the study because they declined to participate (36 women), were lost to follow-up during the recruitment process (48), were younger than 18 years of age (16), or were living outside French territories in the Americas (8); 458 had a negative result on ZIKV RT-PCR; and 25 had none of the qualifying symptoms. Thus, 561 women with symptomatic, PCR-confirmed, ZIKV infection were included in the analysis in this study. Of these, 6 women (1.1%) were excluded after it was determined that they did not meet specific eligibility criteria regarding clinical or PCR results, and 9 women (1.6%) were lost to follow-up. Among these 9 women, the last available ultrasonographic data were from the third trimester for 5 women and from the second trimester for 4 women; these fetal ultrasonographic examinations were normal. Among the 546 women whose pregnancy outcome was known, there were 9 twin pregnancies. We were therefore able to describe outcomes in 555 fetuses and infants (Fig. 1). The 9 twin pregnancies resulted in 17 live births and 1 miscarried fetus. No abnormalities were detected in any of the live-born infants from twin pregnancies. In the twin pregnancy that resulted in 1 live birth and 1 miscarried fetus, the mother had been infected with ZIKV during the sixth week of pregnancy; the loss of 1 fetus occurred at 10 weeks of gestation, and the other fetus was carried to 41 weeks of gestation and was born healthy, without any abnormalities. Table 1 shows the main characteristics of the 546 women (mean age, 29.7 years) with known pregnancy outcomes, and Table 2 shows the main characteristics of ZIKV infection in these women. Coinfections with TORCH microorganisms are shown in Table 3.

PREGNANCY OUTCOMES

Pregnancy outcomes are shown in Table 4 and in Table S2 in the Supplementary Appendix. Of the 546 women with known outcomes, 185 (33.9%) were infected with ZIKV in the first trimester of

pregnancy, 249 (45.6%) in the second trimester, and 112 (20.5%) in the third trimester. Overall, the mean number of fetal ultrasonographic examinations performed between the date of ZIKV infection and pregnancy outcome was 3.5 when ZIKV infection occurred during the first trimester and 2.2 when it occurred during the second trimester. A total of 28 fetuses (5.0%) were not carried to term or were stillborn; there were 11 miscarriages, 10 terminations of pregnancy for medical reasons, 6 stillbirths, and 1 voluntary abortion. Among the 527 live births, 69 infants (13.1%) were small for gestational age, and 75 infants (14.2%) were delivered through emergency cesarean section. A total of 31 infants (5.9%) were hospitalized immediately after birth, and 7 of these infants (1.3%) were admitted to the neonatal intensive care unit. A total of 8 infants (1.5%) had an Apgar score of less than 7 at 5 minutes after birth. These percentages did not differ by trimester of infection.

Neurologic and ocular abnormalities possibly associated with ZIKV infection were observed in 39 fetuses and infants (7.0%; 95% confidence interval [CI], 5.0 to 9.5): 28 live-born infants, 10 fetuses that were not carried to term because of termination of pregnancy for medical reasons, and 1 stillborn baby. Microcephaly was detected in 32 fetuses and infants (5.8%): 9 cases (1.6%) were severe, 9 (1.6%) were moderate-disproportionate, and 14 (2.5%) were moderate-proportionate. Additional defects were observed in only 1 of the 23 infants with moderate microcephaly — a case involving medical termination of pregnancy in which the fetus had moderate-disproportionate microcephaly. Severe microcephaly or other brain abnormalities included in the current definition of the congenital Zika syndrome were seen in 17 fetuses and infants (3.1%). In 3 of the 527 live births (0.6%), clinical abnormalities other than microcephaly were detected at birth. Neurologic and ocular abnormalities were more common when ZIKV infection had occurred during the first trimester (24 of 189 fetuses and infants [12.7%]) than when it had occurred during the second trimester (9 of 252 [3.6%]) or third trimester (6 of 114 [5.3%]) ($P=0.001$). The same was true for severe microcephaly (3.7%, 0.8%, and 0.0%, respectively; $P=0.02$) and the congenital Zika syndrome (6.9%, 1.2%, and 0.9%, respectively; $P=0.002$). The risk of birth defects possibly associated with ZIKV infection was similar in Guadeloupe and Martinique (7.2% and

Table 1. Characteristics of the Women with Symptomatic, PCR-Confirmed ZIKV Infection.*

Characteristic	Study Cohort (N = 546)
Residence — no. (%)	
French Guiana	24 (4.4)
Guadeloupe	245 (44.9)
Martinique	277 (50.7)
Age — yr	
Mean	29.7±6.2
Range	18–46
Occupation — no. (%)	
Student	23 (4.2)
Artisan, merchant, or business owner	30 (5.5)
Professional	111 (20.3)
Employee	177 (32.4)
Laborer, factory worker, or farmer	5 (0.9)
Unemployed	187 (34.2)
Missing data or declined to respond	13 (2.4)
Medical history — no. (%)	
Arterial hypertension	23 (4.2)
Diabetes	8 (1.5)
Sickle cell disease	4 (0.7)
Previous pregnancies — no. (%)	
0	131 (24.0)
1	153 (28.0)
2	126 (23.1)
≥3	136 (24.9)
Previous adverse pregnancy outcomes — no. (%)	
Congenital abnormalities	6 (1.1)
Stillbirth	10 (1.8)
Termination of pregnancy for medical reasons	10 (1.8)
Mean BMI before pregnancy†	26.1±6.3
Lifestyle practices during this pregnancy — no. (%)	
Alcohol consumption	2 (0.4)
Drug use	6 (1.1)
Current smoker	23 (4.2)
Use of mosquito repellents	445 (81.5)
Use of larvicides	337 (61.7)

* Plus-minus values are means ±SD. Percentages may not total 100 because of rounding. PCR denotes polymerase chain reaction, and ZIKV Zika virus.

† The body-mass index (BMI) is the weight in kilograms divided by the square of the height in meters. Data on BMI are missing for 90 women (16.5%).

7.5%, respectively). The risk of birth defects included in the current definition of the congenital Zika syndrome was also similar in the two terri-

Table 2. Characteristics of Infection in the Women with Symptomatic, PCR-Confirmed ZIKV Infection.

Characteristic	Study Cohort (N = 546)
	no. (%)
Trimester of symptomatic ZIKV infection	
First	185 (33.9)
Second	249 (45.6)
Third	112 (20.5)
Number of symptoms at time of ZIKV diagnosis	
1	66 (12.1)
2	111 (20.3)
3	121 (22.2)
4	95 (17.4)
≥5	153 (28.0)
ZIKV symptoms	
Rash	519 (95.1)
Arthralgia	300 (54.9)
Itching	263 (48.2)
Conjunctival hyperemia	199 (36.4)
Headache	161 (29.5)
Myalgia	128 (23.4)
Fever	123 (22.5)
Limb swelling	104 (19.0)
Pain behind eyes	102 (18.7)
Petechiae	38 (7.0)
Bleeding	1 (0.2)

tories (3.6% and 2.8%, respectively). In French Guiana, where the number of participants (24) was small, no birth defects possibly associated with ZIKV infection were observed. There was no significant association between any potentially identifiable toxic prenatal exposures (i.e., larvicides, repellants, alcohol, tobacco, or illicit drugs) and birth defects.

No fetal abnormality or birth defect was observed in any of the women who had coinfection with syphilis (4 women), human immunodeficiency virus (2), toxoplasmosis (3), or cytomegalovirus (1). A total of 31 women underwent amniocentesis during the course of their pregnancy, with 27 instances of karyotyping and 20 ZIKV RT-PCR assays. All karyotypes were normal except for one pericentric inversion of chromosome 2, and RT-PCR for ZIKV in an amniotic-

fluid specimen was positive in 7 cases. In addition, 6 nonneurologic birth defects (in 1.1% of the fetuses or infants) were detected in this cohort (see Table S3 in the Supplementary Appendix for a detailed description of all birth defects).

DISCUSSION

The main findings of this cohort study are twofold. First, we found a 7.0% overall risk of neurologic and ocular defects possibly associated with ZIKV infection that were evident at birth in the offspring of women in French territories in the Americas who had acute, symptomatic, PCR-confirmed ZIKV infection during pregnancy. The overall risk of evident birth defects included in the current definition of the congenital Zika syndrome was 3.1%, and the overall risk of severe microcephaly was 1.6%. Second, although birth defects could be observed as a consequence of ZIKV infection in any trimester of pregnancy, our data showed that the risk of birth defects and the risk of the congenital Zika syndrome were higher when ZIKV infection occurred early in pregnancy — a finding consistent with previous reports.^{5,15} The risk of birth defects was 12.7% when ZIKV infection occurred in the first trimester, 3.6% when it occurred in the second trimester, and 5.3% when it occurred in the third trimester, and the risk of the congenital Zika syndrome was 6.9%, 1.2%, and 0.9%, respectively.

The percentage of fetuses and infants with neurologic birth defects (7%) in this study is similar to the 6% observed in the cohort of women in the United States⁵ and the 5% reported more recently in the U.S. territories,¹⁵ but it is much lower than the 42% observed in the Brazilian cohort.³ The difference is not attributable to the percentage of infants and fetuses with microcephaly — which is similar in the current study in French territories in the Americas, in the study in the United States, and in the study in Brazil (5.8%, 4.1%, and 3.4%, respectively) — but rather to the percentage with wider neurologic birth defects. The percentage of infants who were small for gestational age was similar in French territories in the Americas and in the Brazilian cohort (13.1% and 9%, respectively), but differences between those two cohorts are apparent when we examine the percentage of infants who were admitted to neonatal intensive

Table 3. Results of ZIKV and TORCH Testing in the 546 Women with Symptomatic, PCR-Confirmed ZIKV Infection.*

Test	Time of ZIKV Infection		
	First Trimester	Second Trimester	Third Trimester
	<i>no. of women (%)</i>		
ZIKV RT-PCR			
Positive results	185 (100.0)	249 (100.0)	112 (100.0)
In blood and urine	121 (65.4)	159 (63.9)	66 (58.9)
In blood only†	40 (21.6)	63 (25.3)	23 (20.5)
In urine only‡	24 (13.0)	27 (10.8)	23 (20.5)
TORCH§			
Positive results on any TORCH test	6 (3.2)	2 (0.8)	2 (1.8)
Toxoplasmosis¶			
Tested	165 (89.2)	235 (94.4)	105 (93.8)
Positive	1 (0.6)	0	2 (1.9)
Syphilis			
Tested	150 (81.1)	206 (82.7)	87 (77.7)
Positive	4 (2.7)	0	0
HIV			
Tested	161 (87.0)	210 (84.3)	97 (86.6)
Positive	1 (0.5)	1 (0.4)	0
Rubella¶			
Tested	152 (82.2)	222 (89.2)	97 (86.6)
Positive	0	0	0
Cytomegalovirus¶			
Tested	20 (10.8)	30 (12.0)	14 (12.5)
Positive	0	1 (3.3)	0

* TORCH includes testing for toxoplasmosis, other (syphilis, varicella, parvovirus infection, human immunodeficiency virus infection), rubella, cytomegalovirus infection, and herpes simplex virus infection. In highly febrile women, a reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay for dengue virus was performed on blood specimens. Of the 267 tests performed, 1 was positive. HIV denotes human immunodeficiency virus.

† Results from tests on urine specimens were negative or unknown, or tests were not performed.

‡ Results from tests on blood specimens were negative or unknown, or tests were not performed.

§ In the subcategories of TORCH, the denominators for the percent of women who tested positive are the numbers of women tested.

¶ Toxoplasmosis, rubella, and cytomegalovirus tests were for IgM antibodies.

care immediately after birth (1.3% in the French territories and 21% in Brazil) and the percentage of infants with abnormal neurologic findings from the clinical examination at birth (0.6% and 26.5%, respectively). The termination of 10 pregnancies for medical reasons in the French territories (as compared with none in Brazil) may have resulted in fewer neurologic abnormalities being detected at birth in the French territories than in Brazil, but this cannot explain the entire difference between the two cohorts. In addition,

the extensive use of MRI in the Brazilian cohort may have resulted in isolated abnormal imaging findings that have not been observed in other studies in which the use of MRI has been less frequent. The clinical implications of these findings in Brazil are not yet known and will be determined only through longer-term follow-up of infants.

The strengths of our study include the size and homogeneity of the cohort of pregnant women who were living in a region in which an outbreak

Table 4. Birth Outcomes and Abnormalities Observed in the Fetuses and Infants.*

Variable	Time of ZIKV Infection			
	First Trimester (N=189)	Second Trimester (N=252)	Third Trimester (N=114)	Total (N=555)
	<i>no. of fetuses or infants (%)</i>			
Birth outcome				
Stillborn or not carried to term	24 (12.7)	4 (1.6)	0	28 (5.0)
Miscarried	11 (5.8)	0	0	11 (2.0)
Not carried to term because of voluntary termination of pregnancy	1 (0.5)	0	0	1 (0.2)
Not carried to term because of termination of pregnancy for medical reasons	9 (4.8)	1 (0.4)	0	10 (1.8)
Stillborn	3 (1.6)	3 (1.2)	0	6 (1.1)
Live-born	165 (87.3)	248 (98.4)	114 (100)	527 (95.0)
No prenatal ultrasonography after ZIKV infection†	13 (6.9)	28 (11.1)	55 (48.2)	96 (17.3)
Abnormalities observed				
Neurologic or ocular birth defects‡	24 (12.7)	9 (3.6)§	6 (5.3)	39 (7.0)
Microcephaly¶	19 (10.1)	8 (3.2)	5 (4.4)	32 (5.8)
Severe	7 (3.7)	2 (0.8)	0	9 (1.6)
Moderate: disproportionate	4 (2.1)	2 (0.8)	3 (2.6)	9 (1.6)
Moderate: proportionate	8 (4.2)	4 (1.6)	2 (1.8)	14 (2.5)
Intracranial calcifications	8 (4.2)	0	0	8 (1.4)
Ventriculomegaly	7 (3.7)	1 (0.4)	0	8 (1.4)
Lissencephaly	2 (1.1)	0	0	2 (0.4)
Other brain abnormalities	8 (4.2)	1 (0.4)	0	9 (1.6)
Neural-tube defects	1 (0.5)	0	0	1 (0.2)
Eye abnormalities	0	0	0	0
Consequences of central nervous system dysfunction	1 (0.5)	0	1 (0.9)	2 (0.4)
Other birth defects	2 (1.1)	3 (1.2)	1 (0.9)	6 (1.1)
Chromosomal defects	0	1 (0.4)	0	1 (0.2)
Skeletal abnormalities	2 (1.1)	1 (0.4)	1 (0.9)	4 (0.7)
Other	0	1 (0.4)	0	1 (0.2)
Congenital Zika syndrome	13 (6.9)	3 (1.2)	1 (0.9)	17 (3.1)

* A total of 546 pregnancies were included in the study; there were 9 twin pregnancies, which brought the total number of fetuses and infants to 555.

† Among the 527 live births, 96 infants (18.2%) did not undergo prenatal ultrasonography after the mother had been infected with ZIKV (7.9% when ZIKV infection occurred in the first trimester, 11.3% when infection occurred in the second trimester, and 48.3% when infection occurred in the third trimester).

‡ These results are possibly associated with ZIKV infection. Fetuses or infants may have had more than one neurologic or ocular defect.

§ The mother of one of these infants also had parvovirus B19 infection.

¶ In the case of live birth, microcephaly was defined as a head circumference more than 2 SD below the mean, on the basis of INTERGROWTH-21st standards (<http://intergrowth21.ndog.ox.ac.uk/>) for sex and gestational age. Microcephaly was considered moderate when the head circumference was between 3 SD and 2 SD below the mean and severe when the head circumference was more than 3 SD below the mean. Moderate microcephaly was further defined as proportionate or disproportionate — proportionate if the neonate was small for gestational age (a weight more than 1.28 SD below the mean for sex and gestational age) and disproportionate if the neonate was not small for gestational age. In the case of pregnancy loss or termination of pregnancy for medical reasons, autopsy measurements when available and findings from the last ultrasonographic examination were used to assess for microcephaly. When ultrasonographic findings were used instead of autopsy data, microcephaly was defined as a head circumference more than 3 SD below the mean.

|| This infant had Down's syndrome with severe microcephaly.

of ZIKV occurred and who were prospectively followed from the time that acute symptoms developed and ZIKV infection was confirmed by PCR until the pregnancy outcome. The diagnosis of ZIKV infection was made on the basis of PCR testing of specimens of blood or urine or both, and the date of infection could be ascertained because the date of symptom onset was close to the date of ZIKV PCR testing. The study was conducted in well-defined geographic areas, and high standards of care were available to all pregnant women living in these territories. Linkage to care of pregnant women with ZIKV infection was effective, with a low rate of loss to follow-up (1.6%). In addition, the results were consistent across the two territories in which the largest numbers of women were recruited (Martinique and Guadeloupe).

We acknowledge that our study has limitations. First, it focused only on pregnant women who had acute, symptomatic ZIKV infection. Although the rate of complications would be expected to be higher among women with symptomatic infection than among those who were asymptomatic, an observational study involving U.S. women did not show any significant difference in the rate of birth defects between the offspring of women who had symptomatic ZIKV infection and the offspring of women who had asymptomatic ZIKV infection during pregnancy.⁵ A recent study also showed no significant association between disease severity or viral load and adverse outcomes.¹⁶ Second, we were not able to fully assess the presence of birth defects possibly associated with ZIKV infection in the case of the 11 miscarriages, 2 of the 6 stillbirths, and the 1 voluntary abortion, as well as in the 96 live-born infants (18.2% of the 527 live-born infants) who did not undergo prenatal ultrasonography after ZIKV infection. Although missing ultrasonographic data may have led to underdiagnosis of ZIKV-related birth defects, it should be noted that in our cohort, only 1 live-born baby had an isolated brain abnormality (ventriculomegaly), detected by MRI, in the absence of clinical abnormalities, after infection during the second trimester of pregnancy. All other live-born babies with ZIKV-related defects had at least one abnormality that would have been detected during the clinical examination at birth (e.g., microcephaly, clubfoot, or a neural-tube defect such as spina bifida). Also, the majority of

missing ultrasonographic data involved pregnancies in which infection occurred during the third trimester, and the consequences of infection during the third trimester were found to be limited in the other infants of the same cohort. Third, our end point was based on fetal ultrasonography and on neonatal clinical examinations and did not include postnatal ultrasonography or specialized hearing and ophthalmologic examinations. We believe that this aspect of the study design had a limited effect on the rate of birth defects that could have been identified if all neonates had undergone brain imaging soon after birth. Indeed, it has been reported that when ZIKV infection occurs during the first trimester or early second trimester, all brain abnormalities can be detected with ultrasonography before 28 weeks of gestation.¹⁰ Another study showed that none of 103 infants with normal prenatal ultrasonographic findings and normal clinical examinations at birth had anomalies attributable to ZIKV when MRI of the head was performed after birth.¹⁷ Still, the absence of microcephaly at birth does not exclude the possibility of delayed development of microcephaly or other ZIKV-related brain and other abnormalities.¹⁸ This information is now being collected as part of a cohort study of the infants (ClinicalTrials.gov number, NCT02810210); the study includes regular clinical examinations with specialized hearing and ophthalmologic testing. Only the longer-term follow-up of the children born to the women in the current study will help identify the full spectrum of ZIKV-related complications.

In conclusion, among pregnant women with PCR-confirmed, symptomatic ZIKV infection, birth defects possibly associated with ZIKV infection were present in 7% of fetuses and infants. Defects were more common among fetuses and infants whose mothers had been infected early in pregnancy. Longer-term follow-up of infants is required to assess for late-onset manifestations not detected at birth.

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APPENDIX

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Comparison of the risk of birth defects in live births of pregnant women infected and not infected by Zika virus in Guadeloupe, 2016-2017

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Manuscripts

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3 **Comparison of the risk of birth defects in live births of pregnant women infected**
4 **and not infected by Zika virus in Guadeloupe, 2016-2017**
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Study design: cross-sectional study, and comparison with previously reported prospective cohort study

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Abstract (220 words)

Objectives: To compare the risk of Zika virus (ZIKV)-related congenital abnormalities among exposed and non-exposed live born infants in French territories in the Americas.

Design: Cross-sectional study of pregnant women and live born infants without exposure to ZIKV, compared to those from a previously reported prospective cohort with confirmed ZIKV exposure. **Setting:** Guadeloupe (France) during the 2016 ZIKV epidemic period. **Participants:** 484 ZIKV negative pregnant women and their 490 live born infants. **Main outcome measures:** ZIKV-related congenital abnormalities as measured by clinical examination at birth and foetal ultrasound imaging during pregnancy **Results:** Of the 490 live born infants without in-utero exposure to ZIKV, 42 infants (8.6%) had indication of neurological abnormalities known as ‘potentially linked to ZIKV infection’; all but one of these were microcephaly without any other brain or clinical abnormalities. When compared to the 241 live born infants from pregnancies with ZIKV exposure, the proportion of such abnormalities was similar (6.6%, $p=0.36$).

Conclusions: Isolated microcephaly and other mild neurological conditions were as prevalent among infants with and without ZIKV exposure in-utero. As a result, when considering 249 fetuses and infants of women with confirmed ZIKV infection in Guadeloupe, only one (0.4%) live born infant and three (1.2%) medically-aborted fetuses had birth defects that could be linked to ZIKV infection. **Trial registration:** This study is registered at ClinicalTrials.gov (NCT02916732).

What is already known

- Based on different studies, the risk of birth defects following ZIKV infection during pregnancy varies from as low as 5 to as high as 46%.
- The most precise estimate obtained through a prospective cohort study with PCR-confirmed diagnosis of infection in the pregnant mother comes from French territories in the Americas, with a risk estimated at 7.0% (95%CI: 5-10%).
- However, in the absence of a control group of ZIKV non-infected pregnant women, it is difficult to attribute all observed birth defects to ZIKV infection.

What this study adds

- This study found no difference in the prevalence at birth of anthropometric and other mild abnormalities that may be potentially associated with ZIKV infection when comparing a group of ZIKV-infected and ZIKV non-infected women.
- In both exposure groups, most abnormalities described at birth were isolated microcephaly, i.e. without any additional clinical or imaging abnormalities.
- This study leads to a reduction of our estimate of the risk of ZIKV-related birth defects among women infected during pregnancy down to 1.6% (95% CI: 0.4% - 4.1%), with no risk related to ZIKV infection in the 3rd trimester of pregnancy.

INTRODUCTION

Since the first evidence surfaced that linked Zika virus (ZIKV) to foetal microcephaly and other brain abnormalities,^{1,2} key research priorities have been to define the range of defects associated with ZIKV infection during pregnancy, as well as to establish the risk of a foetus or infant being affected by them following infection during pregnancy. A multitude of case-series and case-control studies of foetuses and infants exposed to ZIKV in-utero have now been summarized to establish a preliminary definition of Zika congenital syndrome (ZCS) that includes a range of ocular abnormalities and neurological defects, such as microcephaly, structural brain abnormalities (e.g. calcifications, lissencephaly, ventriculomegaly), consequences of central nervous system dysfunction (e.g. congenital contractures, abnormal muscle tension, hearing impairment), swallowing disorders, irritability, seizures, neurodevelopmental issues, and others.³⁻⁹ In addition, studies from Brazil, the United States of America, and the French Territories in the Americas, have attempted to answer the question of how likely it is for the foetus or infant to be affected by any of these negative outcomes after infection during pregnancy, deriving differing risks of 46% (95%CI: 37-56%), 5% (95%CI: 4-6%), and 7% (95%CI: 5-10%), respectively.¹⁰⁻¹² In addition, a retrospective cohort study of 86 pregnancies in Colombia found a 2.4% (95%CI: 0.3-8%) risk of adverse foetal outcomes; all of these were microcephaly cases without indication of brain abnormalities or clinical signs.¹³

For the purpose of determining those birth defects that can actually be attributed to ZIKV in an exposed population, the estimation of the baseline level of birth defects in an appropriate ZIKV non-infected control group is necessary. In Brazil, a prospectively

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3 followed control group of 61 symptomatic pregnant women negative for ZIKV by RT-
4 PCR was compared to 125 symptomatic pregnant women showing evidence of ZIKV
5 infection; they found that total adverse outcomes were significantly less in women
6 without evidence of ZIKV (11.5% versus 46.0%, $p < 0.001$), although for some specific
7 outcome categories, such as foetal demise and proportion of infants with microcephaly,
8 there were no differences between the two groups.¹⁰ Two other prospective studies have
9 used control groups: a prospective ultrasound study examined foetopathy in French
10 Guiana and found central nervous system abnormalities in 9.0% and 4.3% of the ZIKV-
11 exposed and non-exposed foetuses, respectively, and a prospective study of 29 ZIKV
12 exposed pregnancies compared to 518 ZIKV non-exposed in the United States found no
13 difference in outcomes between the two.^{14,15}

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31 In the French Territories in the Americas, in the absence of co-circulation of viral
32 infections with similar symptoms at the time of the Zika epidemic, it was not possible to
33 enrol a non-ZIKV symptomatic control group as done in Brazil. We therefore enrolled
34 women and their live born infants at the time of delivery, known for not being infected
35 with ZIKV during pregnancy. Thus, we were able to compare the proportion of live
36 births with anthropometric abnormalities, including microcephaly and small weight for
37 gestational age, and other neurological abnormalities in the recently published
38 prospective cohort of ZIKV symptomatic women¹² with that of a control group of ZIKV
39 non-infected pregnant women and their live born infants. To optimize the comparability
40 of the two groups and study environment, the analysis was restricted to the women from
41 Guadeloupe where sufficient numbers were available.

METHODS

Study design and participants

The ZIKA-DFA-FE cohort study (a French acronym representing “Zika in the French Territories in the Americas in Pregnant Women”), which has been described elsewhere,¹² used four different recruitment methods in an attempt to capture all women whose pregnancies overlapped with the ZIKV epidemic period, 2016-2017, in the French Territories in the Americas (Guadeloupe, Martinique, French Guyana). These included: enrolment of women presenting to hospital with symptoms consistent with ZIKV infection, enrolment of pregnant women for which a foetal abnormality was detected during routine ultrasound, enrolment of pregnant women for which there was foetal demise, and finally, enrolment of pregnant women not yet included through other methods who presented at participating hospitals to deliver during and up until nine months following the ZIKV epidemic period. Those final women recruited at delivery in Guadeloupe, and their live born infants, were used for the study presented in this article.

Procedures

At the time of admission to hospital for labour, each eligible woman was informed of the study and invited to participate; oral consent was obtained before delivery and written informed consent was obtained before delivery whenever possible or within 24 hours after delivery otherwise. A questionnaire including socio-demographic data, such as age, ethnic origin, residence, education, professional activity, and lifestyle factors, was administered. Clinical information, including the number of previous pregnancies, history

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3 of adverse pregnancy outcomes, significant medical history, symptoms of ZIKV
4 experienced during pregnancy, and any clinically significant medical event during
5 pregnancy, was also collected at this time. From the live born infants of participating
6 women, clinical data such as gestational age, length, weight, and head circumference,
7 APGAR score at 1, 5 and 10 minutes of life were collected on the day of birth, and a
8 standardized clinical examination was carried out in the first four days of life. After
9 enrolment, the medical files of participating women were retrospectively reviewed and
10 data were collected on clinical and ultrasound examinations that had been performed
11 during the pregnancy.
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26 Blood samples were collected from all participating women recruited at the time of
27 delivery. These were tested for serological presence of ZIKV, including immunoglobulin
28 G (IgG), using the Euroimmun ZIKV IgG immunoassay (Euroimmun, Medizinische
29 Labordiagnostika AG, Lübeck, Germany). In addition, some women had had other
30 biological samples collected during the pregnancy that were tested for the presence of
31 ZIKV by serological tests and/or by RT-PCR; when available these results were also
32 taken into account. Results of TORCH serologic tests that were routinely performed
33 during pregnancy in the French territories in the Americas were recorded, which included
34 syphilis, toxoplasmosis, rubella, and HIV.^{16,17} CMV serology was performed only on an
35 elective basis, for high-risk pregnancies or when foetal abnormalities were detected.
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51 *Statistical analysis*

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3 Out of the women enrolled in the study, data was included in this analysis if they gave
4 birth in Guadeloupe, had a confirmed negative IgG serology test for ZIKV from maternal
5 blood taken at time of delivery as well as no other positive ZIKV tests during pregnancy,
6 and if their infant was live born. Evidence (ie. seen and reported by a clinician) or
7 recollection of symptoms evocative of ZIKV infection during the pregnancy was not an
8 exclusion criterion.
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12 Microcephaly was defined as moderate when head circumference was between -2 SD
13 and -3 SD and severe when head circumference was less than -3 SD, based on the
14 INTERGROWTH-21st standards (<http://intergrowth21.ndog.ox.ac.uk/>) for gestational age
15 and sex. Moderate microcephaly was further defined as proportionate or disproportionate
16 depending on whether the neonate was small for gestational age.^{10,12,18} Small for
17 gestational age was defined as having a weight less than -1.28 SD according to the
18 INTERGROWTH-21st standards for gestational age and sex.^{10,12}
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38 In addition to determination of anthropometric abnormalities, we reviewed clinical
39 examination records and ultrasound files of participants for evidence of birth defects that
40 are considered to be potentially associated with ZIKV infection according to the current
41 definition of ZCS, including: structural brain abnormalities (e.g. calcifications,
42 ventriculomegaly, lissencephaly), neural tube defects and other early brain
43 malformations, eye abnormalities, hearing impairment, and other consequences of central
44 nervous system dysfunction (e.g arthrogyrosis, clubfoot).^{6,12} Other birth defects that are
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3 not currently considered to be associated with ZIKV infection during pregnancy (e.g.
4 skeletal and other malformations) were also noted.
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10 Baseline characteristics of women with ZIKV infection during pregnancy were compared
11 to those of women without ZIKV infection during pregnancy using the Student's t test for
12 continuous variables and chi-square or Fisher's exact test for categorical variables. The
13 proportion of infants with anthropometric abnormalities and other birth defects was
14 compared to data of live born infants whose mothers had RT-PCR confirmed ZIKV
15 infection during pregnancy within the same setting and timeframe in Guadeloupe,¹²
16 using Fisher's exact test. Data were analyzed using Stata 13 (StataCorp LP Lakeway, TX,
17 USA).
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30 The ZIKA-DFA-FE study is registered with ClinicalTrials.gov (NCT02916732) and
31 received ethics approval by the Comité de Protection des Personnes Sud-Ouest et
32 Outremer III (CEBH2016/03).
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40 *Patient involvement*

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42 The pregnant women and their infants were not involved in the development of the
43 research question or design of the study. Each woman was notified of her Zika virus
44 status following testing by her attending physician. Women have been given information
45 to able them to contact the study investigators to receive information on the results of the
46 study.
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RESULTS

Participants

Of 1484 women enrolled at delivery in Guadeloupe, 1088 had available ZIKV test results. Of these, 588 showed evidence of ZIKV infection through either serological or RT-PCR testing, 16 had negative RT-PCR results but indeterminate ZIKV serological tests, and 484 were confirmed negative by serological tests at the time of delivery with no other positive test seen during pregnancy. Of these 484 ZIKV non-infected women, 6 had twin pregnancies; 490 live born infants were therefore considered in this analysis. In the previously published prospective cohort by Hoen et al,¹² of the 254 pregnant women in Guadeloupe who had symptomatic RT-PCR confirmed ZIKV infection during pregnancy and were enrolled into the prospective cohort, nine pregnancies were excluded either because of loss to follow-up (n=5) or for mistaken inclusion in the cohort (n=4). Of the remaining 245 pregnancies, there were four sets of twins, and eight cases of foetal demise, including: two miscarriages and three stillbirths all without evidence of neurological birth defects, and three cases of medical abortion all with evidence of neurological abnormalities potentially linked to ZIKV infection. The lack of neurological birth defects was confirmed via autopsy for one of the two miscarriages, and all three of the stillborn infants. Therefore, we were able to compare 490 ZIKV-unexposed live born infants with 241 ZIKV exposed live born infants (with ZIKV exposure confirmed by RT-PCR), all born to mothers living in Guadeloupe during the Zika outbreak. *See Figure 1.*

The mean age of ZIKV non-infected women was 30.7 years (SD=6.4), and that of ZIKV infected women was 30.0 years (SD=6.3). There was a higher proportion of reported

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3 smoking in the ZIKV-infected cohort during pregnancy compared to the ZIKV non-
4 infected: 4.2% (95%CI: 2.0-7.6%) versus 0.6% (95%CI: 0.4-1.8%). There were also
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6 more unemployed women in the ZIKV non-infected group compared to the ZIKV-
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8 infected one: 48.1% (95%CI: 43.3-52.4) versus 34.6% (95%CI: 28.6-41.0). *See Table 1.*
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12 13 14 15 *Anthropometric and other birth defects in live born infants*

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17 Of the 490 live born infants of women without ZIKV infection during pregnancy, 66
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19 (13.5%, 95%CI: 10.6-16.8) were small for gestational age, and 41 (8.4%, 95%CI: 6.1-
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21 11.2) had microcephaly. One of these moderate microcephaly cases had a possible
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23 genetic aetiology (Adams Oliver syndrome). Of the remaining infants with microcephaly,
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25 29 (5.9%, 95%CI: 4.0-8.4%) had either moderate-proportionate or moderate-
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27 disproportionate microcephaly with no other structural brain or clinical abnormalities.
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29 Eleven (2.2%, 95%CI: 1.1-4.0) infants had severe microcephaly, seven of which were
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31 proportionate. One infant (0.2%, 95%CI: 0.01-1.1) had an abnormality that could be a
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33 consequence of CNS dysfunction, which was clubfoot. Five infants (1.0%, 95CI: 0.3-
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35 2.4) had skeletal or other abnormalities that are not currently classified as potentially
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37 linked to ZIKV infection, including: skeletal abnormalities of the fingers or toes (n=2)
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39 anal imperforation (n=1), and urinary tract abnormalities (n=2). Besides two small-for-
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41 gestational-age infants whose mothers were HIV-positive, there were no other
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43 abnormalities (including microcephaly) identified in the remaining four infants of
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45 TORCH-positive mothers. *See Table 2.*
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3 There were no significant differences in the anthropometric or other birth defects in live
4 born infants whose mothers had a symptomatic PCR-confirmed ZIKV infection during
5 pregnancy compared to those whose mothers had no evidence of prior ZIKV infection at
6 the time of delivery in Guadeloupe. In two categories, 'neurological or ocular
7 abnormalities without microcephaly' and 'neural tube defects', there were no cases seen
8 in live born infants of women without ZIKV infection during pregnancy, and one case
9 each in infants born to women infected with ZIKV during pregnancy. *See Table 3.*

21 **DISCUSSION**

22 We found no statistically significant difference in the risk of neurological birth defects in
23 live born infants of ZIKV infected and non-infected women in Guadeloupe during the
24 epidemic period in 2016-2017. These findings have important implications for the
25 estimation of the number of congenital birth defects that can be attributed to ZIKV when
26 the mother is infected during pregnancy. Originally, through a prospective cohort of 249
27 RT-PCR confirmed ZIKV infected pregnant women in Guadeloupe, we reported a total
28 of 18 (7.2%) neurological or ocular birth defects that were potentially linked to ZIKV
29 infection; 13 (72%) of these were isolated anthropometric abnormalities (i.e.
30 microcephaly) or isolated mild CNS dysfunction defects (i.e. clubfoot) in live born
31 infants,¹² which on the basis of the current study cannot be linked to ZIKV exposure. In
32 live born infants, we are left then only with two severe neurological abnormalities (i.e.
33 ventriculomegaly and spina bifida) that have no known alternative etiologies; this is,
34 however, reduced to one abnormality when we remove the neural tube defect (ie. spina
35 bifida), which is no longer considered as linked to ZIKV infection.^{19,20} As our ZIKV non-
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3 exposed study looked back retrospectively from the time of delivery, we are unable to
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5 compare the proportion of foetal demise with that of the full 249 ZIKV-exposed fetuses
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7 in the prospective cohort. However, the two (0.5%) miscarriages and three (1.2%)
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9 stillbirths of the ZIKV-exposed cohort are within the generally expected historical
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11 range,^{20,21} and had no evidence of neurological abnormalities during autopsy for the four
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13 that could be examined. There were three (1.2%) further cases of foetal demise in the
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15 prospective cohort, which were all medically indicated terminations of pregnancy due to
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17 ultrasound detection of severe structural brain abnormalities with microcephaly.¹² If
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19 combining the severe neurological abnormalities in fetuses from medically terminated
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21 pregnancies (n=3) and live born infants (n=1) our new adjusted estimate of birth defects
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23 that could be attributed to ZIKV exposure in Guadeloupe would be 1.6% (95%CI: 0.4-
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25 4.1%). See Figure 2. This would translate to a 4.1% (95%CI: 0.9-11.5%), 0.8% (95%CI:
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27 0.02-4.6%), and 0% (one-sided 97.5%CI:0-6.3%) risk of birth defects per first, second,
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29 and third trimester, respectively.
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38 This is the largest study of ZIKV non-infected women from a defined epidemic region
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40 that has been used as a comparative control group against ZIKV-infected pregnant
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42 women followed up during pregnancy. This study was conducted in a resource-rich
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44 setting where the standard of care for pregnant women is high. The exposure statuses of
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46 each of the two groups included in this study were well defined. The ZIKV infected
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48 women from Guadeloupe were confirmed via RT-PCR within days of infection,¹² and the
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50 ZIKV non-infected group was defined so based on the absence of IgG at the time of
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52 delivery. Recent studies support a rapid appearance of ZIKV IgG after infection; it was
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3 100% detectable within 11 days following infection for 15 subjects in Guadeloupe and
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5 within 26 days following infection for 29 returned travelers to the United States of
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7 America.^{23,24} The Guadeloupe study also showed consistently positive IgG results
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9 throughout follow-up for all subjects, which was up to at least 120 days for around 90%
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11 of subjects included. This evidence suggests that subjects infected with ZIKV, even early
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13 in pregnancy, would still have detectable IgG at delivery and would have been excluded
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15 from our ZIKV non-infected control group.
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22 This study has several limitations. As we had no directly comparable prospective cohort
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24 to that of recently published ZIKV confirmed infected women, we used a group of ZIKV
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26 non-infected women delivering at the same hospitals and in the same time period. The
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28 prevalence of TORCH infections did not differ significantly between the two groups and
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30 in terms of most baseline characteristics, the two groups were similar, although ZIKV
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32 non-infected women were more likely to be unemployed, and ZIKV infected women
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34 were more likely to report smoking during pregnancy. This may reflect recall ability and
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36 employment situation differences according to the timing of data collection, as ZIKV
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38 non-infected women were all recruited at the time of delivery and ZIKV infected women
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40 were recruited at various earlier time points during their pregnancy. Furthermore, the
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42 quality of follow-up and collection of data on the course of pregnancy was likely of a
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44 higher quality in the ZIKV-exposed group as this was a prospective cohort where women
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46 had been symptomatic during pregnancy. Highlighting this is the fact that the study team
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48 was able to retrieve ultrasound records for 88.4% of ZIKV infected women and only
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50 51.6% for ZIKV non-infected women. However, such a difference would only lead to an
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3 underestimation of birth defects in the ZIKV non-infected group. Furthermore, the
4 completeness of data at the time of delivery for live births, which was used to determine
5 anthropometric and clinically apparent abnormalities, was very high in both ZIKV-
6 exposed (97·9%) and non-exposed (98·8%) infants. Recruitment of ZIKV non-exposed
7 pregnant women at delivery also prevented us from determining the number of expected
8 miscarriages, stillbirths, and abortions that may occur in the ‘baseline’ population, but
9 this also indicates a potential under rather than over-estimation of birth defects in this
10 group.
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24 Most of the originally reported ‘potentially linked to ZIKV’ abnormalities seen in both
25 the exposed and non-exposed pregnancies of our study represent identification of
26 microcephaly in live births; these cases were defined based only on anthropometric
27 measurements, with known clinical and radiological findings for each infant being
28 normal. This diagnostic approach to microcephaly, which does not require clinician
29 judgment on the appearance of microcephaly, but relies solely on the comparison of a
30 head circumference measurement against a normalized birth curve, has been used in all of
31 the cohort studies describing the risk of birth defects following maternal ZIKV exposure
32 during pregnancy, to date.¹⁰⁻¹⁵ However, defining microcephaly based on ‘metrics’ does
33 not reflect the real-life clinical diagnosis of this condition, and can lead to a false surge in
34 cases if applied to an entire population for surveillance purposes.²⁵ Registries using more
35 stringent definitions (e.g. -3SD) and/or clinician specific criteria indicate that true
36 disease-related microcephaly is very rare; the European Surveillance of Congenital
37 Anomalies (EUROCAT) recently estimated the prevalence of microcephaly in Europe to
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3 be 1.53 per 10,000 births (~0.02%) with data from 2012-2016.²⁶ However, as infant
4 growth is approximately normally distributed, the INTERGROWTH-21st study itself
5 prescribes that approximately 2% and 0.1% of healthy infants should have a head
6 circumference at birth that falls below -2 and -3SD, respectively.²⁷ Corroborating this, a
7 recent study applying this definition of microcephaly to birth cohorts from two Brazilian
8 cities, Ribeirão Preto and São Luís, estimated the baseline prevalence of microcephaly in
9 2010 to be of 2.5% and 3.5%, respectively.²⁸ Our own estimates of the prevalence of
10 moderate and severe microcephaly and small weight for gestational age in ZIKV exposed
11 infants was similar to that which the INTERGROWTH-21st standards prescribes.
12 However, in our larger ZIKV non-infected control group, our estimates for each of these
13 anthropometric abnormalities, while not different from those in the ZIKV-exposed
14 cohort, were higher than what is prescribed by the INTERGROWTH-21st growth
15 standards. This may be due to a 'non-perfect-fit' of the Guadeloupien population to this
16 international pooled growth standard. Other authors,²⁹⁻³¹ as well as the
17 INTERGROWTH-21st study themselves,²⁷ have noted varying levels of fit for individual
18 populations when compared to this pooled standard.
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42 This study highlights the importance of a control group to estimate the baseline risk of
43 anthropometric and other birth defects when determining the risk of severe congenital
44 abnormalities that can be attributed to a given infection during pregnancy. This is
45 particularly true for anthropometric measurements where regional variations may
46 exist.^{27,29-31} Pre-Zika microcephaly baseline prevalence estimates are increasingly being
47 reported.^{25,27,32-34} While such reports can be used to give general clues as to the
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3 magnitude of birth defects seen through cohort studies that can be attributed to ZIKV
4 infection, the definition of microcephaly and Congenital Zika Syndrome used, and
5 whether or not clinician expertise was considered, will be key to their interpretation. As
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7 with other congenital infections that cause neurological abnormalities, such as
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9 cytomegalovirus and rubella,^{35,36} longer term studies that postnatally follow-up infants
10 that are exposed to ZIKV in-utero but who are apparently healthy at birth are needed in
11 order to understand the true overall risk of defects. However, in terms of the risk of
12 immediate severe congenital defects that are potentially linked to ZIKV infection during
13 pregnancy, and that may impact pregnancy outcome, our findings have further
14 diminished our own estimate for pregnant women in Guadeloupe from 7.2% to 1.6%
15 overall. Communication of the most possibly accurate estimate of the risk of severe birth
16 defects linked to this infectious exposure, as well as the likelihood that such
17 abnormalities will be detected early in pregnancy, will have an important influence on the
18 family planning decisions of pregnant women with ZIKV-positive test results.
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38 **Data sharing:** All data is shared with the ZIKAlliance consortium project.
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15 transparent account of the study being reported; that no important aspects of the study
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TABLES

Table 1: Baseline characteristics of ZIKV non-infected and infected¹² women from Guadeloupe who delivered live born infants

Characteristic	ZIKV non-infected (N=484)	ZIKV infected (N=237)
Age — yr (mean and range)	30·7 (18-46)	30·0 (18-46)
<i>Missing</i>	0	0
Occupation — no. (%)		
Student	13 (2·7)	6 (2·5)
Artisan, merchant, or business owner	14 (2·9)	17 (7·2)
Professional	75 (15·5)	39 (16·5)
Employee	148 (30·4)	91 (38·4)
Laborer, factory worker, or farmer	1 (0·2)	1 (0·4)
Unemployed	233 (48·1)*	82 (34·6)*
<i>Missing data or declined to respond</i>	0	1 (0·4)
Medical history — no. (%)		
Arterial hypertension	12 (2·5)	7 (3·0)
Diabetes	12 (2·5)	4 (1·7)
Sickle cell disease	7 (1·5)	2 (0·8)
Previous pregnancies — no. (%)		
0	110 (22·7)	65 (27·4)
1	133 (27·5)	57 (24·1)
2	109 (22·5)	52 (21·9)
≥3	130 (26·9)	63 (26·6)
<i>Missing</i>	2 (0·4)	0
Previous adverse pregnancy outcomes — no. (%)		
Congenital abnormalities	2 (0·4)	3 (1·3)
Stillbirth	6 (1·2)	2 (0·8)
Termination of pregnancy for medical reasons	4 (0·8)	4 (1·7)
Lifestyle practices during this pregnancy — no. (%)		
Alcohol consumption	0	0
Drug use	1 (0·2)	2 (0·8)
Current smoker	3 (0·6)*	10 (4·2)*

*Comparison between Zika non-infected and infected women with p=0.001

Table 2: TORCH results in ZIKV non-infected and ZIKV-infected¹² women giving birth in Guadeloupe during the ZIKV epidemic period 2016-2017

		ZIKV non-infected (N=484)	ZIKV infected (N=237)
Positive results on any TORCH test		6 (1·2)	5 (2·1)
Toxoplasmosis	Tested	468 (96·9)	219 (92·4)
	Positive	3 (1·0)	0
Syphilis	Tested	249 (51·6)	184 (77·6)
	Positive	0	2 (0·8)
HIV	Tested	449 (93·0)	188 (79·3)
	Positive	3 (0·6)	2 (0·8)
Rubella	Tested	464 (96·1)	199 (84·0)
	Positive	0	0
Cytomegalovirus	Tested	17 (3·5)	36 (15·2)
	Positive	0	1 (0·4)

Table 3: Abnormalities in *live births* of ZIKV non-infected and infected¹² women in Guadeloupe during the epidemic period in 2016-2017

	ZIKV non-infected (N=490)	ZIKV infected (N=241)
Any neurological or ocular abnormalities	42 (8·6)	16 (6·6)
Microcephaly (<-2SD)	41 (8·4)	12 (5·0)
<i>Severe microcephaly alone</i>	11 (2·2)	1 (0·4)
<i>Moderate-disproportionate alone</i>	10 (2·0)	6 (2·5)
<i>Moderate-proportionate alone</i>	19 (3·9)	4 (1·7)
<i>Severe or moderate microcephaly with other neurological abnormalities</i>	0	0
<i>Severe or moderate microcephaly with a genetic or chromosomal syndrome</i>	1 (0·2)	1 (0·4)
<i>Missing</i>	6 (1·2)	5 (2·1)
Structural brain abnormalities	0	1 (0·4)
Ocular abnormalities	0	0
Neural tube defects	0	1 (0·4)
Consequences of CNS dysfunction	1 (0·2)	2 (0·8) [^]
Other abnormalities	5 (1·0)	2 (0·8)
Skeletal abnormalities	2 (0·4)	2 (0·8) [^]
Other	3 (0·6)	0
Small for gestational age (weight <-1.28 SD) <i>(with or without any of the above abnormalities)</i>	66 (13·5)	33 (13·7)
<i>Missing</i>	1 (0·2)	3 (1·2)

[^] One infant represented in each category as they had both club-foot and polydactyly. **Note:** No significantly different values

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FIGURE LEGENDS

Figure 1: Flow chart of inclusion of ZIKV un-exposed and exposed pregnant women for inclusion of live births in this analysis

Figure 2: Determination of excess risk of birth defects associated with ZIKV infection, based on autopsy findings and comparison with the ZIKV non-infected group

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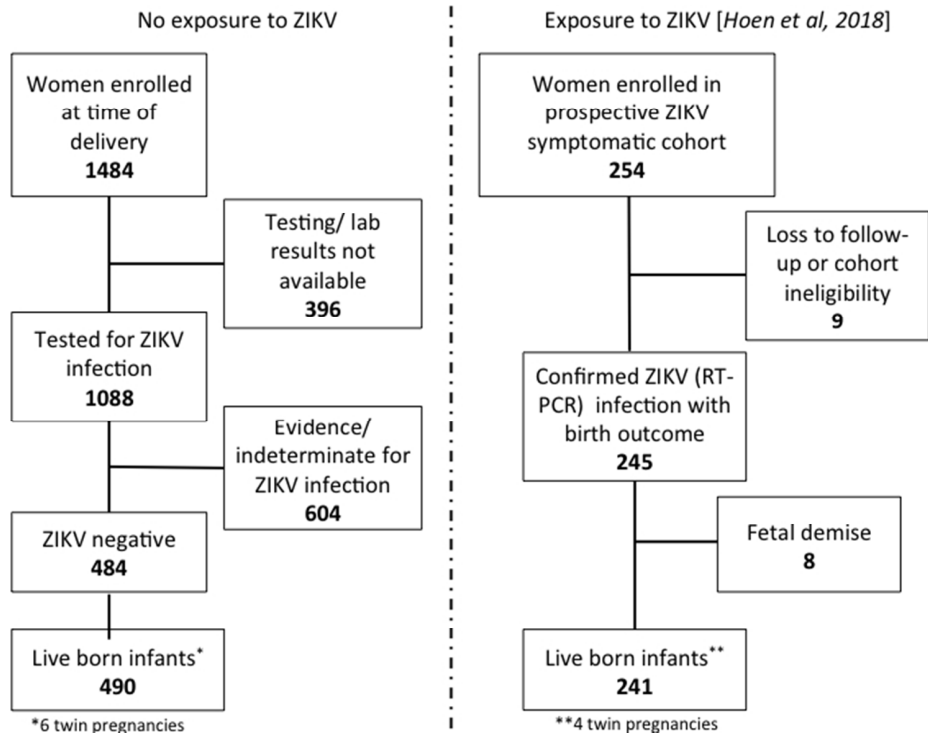


Figure 1: Flow chart of inclusion of ZIKV un-exposed and exposed pregnant women for inclusion of live births in this analysis

254x190mm (72 x 72 DPI)

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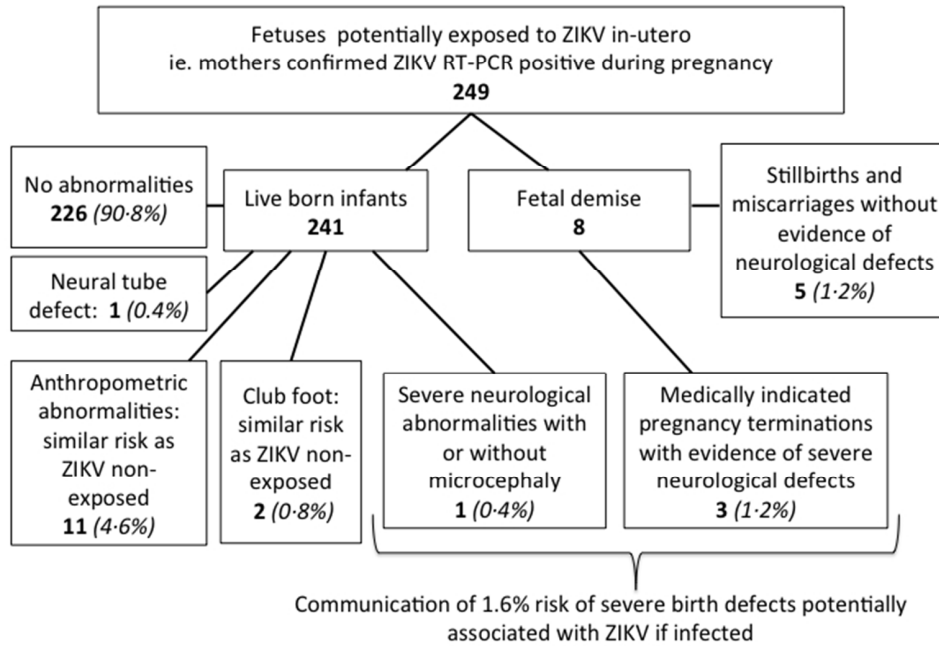


Figure 2: Determination of excess risk of birth defects associated with ZIKV infection, based on autopsy findings and comparison with the ZIKV non-infected group

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PEDIATRICS[®]

Microcephaly surveillance in Africa and Asia using a -2SD cut-off and international growth charts

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3 **Microcephaly surveillance in Africa and Asia using a -2SD cut-off and international growth**
4 **charts**
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37 to disclose
38

39 **Abbreviations:** CDC - Centres for Disease Control and Prevention; ECLAMC - Latin American
40 Collaborative Study of Congenital Malformations; EUROCAT - European Surveillance of
41 Congenital Anomalies; HCZ – head circumference Z score; INTERGROWTH-21st –
42 International Fetal and Newborn Growth Consortium for the 21st Century; NICHD – National
43 Institute of Child Health and Human Development; SD - standard deviations; WHO - World
44 Health Organisation; ZIKV – Zika virus;
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46 **Table of contents summary:** The proportion of live births with microcephaly from four cities in
47 sub-Saharan Africa and Asia varied greatly using the INTERGROWTH-21st growth charts and a
48 -2SD cut-off.
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50 51 **WHAT'S KNOWN ON THIS SUBJECT?** 52

53 In sub-Saharan African and Asian regions suitable for Zika transmission, little information on the
54 baseline prevalence of microcephaly in live born infants is available. However, the prevalence in
55 Europe and the United States was recently estimated at less than 0.1%.
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WHAT THIS STUDY ADDS?

A -2SD cut-off with pooled international growth standards classifies many infants (3 to 35%) as having microcephaly in sub-Saharan Africa and Asia. Consideration of infant body size and regional growth differences are needed in the microcephaly definition used for surveillance.

Contributors Statement: Miss Funk conceptualized and designed the study, analysed the data, drafted the initial manuscript, and reviewed and revised the manuscript. Drs Jayratne, Tejiokem, Qiu, and Soumahoro conceptualized and designed the study, coordinated data collection, and reviewed and revised the manuscript. Drs Saman Kumara, Njom Nlend, Konan-Ble, Gamaathige, Oura, Tano, and Lucas, as well as Miss Xiao and Mr He, supervised data collection and performed data entry and quality checks. Dr Kunkel analysed the data and participated in the drafting, review, and revision of the manuscript. Prof Leung conceptualized and designed the study and critically reviewed the manuscript for important intellectual content. Prof Fontanet conceptualized and designed the study, participated in the drafting of the manuscript, and critically reviewed and revised the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

ABSTRACT (250 words)

Background and Objectives: Little data on microcephaly baseline estimates exists in sub-Saharan Africa and Asia. We aimed to estimate the proportion of live births with microcephaly from four cities in sub-Saharan Africa and Asia using the INTERGROWTH-21st growth charts and a -2 standard deviation (SD) cut-off.

Methods: Large maternities in Yaoundé (Cameroon), Abidjan (Ivory Coast), Colombo (Sri Lanka) and Guangzhou (China) collected data for live born infants through retrospective and/or prospective surveillance, as feasible in each setting. Head circumference, gestational age, and sex were compared to the INTERGROWTH-21st standards to obtain head circumference Z-scores (HCZ) for each infant. Moderate and severe microcephaly were defined as between -2 and -3SD, and less than -3SD, respectively.

Results: Sufficient data for 19914 live births across all study sites were analysed. The proportion of infants with microcephaly, according to the definition, was between 2.9% and 34.8% through retrospective surveillance in three countries. Prospective surveillance in Guangzhou, Colombo, and Abidjan, labelled 3.8%, 7.5%, and 18.6% of infants as having microcephaly, respectively. The retrospective data from Abidjan differed the most from the INTERGROWTH-21st distribution, with a shifted mean HCZ -1.41 SD, whereas the HCZ for infants from Guangzhou, following prospective surveillance, differed the least (mean HCZ -0.02SD).

Conclusions. A cut-off of -2SD will most likely lead to labelling large numbers of healthy babies as having microcephaly, with important regional variations. For the purpose of microcephaly surveillance, we suggest using regional growth charts, increasing consideration of infant body size, and defining the cut-off at -3SD (severe microcephaly).

INTRODUCTION

Microcephaly is a congenital anomaly that can appear in neonates following abnormal brain development due to infectious, genetic or environmental causes.^{1,2} This birth defect has attracted increased attention recently, following the rapid spread of Zika Virus (ZIKV) throughout the Americas in 2015-2016 and the realisation that microcephaly due to abnormal brain development can occur in the infants of women infected with ZIKV during pregnancy.³ Although few recent cases have been reported so far outside of the South-Pacific and Americas, a large proportion of sub-Saharan Africa and Asia is equally suitable for ZIKV transmission.⁴ The lack of understanding of the effects of current or impending transmission of ZIKV in these latter regions further emphasizes the need for strong surveillance systems and clear case definitions for microcephaly.⁵

From 2003 to 2012, the European Surveillance of Congenital Anomalies (EUROCAT) registry estimated the prevalence of microcephaly in Europe to be 1.5 per 10,000 births (~0.02%).⁶ In the United States, between 2009 and 2012, pooled results from 30 birth defects registries, estimated the prevalence of microcephaly to be 9 per 10,000 births (0.09%).⁷ To our knowledge, there are no recent microcephaly registries or causality profiles for most regions of sub-Saharan Africa and Asia. In these regions we may expect different figures than in Europe due to environmental factors, differences in nutrition, and increased circulation of and lower vaccine coverage for infectious agents such as rubella. For the purpose of screening live neonates born to women at risk of ZIKV exposure during pregnancy, at the beginning of the recent outbreak in the Americas,

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3 the Centres for Disease Control and Prevention (CDC) and the World Health Organisation
4 (WHO) recommended defining moderate and severe microcephaly as a head size of less than -
5 2SD (or < 3rd percentile) and -3SD, respectively, for gestational age and sex using the
6 INTERGROWTH-21st or WHO growth standards.^{8,9} The same definition has been used by many
7 of the key cohort and case-control studies defining Zika related birth defects,^{3, 10-12} as well as in
8 some recent estimations of the pre-Zika (ie. <2015) microcephaly prevalence in South
9 America.¹³⁻¹⁵ Therefore, we sought to understand the distribution of newborn head sizes, and in
10 particular the prevalence of microcephaly at birth, that could be expected using data from real-life
11 settings in sub-Saharan Africa and Asia, according to a -2SD cut-off with a pooled international
12 growth standard. Such results could be used as a baseline reference in these regions, for
13 interpretation of disease-related microcephaly surveillance following a Zika epidemic.
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31 **METHODS**

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34 Eight hospitals across four countries, two in sub-Saharan Africa and two in Asia, participated:
35 Essos Hospital Maternity in Yaoundé, Cameroon; the General Hospitals of Yopougon-Attie and
36 Abobo-Sud in Abidjan, Ivory Coast; Guangzhou Women and Children's Medical Center,
37 Guangzhou Huadu Women and Children Health Care Hospital and Guangzhou Liwan Women
38 and Children Health Care Hospital; the Castle Street and De Soyza Hospitals for Women in
39 Colombo, Sri Lanka. These are large hospitals in urban areas suitable for ZIKV transmission,⁴
40 and have many births per day (mean: 22, range: 7-55) in relation to other regional hospitals. Most
41 regions in which the study sites are situated would be considered low-income, besides
42 Guangzhou.
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3 Data from birth registers was collected retrospectively, with an aim to record data for at least
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5 3000 births consecutively moving back from the day at which the collection started, in the
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7 participating hospitals in Yaoundé (January 1st 2015 – December 31st 2016), Abidjan (August
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9 14th, 2016 – November 2nd 2016), and Colombo (June 13th 2016 –December 14th 2016). Head
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11 circumference in these maternities is measured within the first 24 hours of life using vinyl
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13 measuring tapes which are crossed over at the front of the head to get a reading of the head
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15 circumference. Birth register entries that were missing one or more data points were equally
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17 recorded and missing values noted in the data collection form.
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25 In Guangzhou (February 10th 2017 to March 13th 2017), Colombo (December 15th 2016 to April
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27 5th 2017), and Abidjan (April 4th 2018 to 14th July 2018), the participating maternities collected
28
29 data prospectively with the aim to review 3000 consecutive births moving forward from the date
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31 of the collection start. Prior to this prospective data collection, the participating maternities
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33 reinforced their standard procedures for head measurement and birth register data collection.¹⁶
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35 They also introduced use of non-stretch Teflon seca 212 head measuring bands, which remain in
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37 a loop format with a viewing window in order to read head circumference measurements to the
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39 nearest millimetre (<https://us.secashop.com/products/pediatric-measuring-systems/seca-212>).
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47 **Gestational age calculation**

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49 According to standard procedure at each participating maternity, gestational age is calculated
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51 based on the first date of the last menstrual period; for varying proportions of patients within each
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53 country, this estimate is confirmed using ultrasound examination carried out in the first trimester
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3 of pregnancy. In Ivory Coast, women frequently first present at the hospital at a stage very late in
4 pregnancy (e.g. onset of labour or first prenatal visit in the third trimester); in this case, the
5 calculation of gestational age based on the date of last menstruation is still attempted and is often
6 complemented by symphysis fundal height.
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16 **Data analysis**

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18 A newborn was included in the analysis if they had all of sex, gestational age and head
19 circumference recorded in the birth register; this data is needed to calculate the infant's head
20 circumference Z-score (HCZ). Moderate microcephaly was considered as having a HCZ of less
21 circumference Z-score (HCZ). Moderate microcephaly was considered as having a HCZ of less
22 than or equal to -2SD & greater than -3SD and severe microcephaly was considered as having a
23 HCZ of less than or equal to -3SD.^{3,8-12} Data was uploaded into the open access
24 INTERGROWTH-21st online application retrieved at: <http://intergrowth21.ndog.ox.ac.uk>, which
25 gave an exportable HCZ for each infant. We calculated a mean HCZ for each country, by type
26 (retrospective/prospective) of data collection, to compare with the pooled mean from the
27 INTERGROWTH-21st standards (i.e. 0SD). The Student's t-test, or the Chi-squared test were
28 was used to compare continuous data and categorical data, respectively. We excluded stillborn
29 and very preterm (< 33 weeks gestational age) measurements as these were excluded in the
30 elaboration of the growth standards.^{17,18} To facilitate the visual comparison between our data and
31 the INTERGROWTH-21st standards for Figure 1, we recreated the distributions of newborn head
32 circumferences by sex of the INTERGROWTH-21st charts (*Supplementary Material*). Data
33 analysis and graphics were produced using R version 3.4.0.
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Ethical considerations

In Sri Lanka and Ivory Coast, national ethical committee approval was not required in order to publish the aggregated results of the routinely collected data used for this analysis, however, institutional approval from participating hospitals was obtained. In Cameroon and China, institutional ethics committee clearance and approval of hospital authorities was obtained.

RESULTS

In total, data was collected for 21426 births that occurred between January 2015 and July 2018 across eight hospitals in the four participating countries. After exclusion of stillbirths (n=298, 1.4%), very preterm births (n=472, 2.2%), and birth records with missing variables (n=742, 3.5%), data from 19914 (92.9%) live births was analyzed (*Table 1*).

The distribution of HCZ from each country differed from that of the INTERGROWTH-21st standards by varying degrees (see Table 2). Figure 1 compares the HCZ distribution for each country with the HCZ distribution that would be expected based on the INTERGROWTH-21st standards. The prospective data from Guangzhou was the most similar (mean HCZ = -0.02SD, 95%CI: -0.06 – 0.02) to the INTERGROWTH-21st standards, whereas the retrospective data from Abidjan was the least similar (mean HCZ = -1.41SD, 95%CI: -1.45 - -1.37). We found a significant difference in mean HCZ between male and female infants in the retrospectively collected data in Cameroon and Ivory Coast, as well as in the prospectively collected data in Ivory Coast and Sri Lanka (*Table 2, Figure 1*).

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6 Overall, in the retrospectively collected data, the prevalence of moderate microcephaly ranged
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8 from 2.4% (Cameroon, 95%CI: 2.0-2.9%) to 25.8% (Ivory Coast, 95%CI: 24.2-27.4%), and the
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10 prevalence of severe microcephaly ranged from 0.5% (Cameroon, 95%CI: 0.3-0.8%) to 9.0%
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12 (Ivory Coast, 95%CI: 8.0-10.1%). In the prospectively collected data, the prevalence of moderate
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14 microcephaly ranged from 3.6% (China, 95%CI: 2.9-4.3%) to 14.6% (Ivory Coast, 95%CI: 13.4-
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16 15.8%), and the prevalence of severe microcephaly ranged from 0.2% (China, 95%CI: 0.1-0.5%)
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18 to 4.1% (Ivory Coast, 95%CI: 3.4-4.8%) (*See Table 2*).
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26 In Abidjan, where the mean HCZ was the furthest from zero (HCZ=-1.41SD, 95%CI: -1.45 - -
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28 1.37), a sensitivity analysis demonstrated a more shifted HCZ distribution (ie. disaccord with the
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30 INTERGROWTH-21st standards) when mothers presented late in pregnancy (62.4% of women)
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32 and uterine measurements were used to confirm the gestational age estimation, compared to when
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34 this was not needed: -1.48SD (95%CI: -1.53 - -1.42) vs -1.28SD (95%CI: -1.37 - -1.23, p<0.001).
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37 The prospectively collected data from the maternities in Colombo showed less microcephaly and
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39 a mean HCZ closer to 0 (-0.22SD, 95%CI: -0.26 - -0.18) when compared to the retrospectively
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41 collected data from the same site (-0.49SD, 95%CI: -0.54 - -0.44, p<0.001). Similarly, the
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43 prospectively collected data from the maternities in Abidjan showed less microcephaly and a
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45 mean HCZ closer to 0 (-0.85SD, 95%CI: -0.89 - -0.81) when compared to the retrospectively
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47 collected data (-1.41SD, 95%CI: -1.45 - -1.37, p<0.001).
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56 **DISCUSSION**

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5 Defining microcephaly strictly as less than -2SD according to the INTERGROWTH-21st
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7 standards resulted in a prevalence at birth at our study sites in sub-Saharan Africa and Asia that
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9 far exceeded estimates observed in birth defect registries of Europe, the United States, and Latin
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11 America.^{6,7,19} These findings were in part expected, since the approximation of the distributions
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13 underlying these standards suggests that 2.2% of the healthy newborns included in the creation of
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15 the INTERGROWTH-21st standards would be classified as having at least moderate
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17 microcephaly given this definition.²⁰ This prevalence would already be between 25 and 125 times
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19 the proportion of microcephaly as estimated through recent reports from birth defects
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21 registries.^{6,7,19} Supporting this, the ECLAMC (Latin American Collaborative Study of Congenital
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23 Malformations) determined a pre-Zika hospital-based microcephaly baseline prevalence of 0.08%
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25 in Brazil,¹⁹ whereas another study from Brazil that applied a standard definition (-2SD according
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27 to the INTERGROWTH-21st standards) to two hospital populations, without other criteria, found
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29 that more than 2.5% of infants were labelled as having microcephaly.¹³ These differences are not
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31 surprising considering that microcephaly cases in birth defects registries are likely classified as so
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33 by clinicians who have considered additional factors such as proportionality of the infant,
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35 dysmorphic features, and regional norms.
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46 There are many limitations of determining the prevalence of microcephaly in real-life settings if
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48 using pooled international standards that reflect ideal growth under optimal conditions. These
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50 constraints may explain the variation in our own estimates, as well as some of the extreme
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52 estimates for some sites, such as Abidjan, that we obtained. Certain clinical practices and tools
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54 are needed in order for newborn data to best be compared with growth standards, some of which
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3 are not always feasible in real-life settings of low-income countries. First, accurate measurement
4 of the infant head circumference is needed, requiring multiple measurements using a non-stretch
5 measuring tape with correct positioning on the neonate's head. In the analysis we present, these
6 methods were employed in a controlled fashion during the prospective surveillance in China, Sri
7 Lanka and Ivory Coast, but not in the retrospective data from birth registers. In both Sri Lanka
8 and Ivory Coast, the prospectively collected data that followed reinforcement of head
9 measurement techniques and introduction of standard non-stretch headbands demonstrated mean
10 HCZ estimates closer to zero when compared to the retrospective data; this indicates that
11 reduction of measurement error leads to increased, but not total, assimilation with the
12 INTERGROWTH-21st standards. Furthermore, estimation of gestational age, which is best done
13 with an ultrasound assessment in the first trimester, or otherwise using the date of last
14 menstruation,^{17,18} is a measure that is complex to determine for a high proportion of women in
15 our two participating countries in sub-Saharan Africa. In Abidjan, around 60% of the women
16 presented for the first time at the hospital towards the end of their pregnancy, leading to uterine
17 measurements being used to help estimate the gestational age. In Guangzhou, which is not a low-
18 income setting, women typically undergo 4-8 ultrasounds per pregnancy, allowing for precise
19 determination of gestational age and early detection and abortion of infants with any
20 abnormalities. This level of care and availability of tools may partially explain the similarity of
21 the Guangzhou prospectively collected data to that of INTERGROWTH-21st standards.
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50 The differences observed between the populations in each of our study sites and the
51 INTERGROWTH-21st standards likely also reflect regional differences in the head sizes of
52 infants due to environmental and socioeconomic factors, such as poor nutrition and circulation of
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3 infectious agents. These factors are, by definition, limited as much as possible in the creation of
4 prescriptive growth standards, but cannot be teased out when comparing real-life data to the
5 standards on a large-scale for surveillance purposes. Some criteria employed in the creation of the
6 INTERGROWTH-21st standards, such as that for maternal height and body mass index, led to
7 exclusion of more than 10% of otherwise eligible women and disproportionately affected specific
8 countries.¹⁸ In a real-life setting where factors influenced by environmental and socioeconomic
9 factors (e.g. maternal height and weight) are not adjusted for, the distribution of infant head
10 circumference Z scores may be shifted away the pooled standard, further exacerbating extreme
11 microcephaly estimates when using a fixed cut-off. For example, within our two sub-Saharan
12 Africa sites, the included hospitals in Ivory Coast, whose HCZ are shifted left compared to the
13 INTERGROWTH-21st distribution (mean HCZ -1.39SD with retrospective collection, -0.85SD
14 with prospective collection), are public with low-income catchment areas, while the hospital in
15 Cameroon, with data shifted right with a mean HCZ 0.75SD (retrospective collection), is a semi-
16 private institution with most patients coming from a higher socioeconomic status, and therefore
17 likely with different environmental exposures.
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41 Variation in fetal growth has been noted recently across the 10 countries included in the creation
42 of WHO fetal growth standards,²¹ as well as across the four ethnic groups included in the
43 National Institute for Child Health and Human Development (NICHD) fetal growth study in the
44 United States.²² Even after strict inclusion criteria, the INTERGROWTH-21st study noted
45 variance in the mean HCZ for each of their participating countries when compared to their
46 eventual pooled standard, which they call the standardized site discrepancy (SSD). Their eventual
47 range of SSD for head circumference at birth varied from as low as -0.55SD to as high as 0.42
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3 SD depending on the study country,¹⁸ which was within their predetermined limits to justify a
4 pooled growth standard.²⁰ It was demonstrated by Albert and Grantz that this allowed variance
5 means that the probability of falling below the 5th percentile of the INTERGROWTH-21st
6 percentiles would be as high as 12.6% in a setting with an SSD of -0.5 and as low as 1.6% in a
7 setting with an SSD of 0.5.²³ Applied to microcephaly surveillance, in the first case, such pooled
8 standards could lead to over-diagnosis in healthy infants, and in the second, under-diagnosis of
9 potential clinical cases. The mean HCZ (a measure similar to the INTERGROWTH-21st 'SSD')
10 from our study sites had an even wider range, from -1.41 to 0.67SD, which may explain the very
11 high proportion of microcephaly classifications that we observed in some countries, such as Ivory
12 Coast. Indeed some recent studies corroborate this, noting that replacement of regionally specific
13 growth charts with the INTERGROWTH-21st growth standards would reclassify a significant
14 proportion of infants/foetuses as having either macrocephaly or microcephaly in Canada and
15 China, respectively.^{24,25} A systematic review evaluating the WHO pooled international growth
16 standards also corroborates this finding, with individual country means matching particularly
17 poorly to the head circumference standards and leading to misdiagnosis of micro and
18 macrocephaly.²⁶

43 CONCLUSIONS

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46 Our study raises several issues regarding microcephaly surveillance. The use of a -2SD cut-off
47 based on the INTERGROWTH-21st chart implies that by definition, around 2% of healthy
48 newborns will be categorised as having microcephaly. As mentioned by Morris et al,⁶ this will
49 result in a high proportion of babies labelled as microcephalic who will have no detectable
50 neurological impairment, generating unnecessary additional diagnostic costs and anxiety. To this
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3 group will be added those who have underlying morbid or nutritional conditions associated with
4 small body size, and who have been excluded from the elaboration of pooled prescriptive
5 standards that describe ideal growth. These conditions may be particularly common in poor
6 settings of low-income countries. Finally, in regions with high variation in head circumference
7 size due to environmental and socioeconomic factors, the proportion of babies diagnosed with
8 microcephaly may reach proportions so high that any surveillance or diagnostic work-up based
9 on this definition would become very impractical. The Ivory Coast maternities sampled in our
10 study, with 19% of babies diagnosed with microcephaly through reinforced prospective
11 surveillance, may be one example of that situation.
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28 As the ZIKV epidemic has ended,²⁷ the focus of surveillance shifts towards increased specificity
29 in identifying neurological birth defects. As a result, a cut-off of -3SD for microcephaly
30 surveillance should be reconsidered, to conform with EUROCAT as well as guidelines and a
31 systematic review predating the ZIKV epidemic.²⁸⁻³¹ The predictive value of developmental
32 disabilities for infants with an at-birth head circumference Z-score of less than -3SD compared to
33 the norm, as opposed to -2SD, is also much higher,³² and this should be further validated using
34 data from cohorts of infants with ZIKV exposure during pregnancy. In addition to a more specific
35 cut-off, consideration of the proportionality of newborn head circumference length and/or weight
36 should be considered; inclusion of these criteria will mimic the procedures of birth defects
37 registries more closely, and is echoed in updated recommendations from the WHO and CDC.^{33,34}
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50 Finally, there is a need to perform further country-specific and regional studies to develop local
51 standards for foetal and newborn head circumference that can be used on a large-scale for
52 surveillance purposes. A growing body of research, including our own study, challenges the idea
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3 that foetal and newborn growth across the world can be assessed with a ‘one size fits all’
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5 standard.^{21,22,26,35} If the international definitions for microcephaly adapt to be considerate of this
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7 evidence, our global epidemiological understanding of this condition will benefit.
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29 Centre Maternity, Castle Street Hospital for Women, De Soyza Hospital for Women, Guangzhou
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33 Hospital, and Guangzhou Liwan Women and Children Health Care Hospital.
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TABLE LEGENDS

Table 1: Number of births collected and used in this analysis by participating country

Table 2: Mean head circumference Z-score (HCZ), and microcephaly prevalence broken down by study site, type of data collection, and sex

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FIGURE LEGEND

Figure 1: Distribution of Head Circumference Z-Scores (HCZ), by sex, according to the INTERGROWTH-21st standards (in grey), for retrospectively collected data (Cameroon, Ivory Coast, Sri Lanka) and prospectively collected data (Ivory Coast, Sri Lanka, China).

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Table 1: Number of births collected and used in this analysis by participating country

	Cameroon	Ivory Coast	Sri Lanka	China
Data collection method	Retrospective	Retrospective	Retrospective	Prospective
Total births recorded	5031	3212	3147	3229
Still births	84 (1.7%)	83 (2.6%)	3 (0.1%)	12 (0.4%)
Very preterm (<33 weeks gestation)	208 (4.1%)	14 (0.4%)	74 (2.4%)	36 (1.1%)
Live births (missing data)	312 (6.2%)	158 (4.9%)	81 (2.3%)	159 (4.9%)
Live births (sufficient data ^a)	4427 (88.0%)	2957 (92.1%)	3307 (93.8%)	3022 (93.6%)
<i>of which were male^b</i>	<i>2242 (50.6%)</i>	<i>1542 (52.1%)</i>	<i>1698 (51.3%)</i>	<i>1621 (53.6%)</i>

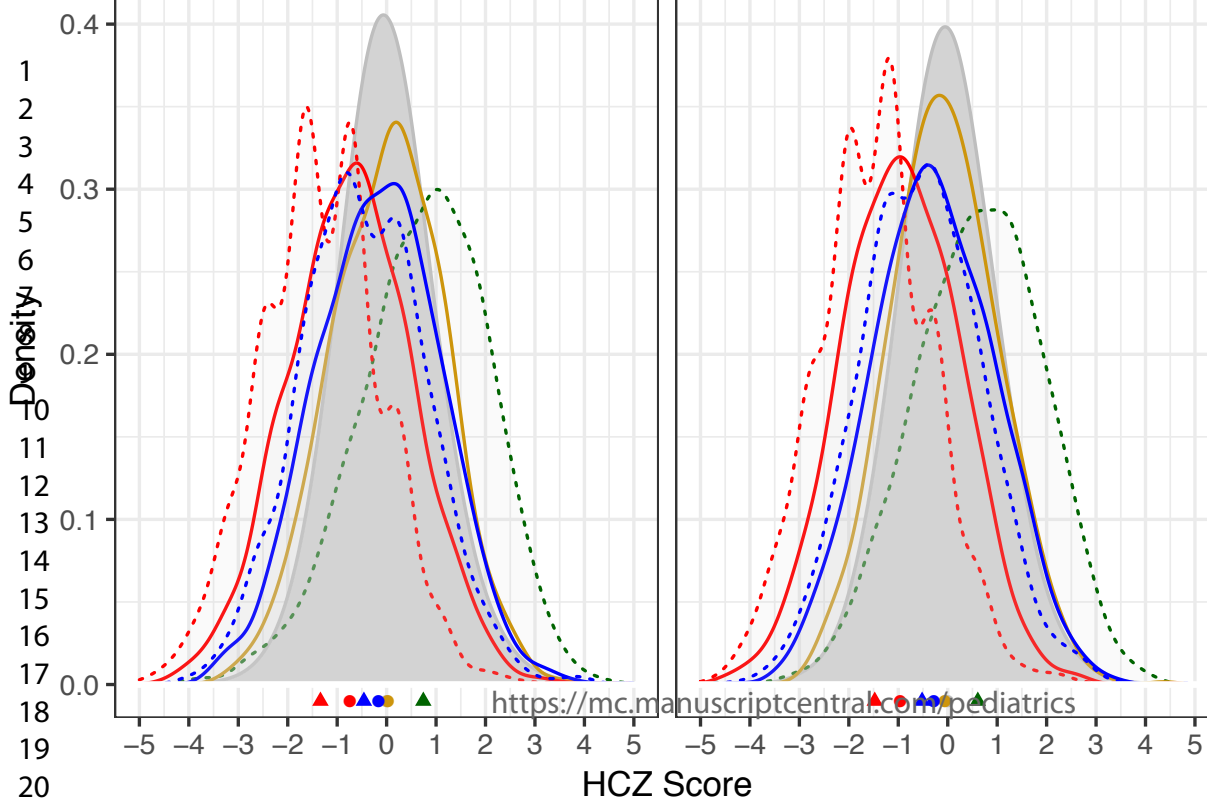
^aWith sufficient data for analysis, including sex, gestational age estimation, head circumference measurement ^bPercentage of live births with sufficient data

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Table 2: Mean head circumference Z-score (HCZ), and microcephaly prevalence broken down by study site, type of data collection, and sex

	Mean HCZ ± SD	Total microcephaly ($\leq 2SD$)	Moderate microcephaly $\leq 2SD$ & $> 3SD$	Severe microcephaly $\leq 3SD$
RETROSPECTIVE DATA COLLECTION				
Cameroon				
Total (n=4427)	0.67 ± 1.33	129 (2.9%)	105 (2.4%)	24 (0.5%)
Male (n=2242)	0.61 ± 1.32 ^a	62 (2.8%)	49 (2.2%)	13 (0.6%)
Female (n=2185)	0.74 ± 1.33 ^a	67 (3.1%)	56 (2.6%)	11 (0.5%)
Ivory Coast				
Total (n= 2957)	-1.41 ± 1.20	1029 (34.8%)	762 (25.8%)	267 (9.0%)
Male (n=1542)	-1.48 ± 1.16 ^b	632 (41.0%)	506 (32.8%)	126 (8.2%)
Female (n=1415)	-1.33 ± 1.25 ^b	397 (28.1%)	256 (18.1%)	141 (10.0%)
Sri Lanka				
Total (n=3059)	-0.49 ± 1.27	335 (11.0%)	280 (9.2%)	55 (1.8%)
Male (n=1592)	-0.52 ± 1.28	193 (12.1%)	165 (10.4%)	28 (1.8%)
Female (n=1467)	-0.46 ± 1.27	132 (9.0%)	115 (7.8%)	27 (1.8%)
PROSPECTIVE DATA COLLECTION				
Ivory Coast				
Total (n=3307)	-0.85 ± 1.24	616 (18.6%)	482 (14.6%)	134 (4.1%)
Male (n=1698)	-0.96 ± 1.20 ^a	341 (20.1%)	272 (16.0%)	69 (4.1%)
Female (n=1609)	-0.74 ± 1.28 ^a	275 (17.1%)	210 (13.1%)	65 (4.0%)
Sri Lanka				
Total (n=3142)	-0.22 ± 1.24	236 (7.5%)	203 (6.5%)	33 (1.1%)
Male (n=1561)	-0.16 ± 1.25 ^c	126 (8.1%)	113 (7.2%)	13 (0.8%)
Female (n=1581)	-0.28 ± 1.23 ^c	110 (7.0%)	90 (5.7%)	20 (1.3%)
China				
Total (n= 3022)	-0.02 ± 1.13	115 (3.8%)	108 (3.6%)	7 (0.2%)
Male (n=1621)	-0.06 ± 1.11	60 (3.7%)	59 (3.6%)	1 (0.1%)
Female (n=1401)	0.02 ± 1.16	55 (3.9%)	49 (3.5%)	6 (0.4%)

*Denotes significant difference between male and female mean HCZ ^ap<0.001 ^bp=0.002 ^cp=0.007



Country

- Cameroon
- China
- Ivory Coast
- Sri Lanka

Study Design

- Prospective
- Retrospective

Mean HCZ Score

- Prospective
- Retrospective

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3 **Supplementary Material: Recreation of INTERGROWTH-21st newborn standards**
4 **distribution**
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9 Following the methods described for the creation of the INTERGROWTH-21st newborn
10 standards, we used a skew t distribution (ST3 in R package GAMLSS) with four
11 parameters and fit separate models for girls and boys.^{1,2,3} For each gestational age, we
12 based the parameters μ and σ on the means and standard deviations from the on the
13 INTERGROWTH-21st z-score charts. In particular, we set the mean equal to the reported
14 value for z-score = 0 and the standard deviation to one-fourth the difference of +2SD and
15 -2SD (similar definitions based on 1 or 3 SD produced similar but not identical results).
16 We assumed the parameters ν and τ were fixed across all gestational ages, and fitted their
17 values so as to minimize the sum of squared differences between the INTERGROWTH-
18 21st head circumferences and modelled head circumferences for each gestational age-
19 percentile combination provided in the INTERGROWTH-21st percentile charts. The
20 INTERGROWTH-21st percentile charts list head circumference in centimetres for 7
21 percentile values (the 3rd, 5th, 10th, 50th, 90th, 95th, and 97th percentiles) and 70
22 gestational ages (33+0 to 42+6); the fit was thus based on 490 points each for boys and
23 girls. We then transformed the resulting distributions of head circumferences into
24 distributions of z-scores, removing the dependence on gestational age.
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48 Our recreation of the distributions underlying the INTERGROWTH-21st newborn head
49 circumference standards matched the INTERGROWTH-21st percentile charts reasonably
50 well, with only 9/490 (boys) and 10/490 (girls) head circumference values differing from
51 the INTERGROWTH-21st standards by more than 0.1 cm, and none by more than 0.15
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3 cm. For comparison, a normal distribution with mean and standard deviation defined
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5 using the same methods resulted in 51/490 (boys) and 79/490 (girls) head circumference
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7 percentile values differing by more than 0.1 cm, and 5/490 (boys) and 16/490 (girls) by
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9 more than 0.15 cm.
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14 **References**

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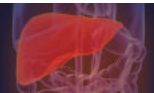
STROBE Statement—Checklist of items that should be included in reports of *cross-sectional studies*

	Item No	Recommendation	Page #(s)
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	1, 4
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5,6
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	6, 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6, 7
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants	8
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7,8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-8
Bias	9	Describe any efforts to address potential sources of bias	N/A
Study size	10	Explain how the study size was arrived at	6,7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	8
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	8
		(b) Describe any methods used to examine subgroups and interactions	8
		(c) Explain how missing data were addressed	8
		(d) If applicable, describe analytical methods taking account of sampling strategy	N/A
		(e) Describe any sensitivity analyses	8
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9, Table 1
		(b) Give reasons for non-participation at each stage	9, Table 1
		(c) Consider use of a flow diagram	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential	N/A

		confounders	
		(b) Indicate number of participants with missing data for each variable of interest	9, Table 1
Outcome data	15*	Report numbers of outcome events or summary measures	9, 10, Table 2
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9, 10
		(b) Report category boundaries when continuous variables were categorized	N/A
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	10
Discussion			
Key results	18	Summarise key results with reference to study objectives	10, 11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	11, 12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12, 13
Generalisability	21	Discuss the generalisability (external validity) of the study results	14, 15
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.



VIRAL HEPATITIS

The prevalence of hepatitis C virus infection in Egypt 2015: implications for future policy on prevention and treatment

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Abstract

Background & Aims: In 2015, a national Egyptian health issue survey was conducted to describe the prevalence of hepatitis C virus (HCV) infection. In this paper, we describe the HCV burden in 2015, compare the results with the national survey conducted in 2008, and discuss the implications of the new findings on prevention of HCV in Egypt. **Methods:** A multistage probability sampling approach was used, similar to the national demographic survey conducted in 2008. More than 90% of sampled individuals complied with the interview and provided blood samples. **Results:** In the 15–59-year age groups, the prevalence of HCV antibody was found to be 10.0% (95% CI 9.5–10.5) and that of HCV RNA to be 7.0% (95% CI 6.6–7.4). In children, 1–14 years old, the prevalence of HCV antibody and HCV RNA were 0.4% (95% CI 0.3–0.5) and 0.2% (95% CI 0.1–0.3) respectively. Approximately, 3.7 million persons have chronic HCV infection in the age group 15–59 in 2015. An estimated 29% reduction in HCV RNA prevalence has been seen since 2008, which is largely attributable to the ageing of the group infected 40–50 years ago during the mass schistosomiasis treatment campaigns. Prevention efforts may have also contributed to this decline, with an estimated 75% (95% CI 6–45) decrease in HCV incidence in the 0–19 year age groups over the past 20 years. **Conclusions:** These findings can be used to shape future HCV prevention policies in Egypt.

Keywords

hepatitis C Egypt – incidence HCV – nation-wide surveys – prevalence HCV

Hepatitis C Virus (HCV) infection is a major global health challenge; it is estimated that more than 80 million people are chronically infected worldwide, with 3–4 million new infections and 350 000 deaths occurring each year because of HCV-related complications (1–3).

Egypt is the country with the highest HCV prevalence in the world; in 2008, the Egyptian Demographic Health Survey (EDHS), which was conducted on a large nationally representative sample, estimated the prevalence of HCV antibodies and HCV RNA, among the 15–59 year

Abbreviations

CIA, chemiluminescent microplate immunoassay; CPHL, Central Public Health Laboratory; DAA, direct-acting antivirals; EDHS, Egyptian Health Demographic Survey; EHIS, Egyptian Health Issue Survey; ELISA, third-generation enzyme immunoassay; FDA, Food and Drug Administration; HCV, hepatitis C virus; PPU, Pasteur-Paris University.

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Key points

- There has been an approximate 30% decrease in HCV prevalence in Egypt between 2008 and 2015.
- This decline is mostly related to the ageing of the initially infected cohort; this phenomenon will be seen in most countries as the bulk of worldwide HCV infections took place between 1960 and 1980.
- Still, a 28% decline in incidence in younger age groups is estimated, most likely related to prevention efforts such as injection safety and awareness programmes.
- Treatment has not yet demonstrated an impact on decline of HCV prevalence in Egypt.

age group, to be 14.7 and 9.8% respectively. Based on the population census and the EDHS done in 2008, it was estimated that more than 6.8 million persons aged 15–59 years had HCV antibodies, of which more than 4.5 million individuals had active HCV infection (4). In 2015, the Egyptian Health Issues Survey (EHIS) was done to re-estimate the prevalence of HCV infection in Egypt. In this paper, we describe the prevalence of HCV in Egypt in 2015, using measures of both HCV RNA, which indicates the burden of disease, and of HCV antibody, which provides an estimate of past infection. We also compare the results of the two national surveys conducted in 2008 and 2015 in order to estimate national changes in prevalence and incidence, and discuss the implications of these findings on the national policy for HCV prevention and treatment in Egypt.

Materials and methods**Source of data**

We obtained data from the EDHS 2008 (4) and EHIS 2015 (5), both of which were conducted by El-Zanaty and Associates with support from the United States Aid of International Development-sponsored DHS-7 project.

Sampling strategy

The national surveys in 2008 and 2015 were cross-sectional household surveys, where sampling weights were used to provide estimates considered representative of the Egyptian population on the basis of a complex, three-stage probability sampling approach. The two surveys provide estimates of HCV prevalence in Egypt for the country as a whole and broken down for the major administrative regions (Urban Governorates, Lower Egypt, Upper Egypt and the Frontier Governorates). In both EDHS 2008 and EHIS 2015, those aged 15–59 years were invited to participate, however, in EHIS 2015, children aged 6 months to 14 years were also included (4, 5).

Data collection

Individuals within sampled households were invited and consented for participation. Basic demographic information was collected, including: age, gender, marital status, place of residence, level of education, work status and wealth status.

Laboratory procedures

The laboratory procedures applied in 2015 were similar to those in 2008 (4, 5). In 2015, consented individuals provided 7 ml of venous blood added to an EDTA vacutainer tube. In the field laboratory, the 2015 EHIS biomarker staff centrifuged the blood and transferred the serum to five microvials that were stored in liquid nitrogen tanks before being transferred to the Central Public Health Laboratory (CPHL) in Cairo. A hepatitis C testing algorithm used third-generation enzyme immunoassay (ELISA) to determine the presence of HCV antibodies. A more specific assay, the chemiluminescent microplate immunoassay (CIA) was used to confirm HCV antibody status for ELISA-positive samples and 5% of the ELISA-negative samples. Quantitative real-time PCR was used to test for HCV RNA in HCV antibody-positive samples to confirm active infections. A quality control procedure of retesting of approximately 10% of all samples was undertaken at the CPHL and a further external quality control was done at the Theodor Bilharz Research Institute, in Cairo, by retesting approximately 5% of the samples tested at the CPHL (5). The only key difference between this procedure and that of the previous survey is that, in 2008, the quality control measure (10% of samples retested) at CPHL was not carried out.

Statistical analysis

Comparisons were carried out for HCV antibody- and HCV RNA-positive tests, by estimating the absolute and relative risk reductions between the two surveys in comparable age groups. To calculate the 95% confidence interval for the risk difference, Newcombe–Wilson's method without continuity correction was applied (6). In order to estimate confidence intervals for the relative risk, we used the methods described by Armitage and Berry (7). The level of significance (*P*-value) was inferred to be less than 0.05 if the corresponding 95% confidence intervals for the relative and absolute effect measures were devoid of 'zero' (8). HCV virus clearance was estimated by finding the absolute difference between the proportion of HCV antibody and HCV RNA-positive participants, and dividing this by the proportion of HCV antibody-positive participants. Comparisons of HCV clearance between combined age groups in 2008 and 2015 were done using the chi-square test. In order to demonstrate the cohort effect, the 2008 DHS data were shifted forward; 7 years were added to

each participant's age from the 2008 DHS survey and the HCV antibody and HCV RNA prevalence estimates were re-calculated using the new numerator and denominator in each age category. The estimated number of population positive for HCV antibodies and HCV RNA was calculated by multiplying the age- and gender-specific prevalence of 2015 by the population census of January 2015 categorised by age and gender.

Ethical considerations

No ethical approval was needed for the data analysis presented in this paper; the anonymised data is publicly available online. El-Zanaty and Co is the responsible party for the ethical considerations of the EDHS 2008 and EHIS 2015; verbal informed consent was obtained from all individuals aged 18 years and older and from married minors aged 15–17 years. For children less than 18 years, consent was obtained from the parent or child caretaker.

Results

In EDHS 2008, 4757 households including 12 780 individuals aged 15–59 years were identified for interview and blood testing. A total of 4662 households complied (98%), 12 008 persons were interviewed (93.9%) and out of these, 11 126 persons provided blood samples for testing (92.7%). In EHIS 2015, 7649 households including 28 079 individuals were identified. A total of 7516 households complied (98.3%), and 27 549 persons (age: 1–59 years) were interviewed (98.1%). Of the total 28 079 persons identified, 17 182 were aged 15–59 years, of which 16 671 were interviewed (97.0%) and out of these, 16 003 provided blood samples for testing (96.0%). The remaining 10 897 persons identified were children aged 1–14 years, and of these, 10 878 (99.8%) were interviewed through their caregivers and 10 044 (92.3%) children provided blood samples for testing. Children therefore represented 39.5% of the total study population (those interviewed) in EHIS 2015. The characteristics of the two populations surveyed in EDHS 2008 and EHIS 2015 are presented in Table 1. For comparison purposes, we describe the age group of 15–59 years who were targeted in both surveys.

There was an overall significant reduction of 32 and 29% in the prevalence of HCV antibody and HCV RNA-positive individuals, respectively, between the DHS in 2008 and the EHIS in 2015 (Table 2). The age-specific prevalence of HCV antibody and HCV RNA-positive individuals in 2008 and 2015 is presented in Table 2 and Fig. 1. The pattern of increased prevalence of HCV antibody and HCV RNA-positive persons with age was observed in both the 2008 and the 2015 survey. A statistically significant reduction in HCV antibody prevalence was observed in all age groups, and the greatest relative prevalence reduction (75%) was observed among those aged 15–19 years. A statistically significant

Table 1. Participant characteristics of Egyptian Demographic Health Surveys in 2008 compared to EHIS 2015

	2008		2015	
	No.	%	No	%
Study population (15–59 years), total interviewed	12 008	100	16 671	100
Laboratory tested				
Yes	11 126	92.7	16 003	95.9
No	882	7.3	668	4.1
Households, total sampled	4953	100	7813	100
Interviewed				
Yes	4662	94.1	7516	96.2
No	291	5.9	297	3.8
Age group (years), total 15–59				
15–19	2151	17.9	2713	16.3
20–24	1960	16.3	2044	12.3
25–29	1635	13.6	2433	14.6
30–34	1322	11.0	2118	12.7
35–39	1209	10.1	1917	11.5
40–44	1148	9.6	1550	9.3
45–49	1044	8.7	1424	8.5
50–59	1539	12.8	2472	14.8
Gender, total				
Males	5718	47.6	7462	44.8
Females	6290	52.4	9209	55.2
Marital status, total				
Never married	3863	32.2	4375	26.2
Married	7588	63.2	11 372	68.2
Widowed	400	3.3	329	2.0
Divorce/separated	157	1.3	595	3.6
Residence, total				
Urban	5288	44.0	6206	37.2
Rural	6720	56.0	10 465	62.8
Place of residence				
Urban governorates	2445	20.4	2267	13.6
Lower Egypt	5213	43.4	8204	49.2
Upper Egypt	4168	34.7	6081	36.5
Frontier governorates	182	1.5	119	0.7
Education				
No education	2588	21.6	2652	15.9
Some primary	1084	9.0	1459	8.8
Primary complete/ some secondary	2919	24.3	4552	27.3
Secondary complete/ higher	5417	45.1	8008	48.0
Wealth quintile				
Lowest	2042	17.0	3268	19.6
Second	2442	20.3	3234	19.4
Middle	2425	20.2	3212	19.3
Fourth	2440	20.3	3436	20.6
Highest	2659	22.1	3521	21.1

reduction in HCV RNA-positive individuals was observed in all except the two age groups encompassing those 20–29 years of age. The age-specific prevalence of HCV antibody and HCV RNA-positive individuals from 2008 shifted forward by 7 years is presented alongside the 2015 prevalence estimates (Fig. 1). The current data

Table 2. Prevalence of hepatitis C virus (HCV) antibody and HCV RNA positive persons (age 15–59 years) by age, gender and region (EDHS 2008 and EHIS 2015 surveys)

Characteristic	HCV antibody positive				HCV RNA positive			
	% Positive 2008	% Positive 2015	Prevalence difference (95% CI)	% Prevalence reduction (95% CI)	% Positive 2008	% Positive 2015	Prevalence difference (95% CI)	% Prevalence reduction (95% CI)
Overall prevalence	14.7	10.0	4.7 (3.9–5.5)*	32 (60–69)*	9.9	7.0	2.9 (2.2–3.6)*	29 (23–35)*
Age group								
15–19	4.1	1.0	3.1 (2.2–4.1)*	75 (64–85)*	2.8	0.8	2.2 (1.2–2.8)*	73 (55–83)*
20–24	4.9	3.2	1.6 (0.4–2.9)*	34 (10–52)*	3.0	2.2	0.8 (–0.2 to 1.8)	27 (–8.8 to 51)
25–29	6.1	4.4	1.7 (0.3–3.2)*	28 (6–45)*	3.9	3.0	0.9 (–0.2 to 2.1)	24 (–7 to 45)
30–34	11.8	7.1	4.5 (2.5–6.7)*	40 (25–51)*	8.3	4.9	3.3 (1.6–5.2)*	41 (23–55)*
35–39	13.8	8.2	5.5 (3.2–7.9)*	40 (26–52)*	9.9	6.0	3.8 (1.9–6.0)*	39 (22–53)*
40–44	23.0	11.6	11.4 (8.4–14.5)*	50 (40–58)*	15.0	8.9	6.1 (3.5–8.7)*	41 (26–52)*
45–49	28.6	16.3	12 (8.6–15.5)*	43 (33–51)*	18.9	11.5	7.3 (4.3–10.4)*	39 (26–50)*
50–54	38.3	27.9	10.6 (6.3–14.8)*	27 (17–36)*	25.3	19.7	5.6 (1.8–9.4)*	22 (8–34)*
55–59	39.4	33.9	5.1 (0.4–9.8)*	14 (2–24)*	27.4	22.3	5.0 (0.8–9.2)*	18 (3–31)*
Gender								
Males	17.4	12.4	5 (3.7–6.2)*	29 (22–35)*	12.1	8.9	3.1 (2.1–4.3)*	26 (18–34)*
Females	12.2	8.1	4 (2.9–5.0)*	33 (26–39)*	7.8	5.5	2.3 (1.4–3.2)*	30 (20–38)*
Work status								
Working for cash	18.8	13.3	5.5 (4.1–6.8)*	29 (23–35)*	13.0	9.5	3.5 (2.3–4.6)*	27 (19–34)*
Not working for cash	11.3	7.4	3.9 (2.9–4.8)*	35 (28–41)*	7.3	5.1	2.2 (1.4–3.0)*	30 (21–38)*
Region								
Urban governorates	9.5	6.9	2.6 (0.98–4.21)*	27 (11–41)*	6.2	4.4	1.8 (0.4–3.0)*	29 (8–45)*
Lower Egypt	17.5	12.2	5.3 (4.0–6.6)*	30 (24–36)*	11.5	8.7	2.8 (1.7–3.9)*	24 (16–32)*
Upper Egypt	14.7	8.2	6.5 (6.9–9.5)*	44 (37–50)*	10.2	5.8	4.4 (3.2–5.5)*	43 (35–50)*
Frontier governorates	3.0	3.5	–0.2 (–5.7 to 5.0)	–17 (–369 to 71)	2.5	2.6	–0.1 (–5.2 to 4.6)	–4 (–402 to 78)

*Statistically significant ($P < 0.05$).

(EHIS 2015) for the three oldest age groups (45–59 years) show a similar HCV clearance percentage when compared to the same age groups in the shifted-forward 2008 data (31.2% in 2015, 32.0% in the shifted 2008, $P = 0.756$). For the cohort of children aged 1–14 years in 2015, the overall prevalence of HCV antibody and HCV RNA-positive individuals was 0.4% (95% CI 0.3–0.5) and 0.2% (95% CI 0.1–0.3) respectively (Table 3).

A significant reduction of HCV antibody and HCV RNA-positive individuals was observed when looking at the overall prevalence in the Urban Governorates and, and for Lower and Upper Egypt Governorates (Fig. 2). No significant change in prevalence of either HCV antibody or HCV RNA positivity was observed in the frontier governorates.

Table 3 shows the age- and gender-specific prevalence of HCV antibody and HCV RNA-positive persons in 2015, as well as the estimated total number of persons positive for HCV antibody and HCV RNA in Egypt at this time. In the population aged 0–59 years, we estimated a total of 3 693 180 persons with chronic HCV infection (HCV RNA positive), and 5 309 555 persons with HCV antibodies.

Discussion

In 2015, HCV still affects a substantial proportion of the Egyptian population, where it is estimated that, in the 1–59-year age group, 5.3 million persons are positive for HCV antibodies and, of these, approximately 3.7 million (69.5%) are HCV RNA positive. This is an underestimate of the total human HCV reservoir in Egypt because older age groups (> 59 years) were not included in the EHIS 2015. This recent survey shows a similar epidemiological pattern of increased HCV antibody prevalence with age as did the EDHS 2008. This phenomenon was described in many studies (9, 10) and is because of the continuing exposure and risk of infection with age (11), while the proportion of persons with HCV infection who go on to develop severe disease and die of it remains low until 30 years after infection; the rate of progression to cirrhosis is estimated at 7% after 20 years of being infected (12). The increase in HCV antibody prevalence with age is therefore demonstrating the cumulative HCV incidence over time until HCV-related mortality becomes manifest.

This study shows a significant reduction in the overall prevalence of HCV antibody from 14.7 to 10.0%, and

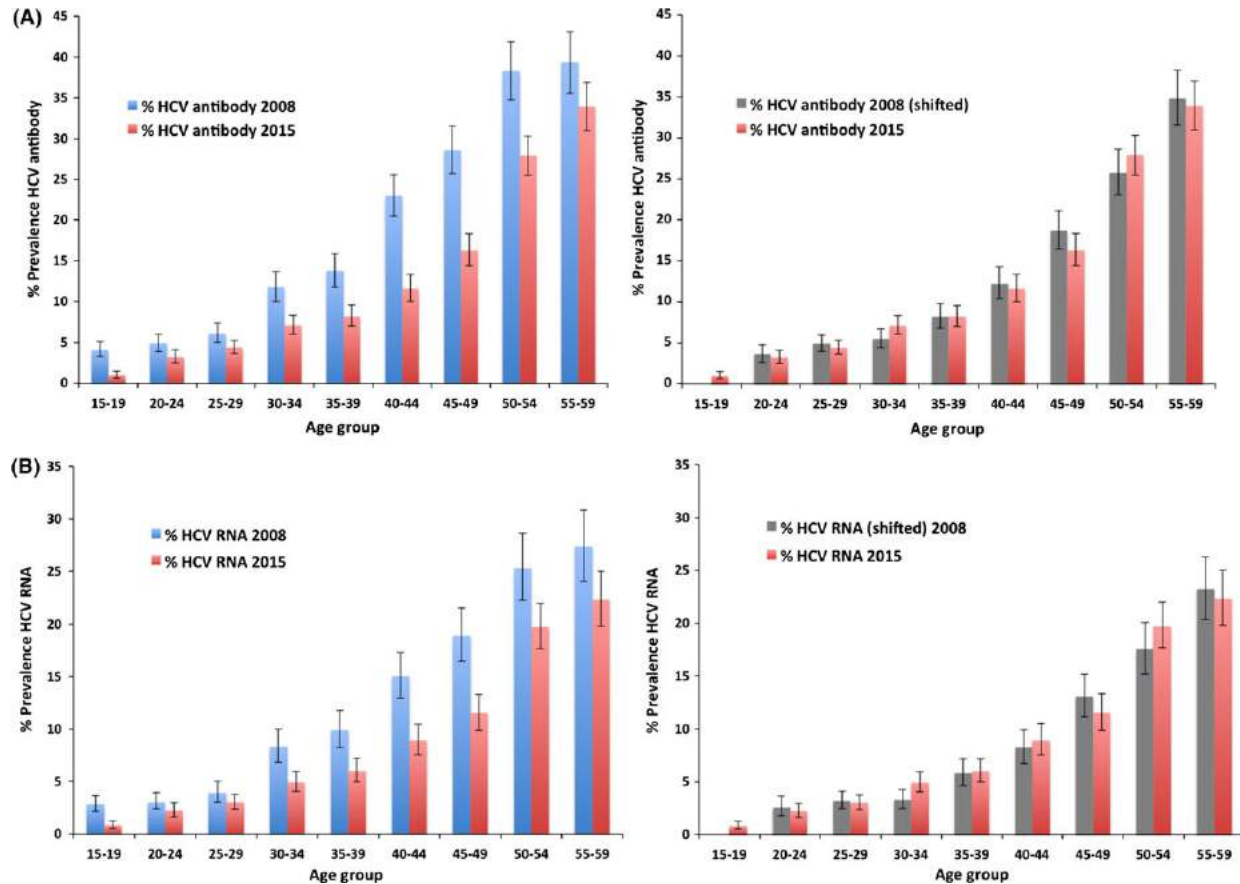


Fig. 1. (A) Age-specific prevalence of hepatitis C virus (HCV) antibody-positive persons in 2008 and 2015 (left), then shifted (by 7 years) 2008 and 2015 (right). (B) Age-specific prevalence of HCV RNA-positive persons in 2008 and 2015 (left), then shifted (by 7 years) 2008 and 2015 (right).

HCV RNA from 9.9 to 7.0%, between 2008 and 2015 among those aged 15–59 years. The main explanation for this marked reduction in HCV prevalence is the disappearance of the group infected during the mass schistosomiasis treatment campaign with reused syringes (1960s through early 1980s) to outside the age range covered by the survey (i.e. those older than 59 years) (13, 14). Indeed, as demonstrated in Fig. 1, the age-specific HCV antibody and HCV RNA prevalence for 2015 matches well with the 2008 prevalence estimates shifted by 7 years, suggesting that the ageing of the infected cohort, the so-called ‘cohort effect’, is the driving mechanism underlying the HCV age distribution; this was also demonstrated in the earlier reduction of HCV antibody prevalence from 30% in 1996 (14) to 14.7% in 2008 (4).

In order to see whether or not a decrease in HCV incidence may have also been a contributor to the reduction in HCV prevalence, the most interesting age groups to look at are those less than 20 years of age; persons who were not affected by the mass treatment campaigns and for whom HCV-related mortality remains

low. If we assume that mother-to-child HCV transmission is a negligible source of infant infection, as suggested by the <0.5% HCV antibody prevalence in the 1–4-year age group, then the HCV antibody prevalence at age 19 indicates the cumulative incidence over the past 20 years. In 2008 and 2015, the prevalence of HCV antibodies in those aged 15–19 years was 4.1 and 1.0%, respectively; the percentage of relative risk reduction was 75% (95% CI 64–85), implying a very substantial reduction in HCV incidence in the past 20 years in this age group. Furthermore, the prevalence of HCV antibody (0.4%) and HCV RNA-positive persons (0.2%) observed in EHIS 2015 among the age group 1–14 years is low compared to several studies conducted in early 2000s, which described the HCV antibody prevalence to be ranging from 2 to 7% in children under 10 years in rural areas of the Nile Delta (15–17).

This change in incidence in the younger age groups could possibly be because of the various public health interventions implemented by the Ministry of Health and Population and its partners since 2008. In this time, several efforts have been made to promote and expand

Table 3. Estimated population numbers who are hepatitis C virus (HCV) antibody and HCV RNA positive (EHIS 2015)

Age groups (years)	Males					Females					Total	
	Male population Jan 2015	% Positive HCV antibodies	Estimated number of positive HCV antibodies	% HCV RNA positive	Estimated number of HCV RNA positive	Female population Jan 2015	% Positive HCV antibodies	Estimated number of positive HCV antibodies	% HCV RNA positive	Estimated number of HCV RNA positive	Estimated population with HCV RNA	Estimated population with HCV antibodies
1-4	4 176 030	0.5	20 880	0.2	8352	3 871 877	0.4	15 487	0.3	11 616	19 968	36 367
5-9	4 790 338	0.5	23 952	0.2	8580	4 456 493	0	0	0	0	8580	23 952
10-14	4 279 072	1.1	47 070	0.1	4279	4 013 008	0.2	8026	0.2	8026	12 305	55 096
15-19	4 367 988	0.9	39 312	0.6	26 208	4 131 991	1.2	49 584	0.9	37 188	63 396	88 896
20-24	4 623 621	4.8	221 934	3.1	143 332	4 424 043	2.1	92 905	1.5	66 361	209 693	314 839
25-29	4 334 645	6.8	294 756	4.7	203 728	4 175 258	2.8	116 907	1.9	79 330	283 058	411 663
30-34	3 456 601	9.0	311 095	7.1	245 419	3 364 004	5.6	188 384	3.2	107 648	353 067	499 479
35-39	2 711 932	9.4	254 922	6.9	192 547	2 639 282	7.1	187 389	5.3	139 882	332 429	442 311
40-44	2 422 954	14.4	348 905	10.8	256 833	2 379 682	9.0	214 171	7.3	173 717	430 550	563 076
45-49	2 222 893	17.8	395 675	12.4	282 307	2 184 980	14.8	323 377	10.4	227 238	509 545	719 052
50-54	1 922 803	31.5	605 683	23.7	448 013	1 903 745	24.3	462 610	16.1	306 503	754 516	1 068 293
55-59	1 567 139	41.9	656 631	27.8	441 933	1 557 610	27.6	429 900	17.6	274 139	716 072	1 086 531
Total	40 876 016	7.5	3 220 815	5.3	2 261 532	39 101 973	5.3	2 088 740	3.6	1 431 648	3 693 180	5 309 555

the infection prevention and control programmes beyond Ministry of Health and Population hospitals, particularly to the university hospitals. Auto disabled syringes were introduced to the routine immunisation sector in 2008 in order to promote safe injection practices among children. Safe blood transfusion activities, including policies and guidelines, have been intensified since 2009. Raising the awareness of the public, by targeting universities and schools to improve their understanding on the epidemiology and prevention of viral hepatitis, was also carried out. Pre-service education targeting healthcare staff has been carried out since 2008 to enforce the concepts of safe healthcare and prevention of blood-borne pathogens.

One may question whether the national treatment programme, which managed to treat more than 350 000 persons in the past 7 years (18) using pegylated interferon and ribavirin (19), has had an impact on HCV prevalence figures. In such a case, it would be expected that cured patients would have cleared HCV RNA, but kept HCV antibodies. Therefore, the impact would be seen through an increase in HCV clearance percentage in the age groups most targeted by the national treatment programme. Owing to established approximate 50% cure rates of the combination of pegylated interferon and ribavirin (20), only half of these persons (i.e. 175 000) would have cleared their HCV infection after treatment. The national treatment programme prioritises persons with more advanced forms of liver disease, and therefore, at least half of the beneficiaries of this service are in their 40s or 50s. Indeed, in a group of 3235 patients treated for HCV in a hospital run by the Ministry of Health in Cairo, between 2007 and 2011, the mean age was 41 years with a standard deviation of approximately 10 years (21). In order to see whether or not treatment has, as of yet, had an impact on lowering the prevalence of persons with HCV RNA in Egypt, we can see whether or not there has been increased clearance in the four older age groups (i.e. encompassing 40–59 years) over the past 7 years. If we assume that around half (87 500) of the total persons cured was in the 40–59-year age group, and considering that we have estimated 2.87 million persons with HCV antibodies in the same age group in 2015 (Table 3), we would have expected to see around a 3% higher clearance percentage in this group, in comparison with 2008. However, an increase in clearance in these older age groups was not observed when comparing data from 2015 and the shifted-forward 2008 data. Indeed, it would require around 4000 anti-HCV-positive persons in this age group, in each data set, in order to have enough power to demonstrate this expected 3% difference; unfortunately, the anti-HCV population examined in this survey was much lower than that.

Treatment may show a larger impact in the near future as it is expected that emphasis will be put on an upscale of treatment of infected persons to prevent long-term complications (22), particularly considering

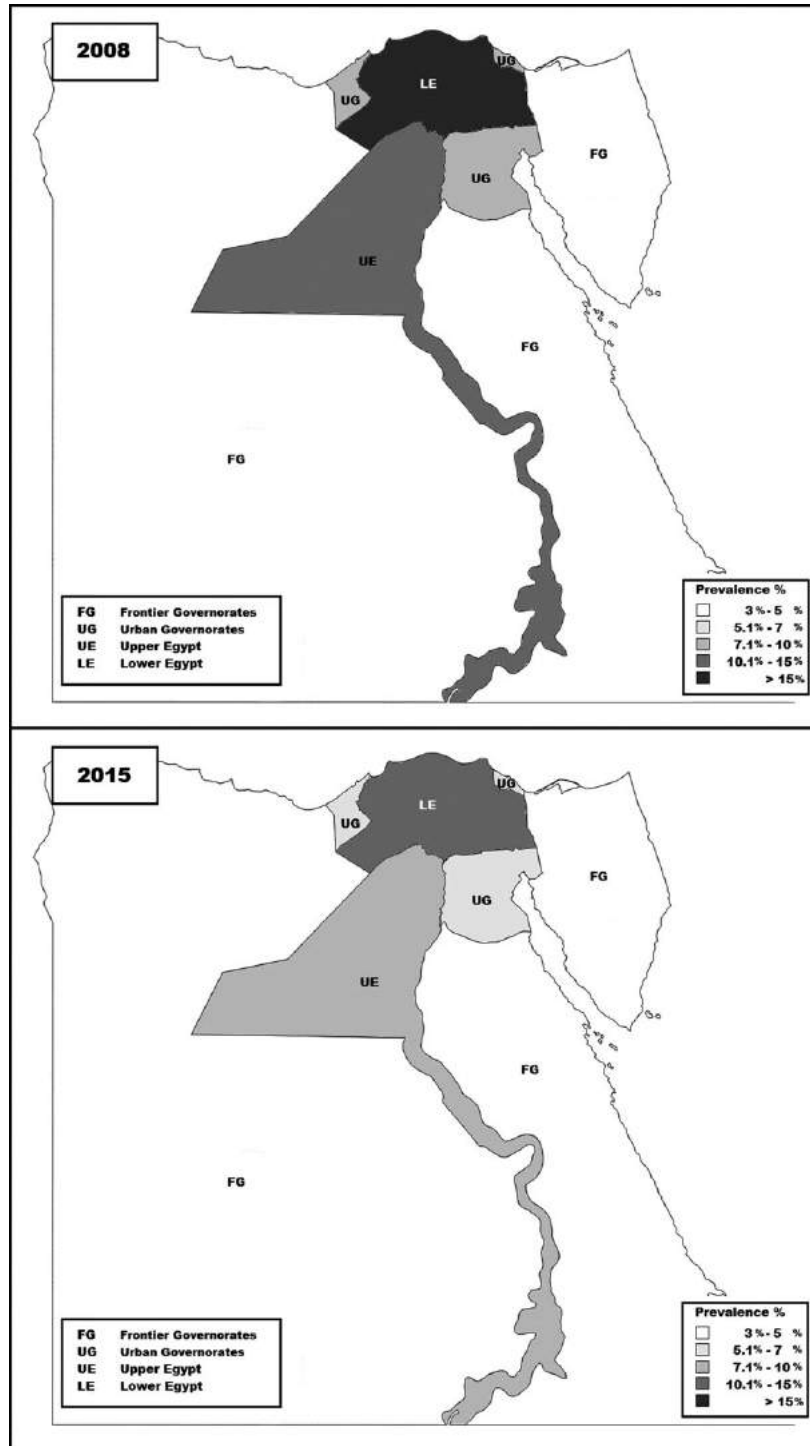


Fig. 2. Prevalence of hepatitis C virus antibody in the 2008 and 2015 Egyptian Demographic and Health Surveys.

that new direct-acting antiviral (DAA) drugs were approved for the treatment of HCV by the U.S. Food and Drug Administration (FDA) in late 2013, and then introduced into Egypt in late 2014. These new

treatment regimens have reduced treatment duration to 12–24 weeks, decreased side effects and improved outcomes, with cure rates of 85–95% across all patient populations (23, 24).

Finally, differential migration and mortality of persons infected with HCV could have contributed to the reduced prevalence of both HCV antibody and HCV RNA-positive persons in 2015, as long as these rates are higher than infection incidence (25–27); these topics need to be explored further.

The prevalence of HCV antibody and HCV RNA-positive individuals varied among governorates and was reduced in several geographical regions. Lower Egypt governorates, which are mostly rural in nature, still show a higher prevalence of HCV antibodies and HCV RNA when compared to urban governorates. This pattern of high HCV prevalence in rural areas is similar to previous multiple studies conducted in rural Lower Egypt governorates which showed a prevalence ranging from 14.4 to 18.5% (28–30). The frontier governorates did not show any significant change, however, this is possibly owing to the fact that the sample size was very low ($n < 200$), in both the 2008 and 2015 surveys.

Based on our findings in this study, we recommend the expansion of national health surveys in Egypt in order to include older age groups and allow further follow-up of elderly persons who have been the most affected by HCV and who, by the cohort effect, are being pushed out of view. Furthermore, there should be continued prioritisation of prevention programmes to increase the effects we are seeing in incidence in young age groups, with a focus on interventions which promote injection safety by reducing the frequency of unnecessary injections and syringe reuse. Interventions could include introduction of single-use materials and engineered safety devices, such as auto-disabled or auto-destructive syringes into the curative sector. Expansion of infection prevention and control programmes are of utmost importance, along with development of elaborate systems for delivering and renewing licenses of healthcare facilities to ensure continuity of safe procedures and application of standard precautions. Other analyses of the EHIS data should be done, using tools such as mathematical modelling, in order to properly estimate and explain changes in HCV incidence in Egypt; this could lead to further evidence-based recommendations related to prevention and control efforts. Finally, access to treatment should be a priority, and although economic constraints are faced by the country, treatment has been shown to be cost-effective in this context (31, 32) and focused, early treatment strategies may be effective in supporting prevention measures and reducing transmission (33).

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Increased recurrence rates of hepatocellular carcinoma after DAA therapy in a hepatitis C-infected Egyptian cohort: A comparative analysis

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Summary

In Egypt, hepatocellular carcinoma (HCC) is the most common form of cancer and direct-acting antivirals (DAA) are administered on a large scale to patients with chronic HCV infection to reduce the risk. In this unique setting, we aimed to determine the association of DAA exposure with early-phase HCC recurrence in patients with a history of HCV-related liver cancer. This was a prospective cohort study of an HCV-infected population from one Egyptian specialized HCC management centre starting from the time of successful HCC intervention. The incidence rates of HCC recurrence between DAA-exposed and nonexposed patients were compared, starting from date of HCC complete radiological response and censoring after 2 years. DAA exposure was treated as time varying. Two Poisson regressions models were used to control for potential differences in the exposed and nonexposed group; multivariable adjustment and balancing using inverse probability of treatment weighting (IPTW). We included 116 patients: 53 treated with DAAs and 63 not treated with DAAs. There was 37.7% and 25.4% recurrence in each group after a median of 16.0 and 23.0 months of follow-up, respectively. Poisson regression using IPTW demonstrated an association between DAAs and HCC recurrence with an incidence rate ratio of 3.83 (95% CI: 2.02-7.25), which was similar in the multivariable-adjusted model and various sensitivity analyses. These results add important evidence towards the possible role of DAAs in HCC recurrence and stress the need for further mechanistic studies and clinical trials to accurately confirm this role and to identify patient characteristics that may be associated with this event.

KEYWORDS

DAAs, Egypt, hepatitis c, hepatocellular carcinoma, recurrence

Abbreviation: BCLC, Barcelona clinic liver cancer; CT, computerized tomography; DAA, direct-acting antivirals; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IPTW, inverse probability of treatment weighting; IRR, incidence rate ratio; MWA, microwave ablation; PEI, percutaneous ethanol injection; RFA, radiofrequency ablation; SVR, sustained virological response; TACE transarterial chemoembolization

Mohamed El Kassas and Anna L. Funk contributed equally.

1 | INTRODUCTION

Several studies and meta-analyses have concluded that eradication of hepatitis C virus (HCV) with antiviral therapy reduces the risk of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C, independent of their fibrosis stage.¹ During the era of interferon-based therapy, patients with a sustained virological response (SVR) including those on combination therapy with pegylated interferon and ribavirin showed both histological improvement through HCV eradication as well as a decrease in the risk of HCC development.² Other research concluded that patients achieving SVR through interferon-based therapies who had previously received curative HCC treatment, including local ablation therapy and hepatic resection, had favourable outcomes compared with non-SVR patients. The introduction of the new wave of direct-acting antivirals (DAAs) in 2014, with increased tolerance and effectiveness, was seen as a continuing step forward in the treatment of persons with a history of HCC and thought likely to improve their overall prognosis.

The new, highly effective DAAs were expected to dramatically decrease HCV-related liver disease progression to end-stage liver disease and HCC; however, these optimistic expectations were questioned by an initial report from Spain in 2016. Reig³ and colleagues reported a “more than expected” early recurrence rate (27.6%) in patients with HCC who received DAA treatment after an initial good response to HCC treatment. This report represented a red flag and opened the door for a debate about the relationship between DAA treatment and HCC recurrence. Reports from Italy and the United States, both demonstrating and refuting any increase in recurrence following DAAs in varying groups, were released soon after.⁴⁻⁷ These initial reports lacked any form of comparative survival analysis between DAA-exposed and nonexposed groups. Subsequently, a comparison of DAA-exposed and nonexposed groups from the French ANRS Hepather cohort, reported by Pol and colleagues,⁸ treated DAA exposure as time varying and found no increased risk of recurrence in those exposed to DAAs (HR: 1.21, 95% CI 0.62-2.34). However, this cohort started following future DAA-exposed patients from a median 23 months after the original HCC diagnosis and was criticized for possible underreporting of HCC recurrence.⁹ A recent review by Reig et al¹⁰ presents an overview of the conflicting evidence that has been presented so far in this debate; the types of studies conducted, heterogeneity in the populations included, and variability in the analytical methods used, suggesting that no firm conclusions can yet be drawn on this topic.

HCC is the most common cancer in Egypt,¹¹ largely due to the country having the highest global prevalence of HCV.¹² Due to the availability of low-cost branded and generic DAAs, the Egyptian Ministry of Health and the National Committee for the Control of Viral Hepatitis have treated approximately one million Egyptian patients since 2014, with cure rates over 90% using various DAA combinations.¹³ Therefore, we examined HCC recurrence within 2 years of initial HCC complete radiological response in an HCV-infected Egyptian cohort, for those who either were or were not given DAAs

through use of comparative time-dependent analysis and propensity scoring.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

This study was carried out at one HCC treatment centre in Cairo, Egypt. Patients of all Barcelona Clinic Liver Cancer (BCLC) stages and prognoses were consulted at this centre through clinical and imaging examinations and those with BCLC stages 0 and A were treated through local ablation procedures; these patients had a maximum of three cancerous lesions, with the largest lesion being <5 cm in diameter. Local ablative procedures available at this centre included the following: radiofrequency ablation (RFA), microwave ablation (MWA), percutaneous ethanol injection (PEI), surgical resection and transarterial chemoembolization (TACE). Following the ablative procedure, patients were followed up after one and 3 months with dynamic computerized tomography (CT) and ultrasound imaging to assess tumour response. After complete radiological response according to modified RECIST criteria (hereafter referred to as “complete radiological response”) had been confirmed by a senior radiologist using these imaging techniques at both month one and months three visits, the patients returned for follow-up imaging every three to 6 months, for continued confirmation of complete radiological response until death or loss to follow-up. HCC follow-up imaging at these time intervals was the same for patients both eventually treated and not treated with DAAs.

Following HCC complete radiological response, patients were referred back to local hepatologists at varying National HCV Treatment Centers where they were examined for DAA treatment eligibility. If the patient's clinical indicators fit within the national HCV treatment guidelines, they were given, free of charge, either a three- or six-month regimen of DAA. These patients' viral loads were assessed at the end of their DAA treatment period (EOT) as well as 12 weeks of post-treatment to establish whether or not there has been a sustained virological response (SVR12). The choice of whether or not a patient with a history of HCC will receive DAAs was dependent on the patient's decision to seek treatment and the decision of the consulting hepatologist at the HCV treatment centre. Patient eligibility, in the Egyptian context, refers to the patient having no contraindication to DAAs, a good prognosis and limited liver damage. During the first wave of DAA introduction, this meant that patients with Child-Pugh scores greater than 6 were not eligible for DAA treatment, nor those greater than 65 years of age. The decision of the consulting hepatologist to either treat immediately, wait to treat or never treat, a patient with a history of HCC was based on treatment ineligibility or their own personal practice method with some hepatologists recommending waiting during the first 2 years post-HCC complete radiological response as this is a period when recurrence is common.

According to the current Egyptian national treatment guidelines, all-oral DAAs can be given as soon as 1 month following the HCC ablative manoeuvre, as long as the patient was seen to have a complete

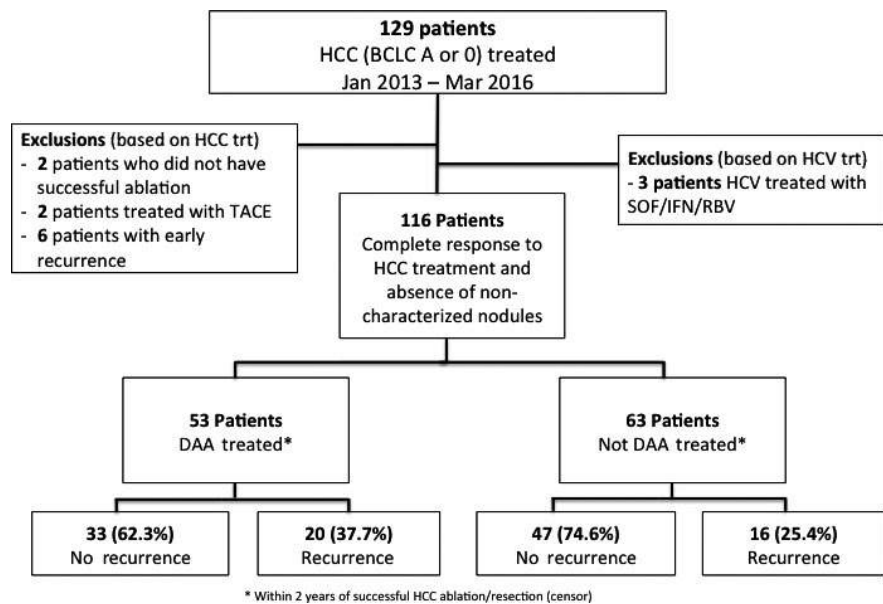


FIGURE 1 Application of inclusion and exclusion criteria to study population with basic recurrence proportions

radiological response through dynamic CT and ultrasound during this visit. Patients with a history of HCC being examined for DAA treatment eligibility need to have had a dynamic CT scan and ultrasound confirming lack of HCC recurrence in the 3 months prior to the DAA start date; any patient who has not had this imaging performed within this time frame through their regular visits at the HCC treatment centre, was required to return to the centre to do so.

2.2 | Procedures

For the presented analysis, we applied various inclusion and exclusion criteria to the total population of BCLC A and 0 patients who visited the HCC treatment centre for local ablation between January 2013 and March 2016 (Figure 1).

We consecutively included patients who achieved HCC complete radiological response according to modified RECIST criteria between 2013 and 2016. All patients needed to be HCV-positive and not coinfecting with HBV or HIV. HCV genotype was not specified for inclusion, and indeed, genotyping was not carried out on all patients; however, most HCV infection in Egypt is with genotype 4.¹⁴ All included patients were treated for their HCC with local ablative procedures including the following: RFA, MWA, PEI and surgical resection.

We excluded patients who were treated using TACE, as this manoeuvre can be considered palliative. For those who were treated with DAAs during the two-year analysis window, we excluded any patients who had received interferon-containing DAA regimens (ie Sofosbuvir/Interferon/RBV). We also excluded any patients with early recurrence within 3 months of the date of complete radiological response, to further ensure that newly detected tumours represented true recurrence and were not residual but previously undetected.¹⁵

This study obtained ethical approval by the Institutional Review Board for Human Subject Research at the National Hepatology and Tropical Medicine Research Institute in Cairo, Egypt, which is organized

and operated according to the Declaration of Helsinki for Human Subject Research (2013). All patients provided written informed consent to have their data included in this analysis.

2.3 | Statistical analysis

After applying the previously described inclusion and exclusion criteria to our original population, persons eventually treated or not treated with DAAs within the two-year follow-up period were compared for their baseline characteristics (ie at the time of HCC ablative manoeuvre) using the Student's *t* test for continuous variables and chi-square or Fisher's exact test for categorical variables. For time-dependent analysis, Poisson regression models were preferred to Cox models because they allow the estimation of incidence rates according to DAA exposure (or nonexposure). Censoring was performed when a patient died, was lost to follow-up or was at the end of their two-year follow-up period. For the main analysis, DAA treatment was treated as time varying, with exposure starting from the date of DAA start and ending at the endpoint/censoring (thus reflecting a current or past exposure to DAA).

Incidence rate ratio for DAA treatment exposure was estimated using univariable and multivariable Poisson regression models. Covariates included in the multivariable model were: time since entry into the cohort (<8 months, 8-16 months and 16-24 months), sex, age (categorized as < or ≥65 years old), baseline Child-Pugh score (categorized as ≤ or >6) and whether or not the patient had ever had gastroesophageal varices (a possible indicator of portal hypertension). Furthermore, to minimize the effect of confounding by treatment indication, we used inverse probability of treatment weighting (IPTW) using propensity scores.¹⁶ The probability of receiving DAA treatment was modelled based on time since entry into the cohort, sex, age, baseline Child-Pugh score and history of gastroesophageal varices. Stabilized weights were calculated using the R package "ipw".¹⁷ The average treatment effect was estimated using a robust variance

estimator to account for the weighted nature of the sample. IPTW diagnostics were conducted as recommended by Austin and Stuart.¹⁸

A sensitivity analysis removed all patients with Child-Pugh score 7 and over the age of 65, as these were DAA treatment ineligibility criteria during the first phase of DAA introduction in Egypt. For a subanalysis, DAA treatment exposure, still treated as time varying, was broken down into three periods: no treatment, the first 6 months after the start of DAA treatment and 6 months after the start of DAA treatment. A further subanalysis examined the difference between exposure to three- and six-month DAA treatment regimens. All statistical analyses were conducted using R.

3 | RESULTS

Between January 2013 and May 2016, 129 HCV-infected HCC patients, all BCLC stage A or O, were consulted and treated with local ablation procedures at the HCC treatment centre in Cairo, Egypt. Of these, the following were excluded: two patients who were treated with TACE; two patients who never achieved a complete radiological

response; six patients who had early recurrence of HCC (two of these were already taking DAAs); three patients who were eventually treated with DAA regimens containing interferon. This left us with a total of 116 patients, 53 of whom eventually received DAAs during the two-year analysis follow-up period and 63 who did not (Figure S1A,B).

The DAA-exposed and nonexposed groups were similar in terms of sex, age and diabetes as well as their number of hepatic focal lesions and size of their largest lesion at baseline (Table 1). No patients in our cohort were alcoholic or had a history of alcoholism.

The Child-Pugh score was lower in those treated with DAAs; 1.9% of the DAA-exposed group had a score of 7, whilst in the DAA nonexposed group, 17.5% ($P = .006$) had a score of 7. The ablative manoeuvres performed on patients in both groups were similar—the majority of patients in both groups underwent radiofrequency ablation. The DAA-exposed group received varying all-oral regimens for either three- or six-month periods. SVR12 was confirmed in 77.4% of the DAA-treated patients overall and in 89.2% of patients when excluding those treated with Sofosbuvir/RBV (Table 2).

Among the 53 patients treated with DAAs, we observed 37.7% recurrence after a median of 16.0 months of follow-up. Among the 63 patients not treated with DAAs, we observed a 25.4% HCC recurrence after a median 23.0 months of follow-up. The association between DAA exposure and recurrence can be seen in Table 3. The unadjusted rate, per 100 person-months, of recurrence was 1.00 (95% Confidence Interval, CI: 0.51-1.49) in the DAA nonexposed group vs 4.06 (95% CI: 2.30-5.85) in the DAA-exposed group, representing a crude incidence rate ratio (IRR) of 4.08 (95% CI: 2.14-7.76) associated with DAA treatment. After controlling for time since HCC complete radiological response (ie time in cohort) as well as sex, age, Child-Pugh score and history of gastroesophageal varices using IPTW, exposure to DAA treatment was associated with a significantly increased risk of recurrence of 3.82 (95% CI: 2.00-7.30), consistently with results found in the multivariate analysis (Table 3). The results of IPTW diagnostics are presented in Supplementary Material.

For patients with recurrence, the location of cancerous lesions was found in a new site for 15 (93.8%) of those not treated with DAAs and 19 (95.0%) of those treated with DAAs. In examination of whether or not patients treated with DAAs had a more aggressive recurrence compared to their non-DAA-exposed counterparts, we observed that six (30.0%) of the recurring patients with DAA exposure had greater than three hepatic focal lesions, but we did not have enough statistical power to show whether or not this was higher than the two (18.0%) patients with this characteristic in the DAA nonexposed recurring population. Among the DAA-treated group, the incidence of recurrence was not different among those who did achieve SRV12 as compared to those who did not (unadjusted IRR = 1.64, 95% IC: 0.64-4.20).

Although three (2.6%) patients were censored due to being lost to follow-up, there was no censorship due to death within the 2 years following inclusion in the study for patients without recurrence. No patients underwent liver transplantation.

When excluding patients who did not achieve SVR12 ($n = 12$), the recurrence IRR was 4.18 (95% CI: 1.64-10.69). The results were

TABLE 1 Baseline characteristics, at the time of HCC complete radiological response, for patients eventually treated and not treated with DAAs

Baseline Characteristic	DAA-treated (N = 53)	Not DAA-treated (N = 63)	P-value
Male, # (%)	35 (66.0)	41 (65.1)	.91
Age, mean (P25-P75)	56.7 (52-62)	57.3 (51-62)	.70
Diabetes, # (%)	17 (32.1)	16 (25.4)	.43
Child-Pugh score			.006 ^a
5	26 (49.1)	30 (47.6)	
6	26 (49.1)	22 (34.9)	
7	1 (1.9)	11 (17.5)	
# Hepatic focal lesions [HFL]			.06
1	50 (94.3)	57 (90.5)	
2	1 (1.9)	6 (9.5)	
3	2 (3.8)	0	
History of gastroesophageal varices	30 (56.6%)	42 (66.7%)	.34
Largest HFL in cm, mean (P25-P75)	2.4 (2.0-3.0)	2.5 (2.0-3.0)	.50
HCC Treatment Manoeuvre, # (%)			.30
RFA	31 (58.5)	48 (76.2)	
PEI or PEI/RFA	13 (24.5)	7 (11.1)	
MWA	7 (13.2)	6 (9.5)	
Surgical Resection	2 (3.8)	2 (3.2)	

^aChild-Pugh score 5 and 6 against Child-Pugh score 7.

TABLE 2 DAA treatment regimens, end of treatment and SVR12 proportions in the DAA-exposed group (N = 53)

DAA Treatment Regimen	Duration of Treatment (mo)	# Treated	% EOT	% SVR12
SOF/RBV	6	16 (30.2)	75.0	50.0
SIM/SOF	3	9 (17.0)	100	88.9
SOF/DCV/RBV	3	8 (15.1)	100	100
	6	1 (1.9)	100	100
SOF/DCV	3	11 (20.8)	90.9	81.8
	6	4 (7.5)	100	75.0
SOF/LDV/RBV	6	2 (3.8)	100	100
SOF/LDV	6	2 (3.8)	100	100
Total	-	53 (100)	90.6	77.4

EOT = undetectable viremia at end of DAA treatment, SVR12 = sustained virological response 12 wk after the end of DAA treatment.

consistent when considering only patients treated with six-month DAA regimens (IRR = 3.39; 95% CI: 1.56-7.37) or when considering only patients treated with three-month regimens (IRR = 3.66; 95% CI: 1.73-7.76). A sensitivity analysis using IPTW and excluding all patients with Child-Pugh score of 7 and aged >65 demonstrated a recurrence IRR of 5.62 (95% CI: 2.52-12.18) for DAA-exposed vs nonexposed patients. A second sensitivity analysis split DAA exposure into two periods: the first 6 months after the start of DAAs and the time following 6 months after the start of DAAs. Using multivariable Poisson regression (nondichotomous nature of this exposure variable prevented the use of IPTW), we found an adjusted recurrence IRR of 3.24 (95% CI: 1.50-7.01) in the first 6 months of DAA exposure and 4.17 (95% CI: 1.73-10.05) in the post six-month DAA-exposed groups, respectively.

4 | DISCUSSION

Our data point to a high (ie almost 4 times) increased rate of recurrence after DAA treatment for patients with a history of successfully treated HCC, when compared to similar patients who were not given DAAs. This significantly higher rate of recurrence in the DAA-exposed group remained after adjustment for baseline factors and time since HCC complete radiological response through inverse probability weighting, as well as across the sensitivity analyses performed. As far as we know, this is the first propensity scored comparative time-dependent analysis for DAA-exposed and nonexposed patients followed from the moment of HCC complete radiological response.

It has been suggested that rapid changes to the immune surveillance system and/or antitumour response following DAA treatment could be the reason for the apparent increase in HCC recurrence.¹⁹ A recent observational study by Villani et al²⁰ supported this idea through demonstration that during treatment with DAAs, an angiogenesis inducer called vascular endothelial growth factor, which supports tumour development, increases significantly and can remain high until 3 months after DAA treatment. However, our own results showed a similar association between DAAs and HCC recurrence for

those with longer (6 months) vs shorter (3 months) DAA exposures (IRR 3.39 vs 3.66, respectively).

The necessary assumption of this comparative analysis is that DAA-exposed and nonexposed patients are similar. However, it can be argued that in an era of highly expensive DAA treatments for HCV, it is possible that clinicians systematically choose to treat patients with certain clinical indicators over others; for example, in Egypt, treating patients with better prognoses has been estimated as more cost-effective based on quality-adjusted life expectancy outcomes.²¹ Our inclusion criteria, especially treatment initiation from 2013 onwards and rapid complete radiological response in response to initial HCC treatment (ie seen already at one-month postablative manoeuvre), were specifically chosen to improve comparability between treated and untreated patients. As a result, baseline characteristics of the DAA-exposed and DAA nonexposed groups in the study presented here appeared to be balanced, except for baseline Child-Pugh score. Moreover, in the analysis, we used IPTW to further balance measured covariates between treated and untreated patients, including possible time-varying confounders such as time since inclusion in the cohort. Our sensitivity analysis, which further controlled the groups through exclusion of all patients with a Child-Pugh score of 7, showed the same magnitudes of effect and significance level as our primary analysis.

It is possible that there are other confounding factors that were not collected as part of this study. Indeed, although baseline characteristics are similar for all patients in our study, we do not have updated records of liver enzyme changes at each imaging follow-up visit. We argue, though, that we can assume that any selection bias in DAA administration would trend towards the treatment of those with better prognosis (eg younger age, limited liver damage), at least in the case of patients with a history of HCC in Egypt. Within the Egyptian National Treatment Program, a patient with a history of HCC would not be given DAAs immediately following HCC complete radiological response because of either ineligibility for treatment according to the Egyptian national treatment guidelines, or due to the personal opinion of the attending hepatologist regarding the need to wait to make sure the cancer is truly gone first.

TABLE 3 Recurrence rates and rate ratios comparing DAA-exposed and nonexposed patients

	Recurrence Rates			Univariable Poisson Regression			Multivariable Poisson Regression			Poisson Regression using IPTW		
	Events	PM	Rate ^a	IRR	95% CI	P	IRR	95% CI	P	IRR	95% CI	P
DAA exposure						<.001			<.001			<.001
No DAAs	16	1605	1.00	1	ref		1	ref		1	ref	
DAAs	20	492	4.06	4.08	2.14-7.76		3.61	1.84-7.11		3.82	2.00-7.30	
Time since cohort entry						.03			.07			
0-8 mo	8	906	0.67	1	ref		1	ref		-	-	-
9-16 mo	19	728	2.39	2.96	1.31-6.69		2.55	1.11-5.86		-	-	-
17-24 mo	9	463	1.69	2.22	0.86-5.65		1.96	0.79-4.86		-	-	-
Sex						.01			.002			
Female	6	802	0.749	1	ref		1	ref		-	-	-
Male	30	1296	2.32	3.09	1.29-7.41		3.45	1.44-8.25		-	-	-
Age						.26			.16			
<65	33	1780	1.85	1	ref		1	ref		-	-	-
≥65	3	318	0.94	0.51	0.16-1.66		1.47	0.53-4.10		-	-	-
Child-Pugh Score						.92			.50			
5/6	32	1875	1.71	1	ref		1	ref		-	-	-
7	4	223	1.80	1.05	0.38-2.90		0.46	0.14-1.48		-	-	-
Gastroesophageal varices						.70			.44			
Never	13	822	1.58	1	ref		1	ref		-	-	-
Ever	23	1275	1.80	1.14	0.58-2.24		1.31	0.67-2.56		-	-	-

PM, person-months; 95% CI, 95% confidence interval; IRR, incidence rate ratio; IPTW, inverse probability of treatment weighting. Bold values indicate key IRR values as well as significant P-values. ^aRate is per 100 person-months; the probability of receiving treatment was modelled using the covariates presented in the table.

TABLE 4 Previous studies evaluating the association between DAA and frequency of recurrence in patients who achieved complete radiological response following HCC treatment

Author	Country	# DAA-exposed	Median time (mo) HCC treatment to DAA exposure	Per cent recurrence in DAA-exposed; (median # mo ^a)	Rate Ratio; (P-value)
A cohort of DAA-exposed patients without a control group (descriptive study)					
Reig et al. ³	Spain	58	11.2	27.6 (5.7 mo)	-
Conti et al. ⁴	Italy	59	12.4	28.8 (not available)	-
Torres et al. ⁶	USA	8	7.5	0 (12 mo)	-
Zavaglia et al. ⁷	Italy	31	19.3	3.2 (8 mo)	-
Time-dependent analysis DAA-exposed and nonexposed patients (analytical study)					
Pol et al. ⁸	France	189	>22.8 ^b	12.7 (20.2 mo)	1.2 (P = .58)
El Kassas et al [Data presented in this article]	Egypt	53	8.0	37.7 (16.0 mo)	3.8 (P < .001)

^aDAA initiation until HCC recurrence in studies of type 1, and median months total follow-up in studies of type 2.

^bMedian 22.8 mo from HCC diagnosis to cohort inclusion, and an additional median 1.4 mo until DAA administration in those eventually exposed to DAAs.

Ineligibility for treatment in this case relates mainly to when a patient had a worse prognosis (eg older age, more deteriorated liver function); this was the case for 11 of our patients who had Child-Pugh score 7, and two patients who were too old (>65 years), who did not receive DAAs according to the applied treatment protocol at that time. For the remaining 50 patients who did not receive DAAs within our two-year analysis period, 14 eventually received DAAs afterwards, 15 had recurrence within this time and were therefore not eligible, and for 21, the attending hepatologist decided not to recommend treatment at all within the viewed follow-up period. Although our results cannot approximate a clinical trial in terms of random treatment assignment, we do assess that any underlying differing factors between the groups would likely be negative confounders; we would expect patients with a better prognosis to be treated with DAAs.

One of the biggest strengths of this study was our ability to follow and analyse recurrence depending on eventual DAA exposure in an entire cohort of HCV-positive patients from the moment of HCC complete radiological response. This has not yet been done in any other robust study; the survival analysis performed by Pol et al⁸ followed patients from time of "cohort inclusion"; this was a median 1.9 and 1.6 years after HCC diagnosis for DAA-exposed and unexposed groups, respectively. All other currently published papers on this subject report recurrence in DAA-exposed groups after varying median number of months following their HCC complete radiological response. A demonstration of these differences, using some of the first studies published within this debate, can be seen in Table 4. The issue with a delayed observation window in these populations is twofold: 1. The biological mechanisms behind early- (within 2 years) and late (> 2 years)-phase recurrence have been reported to differ²² and 2. All patients with recurrence or death prior to the observation window are inherently excluded from the analysis and final recurrence rates. The former indicates that studies observing recurrence in the first 2 years since HCC complete radiological response, such

as the study we present here or that of Reig or Conti are not comparable to those of Pol or Zavaglia.^{3,4,7,8} The second issue with the use of this delayed observation window is that some studies, such as that of Pol and Zavaglia, who have reported "no apparent effect of DAAs" have done so in survivor populations. Recurrence in the first 2 years since HCC complete radiological response has been linked to microscopic vascular invasion, high serum AFP levels and having had nonanatomical resection, whereas after 2 years since HCC complete radiological response, tumours can be considered de novo and are linked to the grade of hepatitis activity, tumour nodule multiplicity and gross tumour classification.²²

In order to draw any interim guidance from the currently published data on this subject, the findings that have been put forth to date should be considered and grouped according to the population included, analysis methods used and the time window of observation for the included patients. Perhaps DAA treatment of surviving patients without any recurrence 2 years after HCC complete radiological response poses no additional risk, whereas administration of DAAs to patients in the first 2 years after HCC complete radiological response should be avoided until clinical trials provide more concrete evidence of their benefit.

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CONFLICT OF INTEREST

The authors declare no personal conflict of interests. Anna Funk is part of the Pasteur-Paris University (PPU) International PhD Program and has received funding from the Institut Carnot Pasteur Maladie Infectieuse (ANR 11-CARN 017-01). This work benefited from the kind contribution of Alice Zuccaire.

AUTHOR CONTRIBUTIONS

MEK, ALF, GE and AF designed the study. MEK, MS, ME, ATS, SA, AET and NFAY provided clinical management to patients and collected the patient data. ALF and KJ performed the statistical analysis. AF and YS advised on statistical methods. ALF and MS drafted the manuscript. All authors approved the final version of this article, including the authorship list.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Risk of hepatitis B virus reactivation with direct-acting antivirals against hepatitis C virus: A cohort study from Egypt and meta-analysis of published data

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Abstract

Background & Aims: Hepatitis B virus (HBV) reactivation in chronic hepatitis C (CHC) patients treated with direct-acting antivirals (DAAs) became an issue. However, its frequency has been poorly estimated, because of the varying definitions used and evaluation of heterogeneous study populations, including those concurrently treated for HBV.

Methods: We prospectively followed HBV surface antigen (HBsAg)-positive Egyptians undergoing interferon-free DAAs, to estimate the risk of HBV reactivation and HBV-related hepatitis. We also conducted a meta-analysis to estimate the reactivation risk using published data obtained from a systematic review of PubMed/Embase, in addition to our Egyptian data. We applied a standard definition of HBV reactivation proposed by the international liver associations (APASL and AASLD).

Results: Of 4471 CHC patients, 35 HBsAg-positive patients started interferon-free DAAs without HBV nucleos(t)ide analogues in our Egyptian cohort. Ten experienced HBV reactivation (28.6%), of whom 1 developed hepatitis (10.0%). Our systematic review identified 18 papers. The pooled reactivation risk in HBsAg-positive patients was 18.2% (95% CI: 7.9%-30.7%) without HBV therapy and 0.0% (95% CI: 0.0%-0.0%) with HBV nucleos(t)ide analogue. The pooled risk of hepatitis in those with HBV reactivation was 12.6% (95% CI: 0.0%-34.7%). The pooled reactivation risk in HBsAg-negative, antibody to HBV core antigen-positive (anti-HBc-positive) patients was negligible (0.1%, 95% CI: 0.0%-0.3%), irrespective of the presence of antibody to HBsAg (anti-HBs).

Conclusions: We confirmed high HBV reactivation risk in HBsAg-positive patients undergoing DAAs, with only a minority developing clinically important hepatitis. The risk is negligible for HBsAg-negative anti-HBc-positive patients.

KEYWORDS

direct-acting antivirals, hepatitis B, hepatitis C, reactivation

Abbreviations: AASLD, American Association for the Study of Liver Diseases; ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to HBsAg; APASL, Asian Pacific Association for the Study of the Liver; AST, aspartate aminotransferase; DAAs, direct-acting antivirals; DCV, daclatasvir; DSV, dasabuvir; EASL, European Association for the Study of the Liver; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; IQR, interquartile range; OBV, ombitasvir; PTV, paritaprevir; ritonavir; RBV, ribavirin; SMV, simeprevir; SOF, sofosbuvir; SVR, sustained virological response.

Mohamed El Kassas and Yusuke Shimakawa contributed equally as joint first authors.

1 | INTRODUCTION

The recent advent of direct-acting antivirals (DAAs) for chronic hepatitis C virus (HCV) infection has dramatically changed the landscape of HCV management. DAA regimens are associated with a sustained virological response (SVR) rate of >90%-95%, and are considered safe; nevertheless, a few serious complications have been reported including reactivation of hepatitis B virus (HBV).^{1,2}

In 2015-2016, several case reports alerted to the risk of HBV reactivation and HBV-related hepatitis during the course of DAAs in patients with chronic HCV infection positive for hepatitis B surface antigen (HBsAg) (chronic HBV infection)^{3,4} and those negative for HBsAg but positive for antibody to hepatitis B core antigen (anti-HBc) (past or resolved HBV infection).^{3,5} Following these reports, including a case of fulminant hepatic failure requiring liver transplantation,⁵ the U.S. Food and Drug Administration and the European Medicine Agency issued a warning about the risk of HBV reactivation with the use of DAAs.^{6,7} Now, both European and American guidelines recommend to screen for HBsAg, anti-HBc and antibody to HBsAg (anti-HBs) in all patients initiating DAAs, and to concurrently start anti-HBV nucleos(t)ide analogue therapy in those who are eligible (ie, high HBV DNA levels ≥ 2000 IU/mL and elevated alanine transaminase (ALT) and/or significant liver fibrosis).^{1,2} However, the recommended management for chronic hepatitis C patients who are ineligible for anti-HBV treatment itself differs considerably between these 2 guidelines. While the European Association for the Study of the Liver (EASL) guidelines recommend to systematically administer concomitant nucleos(t)ide analogue prophylaxis to all the HBsAg-positive patients irrespective of HBV DNA levels until 12 weeks post-DAAs,⁸ the American Association for the Study of Liver Diseases (AASLD) just recommends to monitor inactive HBsAg-positive patients with low HBV viraemia and to start nucleos(t)ide analogues only when their viral load reaches a level fulfilling HBV treatment criteria.² For persons HBsAg-negative but anti-HBc-positive, EASL recommends to closely monitor and test for HBV reactivation in case of ALT elevation, while the AASLD recognizes that there is too little data to make a clear recommendation for monitoring these patients. The discrepancies between these 2 major guidelines highlight the lack of robust evidence in the risk of HBV reactivation in DAA-treated HCV patients.

Few cohort studies have attempted to investigate the frequency of HBV reactivation during DAAs in HBsAg-positive patients and HBsAg-negative anti-HBc-positive patients. These studies have shown that the risk is substantial in the former (>30%, except in 1 study) and minimal in the latter group (<2%).⁹⁻¹³ However, these studies were limited because of: (i) heterogeneity in study participants (inclusion of patients under HBV antiviral treatment); (ii) use of different criteria for HBV reactivation, and (iii) very small sample size in the estimates for the concurrent HBV infection (number assessed in each study was between 7 and 12).⁹⁻¹³ Retrospective analyses of an electronic database of USA veterans provided estimates based on a large number of patients; however, HBV reactivation was not systematically ascertained during and after the course of DAAs, and

Key points

- In a cohort of HBsAg-positive Egyptians with chronic HCV infection treated with DAAs, the risk of HBV reactivation was 28.6% (95% CI: 15.6%-46.4%) and the risk of hepatitis in those who developed reactivation was 10.0% (0.9%-57.8%) in the absence of concurrent HBV treatment.
- In a systematic review that incorporated the Egyptian data, the pooled reactivation risk in HBsAg-positive patients was 18.2% (95% CI: 7.9%-30.7%) and the risk of hepatitis was 12.6% (95% CI: 0.0%-34.7%) in those who had reactivation.
- The pooled reactivation risk in HBsAg-negative anti-HBc-positive patients was negligible (0.1%, 95% CI: 0.0%-0.3%), irrespective of the presence of anti-HBs.

those under anti-HBV nucleos(t)ide analogue therapy were not separated out from the cohort, hampering an accurate estimation of the risk of HBV reactivation.^{14,15}

Here, we prospectively estimated the risk of HBV reactivation and HBV-related hepatitis in a cohort of HBsAg-positive patients treated for HCV using interferon (IFN)-free DAAs in Egypt. We also conducted a systematic review and meta-analysis to estimate the frequency of HBV reactivation in HBV co-infected patients treated for IFN-free DAAs, by applying the well-established definition of HBV reactivation^{16,17} to the previously published data and our new data.

2 | PATIENTS AND METHODS

2.1 | Egyptian cohort study

2.1.1 | Patients

In Egypt, a country with the highest HCV prevalence in the world, the National Treatment Program introduced sofosbuvir (SOF)-based treatment in 2014.¹⁸ As of March 2017, more than one million Egyptian patients with chronic HCV infection have been treated,¹⁹ with >90% achieving a SVR.²⁰

Between March 2015 and March 2016, we prospectively recruited patients co-infected with HBV and HCV who initiated DAAs at the New Cairo Viral Hepatitis Treatment Unit in Cairo, Egypt. According to the Egyptian guidelines, patients with chronic HCV infection (positive antibody to HCV and positive HCV RNA) who were referred to our Unit were systematically assessed for their eligibility for DAA therapy through a standardized clinical and virological assessment, including complete blood count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, albumin, creatinine, alpha-foetoprotein, prothrombin

time, anti-HIV antibody (MINI VIDAS[®], Biomerieux, France), HBsAg (Stat Fax 4200, Dia Sorin, USA), alpha-foetoprotein and abdominal ultrasonography. Some patients underwent liver biopsy. Patients with the following characteristics were ineligible for DAAs according to the national guidelines: Child-Pugh class C cirrhosis, hepatocellular carcinoma, platelet counts $<50 \times 10^9$ cells/L, current pregnancy or breast-feeding. All the patients testing positive for HBsAg during the recruitment period were invited to participate in the current prospective study. After obtaining written informed consent, the patients identified to carry HBsAg at baseline were further assessed for other HBV markers: hepatitis B e antigen (HBeAg, Stat Fax 4200, Dia Sorin, USA) at baseline, and serum HBV DNA measurement (DT Lite Real Time PCR, DNA-Technology, Russia, limit of detection: 25 IU/ml) at baseline, week 4, end of treatment and 12 weeks post-treatment. All the patients received HCV antiviral therapy according to the Egyptian guidelines, and were followed clinically every 2 weeks with complete blood count, AST and ALT. SVR was assessed at 12 weeks after the end of treatment using HCV RNA. Patients co-infected with HIV and those receiving immunosuppressive therapy were excluded from the current analysis. The study was approved by the Institutional Review Board at the National Hepatology and Tropical Medicine Research Institute in Cairo, Egypt.

2.1.2 | Definition

We used the definition of HBV reactivation proposed by the Asian Pacific Association for the Study of the Liver (APASL) guidelines, and by the American Association for the Study of Liver Diseases (AASLD)'s consensus conference: ≥ 2 log increase for those who had detectable HBV DNA at baseline or a new appearance of HBV DNA to a level of ≥ 100 IU/mL for those who had undetectable HBV DNA at baseline.^{16,17} HBV-related hepatitis was defined as ALT ≥ 5 times upper limit of normal or ≥ 2 times of the baseline level in patients who experienced HBV reactivation.^{13,21}

2.1.3 | Statistical analysis

Baseline characteristics were presented using percentage for categorical variables and median and interquartile range (IQR) for continuous variables. Factors associated with HBV reactivation were assessed using logistic regression analysis. Variables found to be associated with HBV reactivation ($P < .25$) were further assessed using multivariable logistic regression using STATA 13.1 (STATA Corporation, College Station, TX, USA).

2.2 | Systematic review and meta-analysis

2.2.1 | Systematic review

After developing a review protocol, we searched PubMed and Embase up to January 2018, using the following search terms and their variation: hepatitis B, hepatitis C, antiviral agents, and reactivation

(Appendix S1). We also conducted a manual search through bibliographies. Without any language restriction, we included cohort studies of chronic HCV patients undertaking IFN-free DAAs who were HBsAg-positive or HBsAg-negative/anti-HBc-positive before starting DAAs. For the studies which also had IFN-treated patients, we only used those within the cohort that were treated with IFN-free regimens. We excluded case series, which described the clinical courses of only those who developed hepatitis B reactivation, because this does not allow us to estimate the risk (frequency) of reactivation. HBV DNA and ALT needed to have been measured at least once during or after (up to 24 weeks post-treatment) DAAs. Since the definition of the primary endpoints, HBV reactivation and HBV-related hepatitis varied substantially between studies, we did not use the risks reported in each paper, but rather we applied the definitions proposed by the APASL and AASLD.^{11,13-15} We only included studies in which the endpoints could be stratified by the concurrent HBV nucleos(t)ide analogue therapy at baseline. Two authors (YS and ALF) independently screened titles and abstracts of all papers identified by the electronic searches. Potentially eligible papers were independently reviewed for their eligibility. Data were extracted for the following variables: study design, setting, baseline characteristics, DAAs regimen, SVR rate and when the outcome (HBV DNA) was measured. The included studies were assessed for the risk of bias (Appendices S2 & S3).²²

2.2.2 | Meta-analysis

The risk of HBV reactivation was calculated as the number of patients with reactivation divided by the total number assessed. The risk of HBV-related hepatitis was calculated as the number with HBV-related hepatitis divided by the number of patients who developed HBV reactivation. The risks were pooled by meta-analysis using "metaprop" command in STATA 13.1.²³ The variance of the proportions was stabilized using Freeman-Tukey double arcsine transformation,²⁴ and the estimates were pooled by the DerSimonian-Laird random-effects model.²⁵ The confidence intervals for the pooled estimates were assessed with the Wald test.²³ The percentage of total variation between studies because of heterogeneity was evaluated by the I^2 statistic. The pooled estimates were presented separately according to the HBV serology at baseline: HBsAg-positive, and HBsAg-negative/anti-HBc-positive. Subgroup analyses were made to explore the sources of between-study heterogeneity in the risk of HBV reactivation using a test of heterogeneity of the "metaprop" command,²³ and the following variables were assessed: concurrent anti-HBV therapy at baseline; HBV genotype; HCV genotype; HIV co-infection; achievement of SVR12; and positivity of antibody to hepatitis B surface antigen (anti-HBs) in those negative for HBsAg but positive for anti-HBc.

3 | RESULTS

3.1 | Egyptian cohort study

Between March 2015 and March 2016, 4471 patients with chronic HCV infection were assessed at the New Cairo Viral Hepatitis

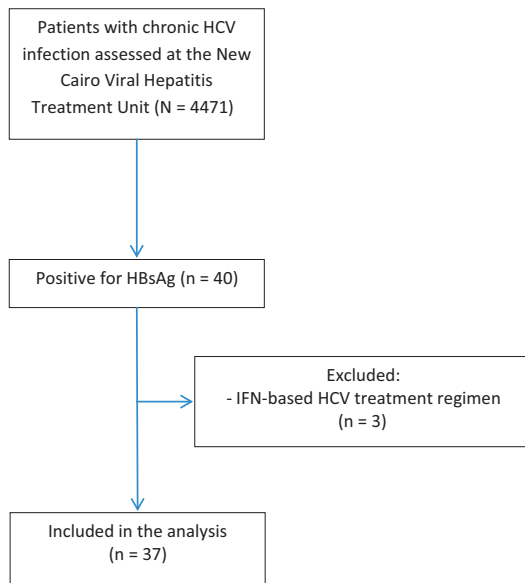


FIGURE 1 Flow chart of study participants in Egyptian cohort study

Treatment Unit for the eligibility for DAAs. All were tested for HBsAg, and 40 were found to carry HBsAg (prevalence: 0.89%, 95% CI: 0.66-1.22%). Of these, 3 patients who received DAA regimens that included IFN were excluded from the analysis (Figure 1). None were co-infected with HIV or receiving immunosuppressive therapy.

Baseline characteristics of the 37 patients with concurrent HBV infection are presented in Table 1. Median age was 52 years (IQR: 48-56), and 62.2% were men. Ten (27.0%) patients had cirrhosis, based on liver histopathology ($n = 5$) or abdominal ultrasonography ($n = 5$). None carried HBeAg, and HBV viral load was detectable in 13 patients (35.1%). The median HBV DNA level was 2.8 log₁₀ IU/ml (range: 1.4-4.7). Two patients were under treatment for HBV at baseline: one treated with lamivudine since 1 year, and another concurrently initiated entecavir because of high HBV viral load (43 600 IU/mL) found at baseline. The following DAA regimens were administered: daclatasvir (DCV)/SOF with or without ribavirin (RBV) for 12 weeks ($n = 19$), SOF/RBV for 24 weeks ($n = 10$), simeprevir (SMV)/SOF for 12 weeks ($n = 7$) and ombitasvir (OBV)/paritaprevir ritonavir (PTV)/dasabuvir (DSV) with ribavirin for 12 weeks ($n = 1$). All patients completed the full course of treatment except for one who was treated with SOF/RBV. This patient, without cirrhosis at baseline, developed fatigue and anorexia at week 12, with HBV reactivation (HBV DNA levels increased from 544 IU/mL at baseline to 400 000 IU/mL), after which entecavir was added to the SOF/RBV regimen. At week 16, the patient developed HBV-related hepatitis with mild ascites, elevated liver enzymes (ALT 465 U/mL, AST 406/mL U/mL) and jaundice (total bilirubin 3.3 mg/dL), which necessitated the cessation of anti-HCV treatment. One month after the cessation of the DAAs, the ascites resolved and liver functions returned to normal. Of the 36 patients who completed DAAs, 34 patients achieved

SVR12 (Table 1). The 2 who did not achieve SVR12 had both received SOF/RBV for 24 weeks, and had undetectable HCV RNA at the end of treatment; however, the relapse was confirmed at 12 weeks post-treatment.

Out of the 35 co-infected patients without HBV therapy at baseline, 10 developed HBV reactivation either during the course of DAAs or in the period up to 12 weeks post-treatment; the risk of reactivation was 28.6% (95% CI: 15.6%-46.4%). The clinical courses of these patients are summarized in Appendix S4. Except for the 1 patient, reported above, who developed HBV-related hepatitis and subsequently started entecavir, none had a clinically important ALT elevation compared to the baseline level and thus required anti-HBV therapy. The risk of hepatitis in those who experienced HBV reactivation was 10.0% (1/10, 95% CI: 0.9%-57.8%). HBV reactivation was not observed in any of the 2 patients under anti-HBV treatment at baseline.

We tried to identify factors associated with HBV reactivation among those without HBV therapy at baseline ($n = 35$). No factor was significantly associated with HBV reactivation (Appendix S5). Neither of the 2 HCV relapsers had HBV reactivation.

3.2 | Systematic review and meta-analysis

The search strategy identified 862 papers. After excluding duplicates, 774 were screened, and 63 papers were assessed in full-text. Finally, 18 articles (comprising 17 unique cohorts) were found to be eligible, and after adding our Egyptian study, a total of 18 cohorts were included in our meta-analysis (Figure 2). The large retrospective studies of USA veterans were not eligible because of the fact that the study reported the overall risk of HBV reactivation regardless of whether or not the patients were under anti-HBV nucleos(t)ide analogue treatments, and we could not stratify the analysis by the concurrent administration of anti-HBV treatment.^{14,15} The characteristics of the included studies and our Egyptian study are summarized in Table 2. A total of 425 patients positive for HBsAg (including the 37 patients from the Egyptian study), and 1900 patients negative for HBsAg but positive for anti-HBc were used to estimate the pooled risks. Nine studies (10 articles) were from East Asia,^{10,12,13,26-32} 6 from Europe,^{11,33-37} 1 from USA³⁸ and 1 from New Zealand.⁹ All of these studies included patients on IFN-free DAAs regimens for 8-24 weeks. While most of studies reported HCV genotype, only 3 studies reported HBV genotype.^{9,31,32} Risk of bias in these studies is summarized in Appendix S3.

3.2.1 | Pooled risk of HBV reactivation

In patients with concurrent chronic HBV infection, the pooled risk of HBV reactivation without anti-HBV therapy was 18.2% (95% CI: 7.9%-30.7%; Figure 3A). This was significantly higher than the risk in HBsAg-positive patients concurrently treated with HBV nucleos(t)ide analogue, in which none experienced HBV reactivation (0.0%, 95% CI: 0.0%-0.0%, $P < .001$; Figure 3B). In HBsAg-positive patients

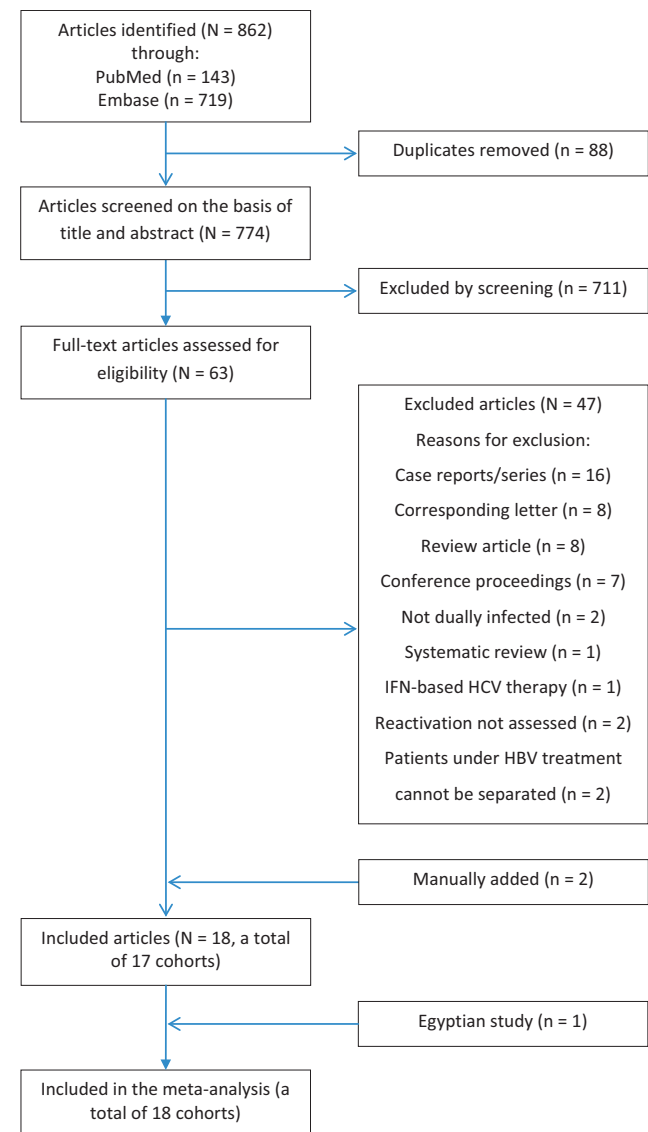
TABLE 1 Baseline and follow-up data (n = 37)^a

Baseline data	
Median age	52 (48-56)
Male gender (%)	23 (62.2)
Median BMI	31 (29-34)
Diabetes (%)	8/36 (22.2)
HCV treatment-naïve	31 (83.8)
HCV treatment regimen	
DCV/SOF±RBV	19 (51.3)
SMV/SOF	7 (18.9)
SOF+RBV	10 (27.0)
OBV/PTV/DSV+RBV	1 (2.7)
HCV RNA (IU/mL)	
<10 ³	1 (2.7)
10 ³ -10 ⁶	26 (70.3)
≥10 ⁶	10 (27.0)
Median HCV RNA (log ₁₀ IU/mL)	5.6 (4.7-6.1)
HBsAg (%)	37 (100)
HBeAg (%)	0
HBV DNA (IU/mL)	
Undetectable	24 (64.9)
<2000	11 (29.7)
≥2000	2 (5.4)
Median HBV DNA (log ₁₀ IU/mL) ^b	2.8 (2.3-3.2)
Concurrent HBV antiviral therapy	2 (5.4)
ALT (U/mL)	
<40	15 (40.5)
40-80	15 (40.5)
≥80	7 (19.0)
AST (U/mL)	
<40	14 (37.8)
40-80	17 (46.0)
≥80	6 (16.2)
Median total bilirubin (mg/dL)	0.7 (0.6-1.0)
Median albumin (g/dL)	4.1 (3.8-4.4)
Median creatinine (mg/dL)	0.8 (0.7-0.9)
Median platelet count (10 ⁹ /L)	146 (116-208)
Fib-4	
<1.45	13 (35.1)
1.45-3.25	12 (32.4)
≥3.25	12 (32.4)
Cirrhosis ^c	10 (27.0)
Follow-up data	
HCV SVR12	
Achieved	34 (91.9)
Not achieved	2 (5.4)
Stopped treatment	1 (2.7)

(Continues)

TABLE 1 (Continued)

Follow-up data	
HBV reactivation in those without HBV therapy	10/35 (28.6)
HBV-related hepatitis in those without HBV therapy	1/35 (2.9)
HBV reactivation in those under HBV therapy	0/2 (0)
HBV-related hepatitis in those under HBV therapy	0/2 (0)

^aMedian values are presented with IQR.^bExcluding those with undetectable HBV DNA.^cThe diagnosis was based on liver histopathology (n = 9) or abdominal ultrasonography (n = 28).**FIGURE 2** Flow diagram of study selection in systematic review

without HBV therapy, the risk of reactivation tended to be higher in those who achieved HCV SVR (21.8%, 95% CI: 11.5%-33.6%) than those without SVR (0.0%, 95% CI: 0.0%-57.0%) (Appendix S6). However, this did not reach statistical significance ($P = .4$) given the

TABLE 2 Characteristics of the studies identified by the systematic review (n = 17) and our Egyptian study

Author	Country	No. included in the current analysis		Baseline characteristics before starting DAAs			
		HBsAg (+)	HBsAg (-) and Anti-HBc (+)	Age ^a	Men (%)	Cirrhosis (%)	HCV genotype (%)
Gane EJ	New Zealand	8	ND	Mean (SD) 53 (7)	75	25	1a: 75 1b: 25
Wang C	China	10	124	54 (20-75)	57	58	1a: 82 2a: 18
Sulkowski MS	Korea Taiwan	0	103	58 (36-75)	42	18	1: 1 1a: 8 1b: 91
Londono MC	Spain	10	64	61 (20-84)	53	44	1a: 14 1b: 70 2: 2 3: 7 4: 7
Yeh ML	Taiwan	7	57	63 (35-81)	27	34	1: 72
Kawagishi N	Japan	1	82	69 (44-87)	47	51	1: 72 2: 28
Liu CH	Taiwan	12	81	Mean (SD) 56 (8)	50	33	1a: 2 1b: 82 2: 16
Doi A	Japan	4	143	73 (36-90)	52	26	1: 80 2: 20
Ogawa E	Japan	0	63	71 (43-82)	41	24	1: 65 2: 35
Mucke VT	Germany	8	249	57 (18-86)	59	36	1a: 28 1b: 35 2: 7 3: 20 4-6: 10
Loggi E	Italy	2	42	62 (48-86)	70	93	1a: 18 1b: 52 2: 9 3: 11 4: 9
Calvaruso V	Italy	8	37	Mean (SD) 64 (9)	69	100	1a: 9 1b: 60 2: 22 3: 4 4: 4
Macera M	Italy	29	ND	61 (38-80)	72	86	NR
Tamori A	Japan	25	765	70 (22-92)	48	28	1: 79 2: 21
Liu CJ	Taiwan	110	ND	Mean (range) 55 (32-76)	38	16	1: 61 2: 39
Preda CM	Romania	15	ND	60 (51-72)	27	100	1b: 100
Yanny BT	USA	139	90	Mean (SD) 60 (7)	58	43	1: 93 4: 7
El Kassas M	Egypt	37	ND	52 (48-56)	62	32	NR

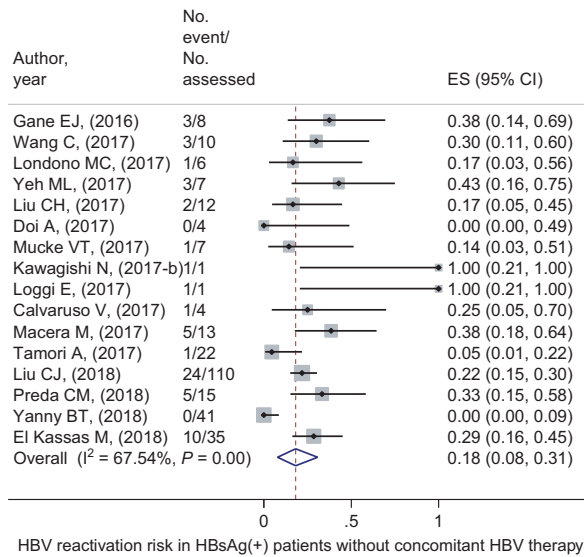
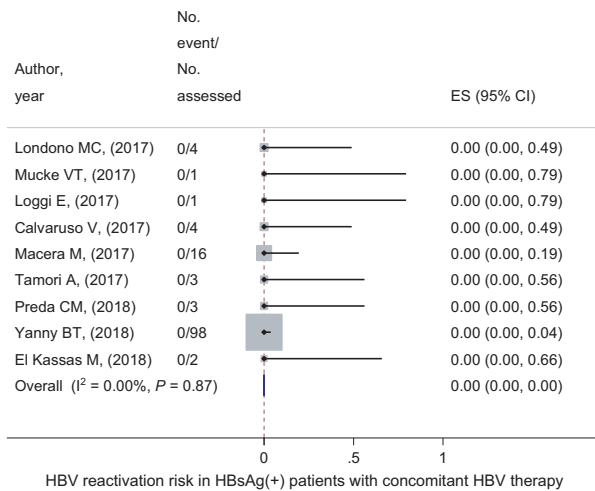
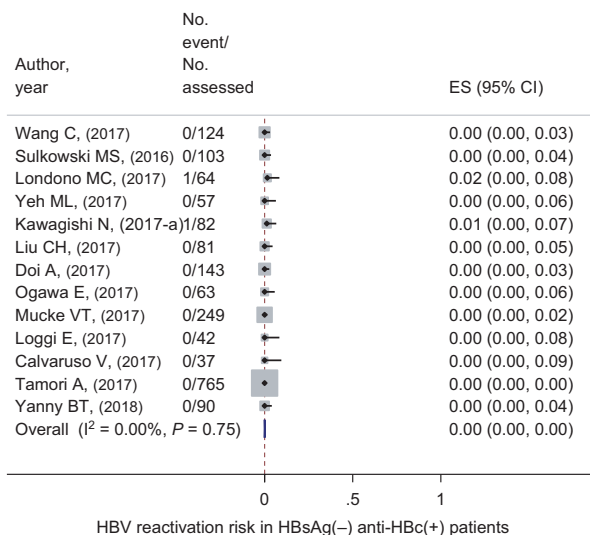
AASLD, American Association for the Study of Liver Diseases; APASL, Asian Pacific Association for the Study of the Liver; DCV, daclatasvir; DSV, dasabuvir; LDV, ledipasvir; ND, not done; NR, not reported; OBV, ombitasvir; PTV, paritaprevir/ritonavir; RBV, ribavirin; SD, standard deviation; SMV, simeprevir; SOF, sofosbuvir.

^aMedian age (range) is presented unless indicated.

^bFor the meta-analysis, we did not use these definitions. We used the definition of HBV reactivation proposed by the AASLD and APASL.

^cSVR 24 is presented.

HBV therapy in HBsAg (+) patients (%)	HIV (%)	DAA regimens	SVR 12 (%)	When the outcome (HBV DNA) measured	Definition of HBV reactivation used in each study ^b
0	0	LDV/SOF	100	Multiple from the start of DAA till 12 wks after the EOT	NR
0	NR	LDV/SOF DCV/SOF OBV/PTV+DSV	100	Multiple from the start of DAA till 12 wks after the EOT	>2.0 log increase in HBV DNA
0	NR	LDV/SOF	98	Once at 24 wks after the EOT	Abrupt reappearance/rise of HBV DNA
40	1	OBV/PTV±DSV±RBV LDV/SOF±RBV DCV/SOF±RBV SOF+RBV DCV/SMV	NR	Multiple from the start of DAA till 12 wks after the EOT	>1.0 log increase in HBV DNA
0	0	LDV/SOF±RBV OBV/PTV+DSV DCV/SOF DCV/ASV SOF+RBV	97	Multiple from the start of DAA till the EOT	>1.0 log increase in HBV DNA for those with HBV DNA(+); Reappearance of HBV DNA for those with HBV DNA(-); Reappearance of HBsAg or HBV DNA >2000 IU/mL for those with HBsAg(-)
0	NR	LDV/SOF DCV/ASV SOF+RBV	95	Once at the EOT	>1.3 log increase in HBV DNA
0	0	LDV/SOF±RBV SOF+RBV OBV/PTV/DSV±RBV	NR	Multiple from the start of DAA till 12 wks after the EOT	AASLD/APASL
0	NR	LDV/SOF SOF+RBV	98	4 and 12 wks after the start of DAA	HBV DNA >20 IU/mL for those with <20 IU/mL; or >1.0 log change in HBV DNA
0	0	LDV/SOF SOF+RBV	94 ^c	Multiple from the start of DAA till 24 wks after the EOT	HBV DNA >20 IU/mL
13	1	LDV/SOF±RBV DCV/SOF±RBV OBV/PTV±DSV±RBV SOF+RBV	94	Once at the EOT	Abrupt reappearance/rise of HBV DNA
50	NR	LDV/SOF±RBV DCV/SOF±RBV SMV/SOF±RBV OBV/PTV/DSV±RBV SOF+RBV OBV+RBV	93	Once at 24 wks after the EOT	AASLD/APASL
50	0	LDV/SOF±RBV DCV/SOF+RBV SMV/SOF+RBV OBV/PTV/DSV+RBV SOF+RBV	94	Multiple from the start of DAA till 12 wks after the EOT	HBV DNA >20 IU/mL for HBsAg(+) with HBV DNA(-); Reappearance of HBV DNA for HBsAg(-) and HBV DNA(-)
55	NR	SMV/SOF LDV/SOF DCV/SOF OBV/PTV/DSV	93	Multiple from the start of DAA till 12 wks after the EOT	>1.0 log increase in HBV DNA for those with HBV DNA(+); Reappearance of HBV DNA for those with HBV DNA(-)
12	NR	LDV/SOF DCV/ASV OBV/PTV SOF+RBV	99	Multiple from the start of DAA till 12 wks after the EOT	>2.0 log increase in HBV DNA for those with HBV DNA(+); Reappearance of HBV DNA >20 IU/mL for those with HBV DNA(-)
0	0	LDV/SOF	100	Multiple from the start of DAA till 108 wks after the EOT	>1.0 log increase in HBV DNA for those with HBV DNA(+); Reappearance of HBV DNA for those with HBV DNA(-)
0	0	OBV/PTV/DSV+RBV	100	Multiple from the start of DAA till 12 wks after the EOT	AASLD/APASL
70	NR	LDV/SOF	91 (G1) and 84 (G4)	At least once from the start of DAA till 12 wks after the EOT	Increase in AST or ALT ≥3 times the ULN and reappearance of HBV DNA detection or HBsAg
5	0	DCV/SOF±RBV SMV/SOF SOF+RBV OBV/PTV/DSV+RBV	92	Multiple from the start of DAA till 12 wks after the EOT	AASLD/APASL

(A) In HBsAg-positive patients without concomitant anti-HBV nucleos(t)ide analogue therapy**(B)** In HBsAg-positive patients with concomitant anti-HBV nucleos(t)ide analogue therapy**(C)** In HBsAg-negative anti-HBc-positive patients**FIGURE 3** Pooled risk of HBV reactivation. A, In HBsAg-positive patients without concomitant anti-HBV nucleos(t)ide analogue therapy. B, In HBsAg-positive patients with concomitant anti-HBV nucleos(t)ide analogue therapy. C, In HBsAg-negative anti-HBc-positive patients

small sample size in the latter group ($n = 3$). Subgroup analysis according to HBV genotype, HCV genotype, and HIV co-infection could not be performed as in most of the studies not enough data was provided to stratify the outcomes (HBV reactivation) by these variables.

In patients with negative HBsAg and positive anti-HBc, only 2 out of 1900 patients developed HBV reactivation; the pooled risk was 0.1% (95% CI: 0.0%-0.3%; Figure 3C). This was significantly lower than the risk in HBsAg-positive patients without HBV treatment (18.2%, 95% CI: 7.9%-30.7%, $P < .001$). The risk of reactivation in HBsAg-negative anti-HBc-positive patients was negligible irrespective of the presence of anti-HBs: 0.0% (95% CI: 0.0%-0.0%) in patients negative for anti-HBs (isolated anti-HBc) and 0.1% (95% CI: 0.0%-0.5%, $P = .5$) in patients positive for anti-HBs (Appendix S7).

3.2.2 | Pooled risk of HBV-related hepatitis

Of 61 HBsAg-positive patients without concurrent HBV nucleos(t)ide analogue treatment who experienced HBV reactivation after DAAs, 11 developed hepatitis. The pooled risk of HBV-related hepatitis in those with HBV reactivation was 12.6% (95% CI: 0.1%-34.7%; Figure 4). Of 2 HBsAg-negative anti-HBc-positive patients who experienced HBV reactivation, none had HBV-related hepatitis; the pooled risk was 0.0% (95% CI: 0.0%-78.7%).

4 | DISCUSSION

In the cohort of chronic hepatitis C patients positive for HBsAg who underwent DAAs in Egypt, we found that in the absence of concurrent HBV treatment the risk of reactivation was 28.6% (95% CI: 15.6%-46.4%) and the risk of hepatitis in those who developed reactivation was 10.0% (95% CI: 0.9%-57.8%). In the subsequent systematic review that also incorporated these Egyptian data, we found that: (i) the pooled risk of HBV reactivation in patients concurrently infected with HBV was 18.2% (95% CI: 7.9%-30.7%) without HBV therapy and 0.0% (95% CI: 0.0%-0.0%) with HBV suppressive treatment; (ii) the pooled risk of reactivation in HBsAg-negative anti-HBc-positive patients was negligible 0.1% (95% CI: 0.0%-0.3%), irrespective of the presence of anti-HBs; (iii) the pooled risk of hepatitis in patients who developed HBV reactivation was 12.6% (95% CI: 0.1%-34.7%) in HBsAg-positive, and 0.0% (95% CI: 0.0%-78.7%) in HBsAg-negative group.

Recently, a systematic review was conducted to estimate the risk of HBV reactivation in HBV/HCV dually infected patients treated with HCV antiviral agents³⁹ and found that 12.2% (95% CI: 0.2%-33.2%) of those treated with IFN-free DAAs developed HBV reactivation. However, the review was limited because most of the

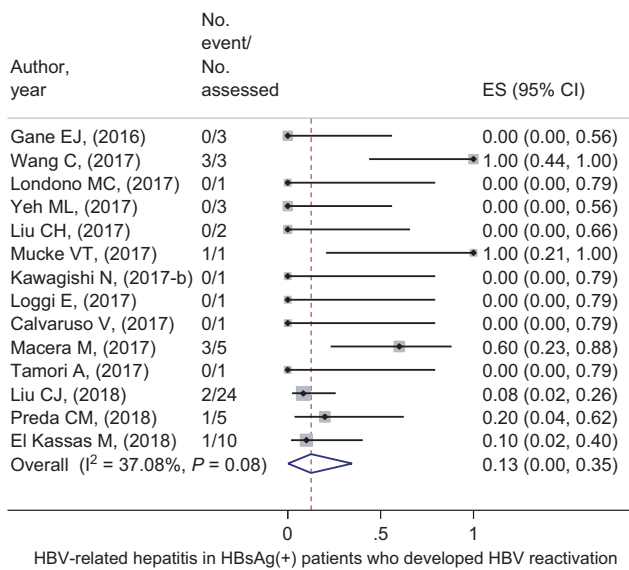


FIGURE 4 Pooled risk of hepatitis in HBsAg-positive patients who developed HBV reactivation

included studies used patients on IFN-based regimens, and there were only 2 studies with patients on IFN-free DAAs. Moreover, the meta-analysis endpoint was HBV reactivation as defined by the authors of each article, which varied considerably.⁴⁰ In the systematic review and meta-analysis that we present here, we were able to include 18 studies with patients on IFN-free DAAs and use a common endpoint definition, proposed by the APASL and AASLD. This standard definition requires ≥ 2.0 log increase in viral load for those who had detectable HBV DNA at baseline.^{16,17} In contrast, many of the studies included in our systematic review used lower cut-off values in their published papers: ≥ 1.0 log,^{11,12,29} or ≥ 1.3 log^{27,28} increase in HBV DNA. Previous longitudinal studies of HBV mono-infected patients have shown that 25%-50% of chronic HBV carriers experienced a spontaneous fluctuation of HBV DNA levels ≥ 1.0 log IU/ml over short period of time,^{41,42} suggesting that the use of these lower cut-off levels might result in overestimation of the frequency of HBV reactivation.

Considering that only around 20% of HBsAg-positive co-infected patients develop HBV reactivation during or after DAAs, it would be useful for clinicians to be able to identify patients at a high-risk for this event who would most benefit from concurrent anti-HBV nucleos(t)ide analogue prophylaxis. A Taiwanese study suggested that higher quantified HBsAg levels at baseline might be associated with higher risk of HBV reactivation during DAA therapy.¹³ In our Egyptian cohort, no factor was found to be associated with HBV reactivation, probably because of lack of power. Although it did not reach statistical significance, our meta-analysis found that the pooled risk of reactivation tended to be higher in those who achieved SVR than non-SVR. The positive association between HCV SVR and higher risk of HBV reactivation has been confirmed in co-infected patients treated with IFN plus RBV.⁴³ These findings support the hypothesis that, in dually infected individuals, the successful control

of HCV may result in an upsurge of HBV that has previously been suppressed by the dominant HCV.

Some studies have reported factors associated with reactivation in HBsAg-negative anti-HBc-positive patients treated with DAAs: lower anti-HBs antibody titre at baseline,^{28,29} higher ALT levels,²⁹ younger age³⁵ and certain HCV genotypes.³⁵ In our meta-analysis, we did not see any difference in reactivation risk between the isolated anti-HBc group (anti-HBs-negative) and the resolved HBV group (anti-HBs-positive); the risk of former group was already very low (0.0%, 95% CI: 0.0%-0.0%).

Although the total number of patients was small ($n = 132$), our systematic review found that no HBsAg-positive patients who had been under HBV nucleos(t)ide analogue therapy had HBV reactivation after the start of DAAs. This is reassuring, as it supports the preventive efficacy of the concurrent nucleos(t)ide analogues in reducing the risk of reactivation in patients receiving DAAs.

In our Egyptian study, we found that 0.9% (40/4471) of patients with chronic HCV infection were co-infected with HBV. This co-infection rate is very similar to the prevalence of HBsAg reported in the general population in Egypt.^{44,45}

Our study has several limitations. Firstly, in the Egyptian cohort we only studied HBsAg-positive patients and not those HBsAg-negative but anti-HBc-positive. This was a result of the fact that anti-HBc screening is not part of the Egyptian national guidelines. Nevertheless, our systematic review found that the risk of reactivation in this group is homogeneously very small throughout other published studies (Figure 3C). Secondly, we could not assess other factors that may modify the effect of IFN-free DAA regimens on the risk of reactivation, such as HIV co-infection, HCV genotype, HBV genotype or quantified HBsAg levels. There were a few studies included in the systematic review which reported these, but it was not possible to stratify the estimates by these variables.

In conclusion, our study confirmed the importance of screening for HBV in patients undergoing DAA therapy. For those negative for HBsAg but positive for anti-HBc, the EASL recommendation of monitoring and testing for HBV DNA in case of ALT elevation seems reasonable as HBV reactivation and particularly HBV-related hepatitis are rare in this group. In contrast, for those positive for HBsAg, the risk of reactivation was substantial (18.2%, 95% CI: 7.9%-30.7%). However, only the minority (12.6%, 95% CI: 0.1%-34.7%) of these HBV reactivation events resulted in clinically important ALT elevation, which may limit the benefit of systematic nucleos(t)ide analogue prophylaxis for all HBsAg-positive patients. In our Egyptian cohort, for example, if we had followed the EASL recommendation (ie, starting nucleos(t)ide analogue prophylaxis for all 35 HBsAg-positive patients irrespective of their HBV DNA levels at baseline), this could have prevented 1 case of HBV-related hepatitis during the course of DAAs, but might have resulted in over-treatment in the rest of the subjects ($n = 34$). As has been studied in lymphoma patients undergoing chemotherapy,⁴⁶ this question deserves to be addressed through a randomized controlled trial assessing the efficacy of systematic nucleos(t)ide analogue prophylaxis (EASL recommendation) in preventing

HBV-related hepatitis, compared to “on-demand” HBV therapy implemented only when HBV DNA levels become elevated (AASLD recommendation), in chronic HCV patients with inactive HBV infection with low HBV viraemia who are administered IFN-free DAAs.

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CONFLICT OF INTEREST

The authors do not have any disclosures to report.

AUTHOR CONTRIBUTIONS

MEK, ZAE, GE, YS and AF designed the Egyptian study. MEK, ZAE, MNW, SZ and FER were in charge of clinical management and data collection. YS performed the statistical analysis. AF and ALF advised on statistical methods. YS designed and YS and ALF conducted the systematic review and meta-analysis. YS and ZAE drafted the manuscript. All authors critically reviewed and contributed to the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Letter: concordance of SVR4 and SVR12 following direct-acting anti-viral treatment in Egypt

EDITORS,

In your recently published paper by Omar and colleagues, we are presented with the results of 18 378 patients treated for chronic hepatitis C (HCV) with direct-acting anti-virals (DAAs) through the Egyptian National Treatment Program.¹ During the same time period within the programme, retention of patients until the assessment of sustained virological response (SVR12) posed a significant logistical hurdle and led to 29%-40% of patients being lost to follow-up.^{2,3} Use of a more prompt endpoint, 4 weeks after the end of treatment (SVR4), could potentially lower these proportions and subsequently support evaluations of Egyptian and other national treatment programmes administering DAAs; however, SVR4 has previously shown to be inadequate.⁴

To compare SVR4 to the conventional endpoint, SVR12, in a real-life setting, the programme carried out a prospective, observational cohort study of patients receiving interferon-free DAAs at the New Cairo Hospital treatment centre in Cairo, Egypt, between September 2014 and December 2016. All patients eligible for treatment according to the Egyptian National Guidelines were invited to participate. Participants received 2 vouchers for free quantitative HCV RNA testing 4 and 12 weeks post-treatment and real-time PCR was done at the centre's laboratory or other selected national

laboratories. Patients had clinical follow-up at the centre every 4 weeks until the end of treatment, then at 4 and 12 weeks post-treatment.

Of the 2899 patients treated at the centre who agreed to participate, 233 (8.0%) were treated with IFN containing DAA regimens, and 196 (6.8%) were lost to follow-up, leaving 2470 (85.2%) eligible for analysis. Patient characteristics are described in the Table 1. The DAAs administered include sofosbuvir and daclatasvir with or without ribavirin (n = 1559, 63.1%), sofosbuvir and simeprevir (n = 586, 23.7%), sofosbuvir with ribavirin (n = 285, 11.5%), ombitasvir/paritaprevir with ribavirin (n = 40, 1.6%). A total of 5 and 15 patients had detectable HCV RNA at 4 and 12 weeks post-treatment, leading to an estimation of SVR4 and SVR12 as 99.8% (95% CI: 99.5%-99.9%) and 99.4% (95% CI: 99.0%-99.7%) respectively. The sensitivity of viral load testing at 4 weeks compared to 12-week post-treatment was 33.3% (5/15, 95% CI: 11.8%-61.6%) and the specificity was 100% (2455/2455; one-sided 97.5% CI: 99.9%-100%). The negative and positive predictive values were 99.6%, (2455/2465, 95% CI: 99.3%-99.8%) and 100% (5/5, one-sided 97.5% CI: 47.8%-100%) respectively.

In this study, viral load testing 4 weeks post-treatment misclassified a small number of individuals (ie 10/2470, 0.4%), but did not

TABLE 1 Baseline characteristics and viral load dynamics for treatment failing discordant and concordant patients, with summary baseline characteristics for all study patients

Baseline evaluation						SVR4 Viral load ^a log ₁₀ IU/mL	SVR12 Viral load ^a log ₁₀ IU/mL
Sex	Age	Viral load log ₁₀ IU/mL	Past HCV treatment	DAA regimen/duration in wk	Fib4 score		
Discordant treatment failures (n = 10), summary: male = 7 (70.0); age = 55.4 (11.4); viral load in log ₁₀ IU/mL = 13.1 (1.5); treatment experienced = 0; fib4 score = 6.6 (4.9)							
F	64	14.3	No	SOF/DCV/RBV; 12	5.7	Not detected	9.8
M	67	10.8	No	SOF/RBV; 24	14.4	Not detected	Detected;UA
M	61	14.7	No	SOF/DCV/RBV; 12	15.8	Not detected	10.7
M	47	12.5	No	SOF/SIM; 12	2.3	Not detected	11.2
M	29	11.5	No	SOF/DCV; 12	0.6	Not detected	11.0
F	57	13.1	No	SOF/DCV/RBV; 12	4.8	Not detected	11.4
M	58	12.9	No	SOF/RBV; 24	5.4	Not detected	Detected;UA
M	59	13.9	No	SOF/SIM; 12	8.8	Not detected	13.2
M	47	15.3	No	SOF/DCV/RBV; 12	4.8	Not detected	10.6
F	65	12.0	No	SOF/SIM; 12	3.9	Not detected	11.7
Concordant treatment failures (n = 5), summary: male = 2 (40.0); age = 56.0 (7.8); viral load in log ₁₀ IU/mL = 13.0 (0.9); treatment experienced = 2 (20.0); fib4 score = 7.6 (5.8)							
M	59	13.7	No	SOF/RBV; 24	4.9	Detected; UA	13.7
F	68	13.0	No	SOF/RBV; 24	17.7	14.0	Detected; UA
F	53	11.8	IFN/RBV	SOF/SIM; 12	7.3	6.9	6.9
M	52	12.2	IFN/RBV	SOF/RBV; 24	4.6	Detected; UA	Detected; UA
F	48	14.0	No	SOF/RBV; 24	3.7	Detected; UA	11.1
Concordant treatment successes (n = 2455), summary: male = 1045 (42.6%); age = 51.8 (11.3); viral load in log ₁₀ IU/mL = 13.0 (1.9); treatment experience = 249 (10.1); fib4 score = 2.4 (2.2)							
ALL PATIENTS (N = 2470), summary: male = 1054 (42.7); age = 51.8 (11.3); viral load in log ₁₀ IU/mL = 13.0 (1.9); treatment experience = 251 (10.2); fib4 score = 2.5 (2.3)							

Summary statistics are presented as n (%) or mean (SD) for categorical and continuous variables respectively.

^aUA indicates a detected viral load, but quantitative value unavailable.

identify the majority (66.6%) of eventual treatment failures. Practically, within the Egyptian National Treatment Program, which aims to treat 350 000 persons annually, this would leave 1400 viremic persons returning to their communities as transmitters, with a higher risk of complications and without a referral for further DAA treatment.⁵⁻⁷ Evaluations of other intermediate endpoints, such as SVR8 as well as studies examining patient factors contributing to relapse between the end of treatment and SVR12, are needed. In the meantime, various other measures have been employed in Egypt to ensure higher proportions of patients undertake viral load testing at SVR12, including text message and phone call reminders as well as delivery of "cure" certificates upon demonstration of SVR12 results.^{2,8}

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Letter: tacrolimus may be hazardous in decompensated autoimmune liver disease with hyperbilirubinaemia

EDITORS,

This letter is with regard to the published article by Liberal et al.¹ The authors described expert clinical management of autoimmune hepatitis in the real world. The article provides useful information managing the therapy of autoimmune hepatitis (AIH); however, there was little data regarding tacrolimus as a second-line treatment. We describe our own experience of the effectiveness and safety of tacrolimus in the treatment of decompensated autoimmune liver disease.

In the last 6 months, 6 patients with autoimmune liver disease in our department have been treated with tacrolimus. One was diagnosed as AIH and hepatitis B cirrhosis, and the others were diagnosed as AIH-primary biliary cirrhosis overlap syndrome. Their basic clinical data are shown in Table 1. As described in Table 1, all

patients were prescribed tacrolimus when they proved refractory to standard treatment (prednisolone alone or a combination with azathioprine). However, no patient achieved a biochemical remission despite the change to tacrolimus treatment. Importantly, 3 patients died shortly after they were treated with tacrolimus.

According to the data shown in Table 1, the median serum tacrolimus concentration of the 6 patients was 4.6 (3.3, 7.2) ng/mL, which conformed to the effective dose described in most other studies,²⁻⁶ but the outcome was not the same. After analysing the basic status of these patients, we found that all 6 patients had hepatic decompensation prior to treatment with tacrolimus. Notably most of them had hyperbilirubinaemia, with a median total bilirubin of 182.7 (49.5, 270.2) µmol/L and a median direct bilirubin of 158.4 (26.6, 219.1) µmol/L. Highest serum bilirubin was present in the 3 patients who



MERS-CoV at the Animal–Human Interface: Inputs on Exposure Pathways from an Expert-Opinion Elicitation

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Nearly 4 years after the first report of the emergence of Middle-East respiratory syndrome Coronavirus (MERS-CoV) and nearly 1800 human cases later, the ecology of MERS-CoV, its epidemiology, and more than risk factors of MERS-CoV transmission between camels are poorly understood. Knowledge about the pathways and mechanisms of transmission from animals to humans is limited; as of yet, transmission risks have not been quantified. Moreover the divergent sanitary situations and exposures to animals among populations in the Arabian Peninsula, where human primary cases appear to dominate, vs. other regions in the Middle East and Africa, with no reported human clinical cases and where the virus has been detected only in dromedaries, represents huge scientific and health challenges. Here, we have used expert-opinion elicitation in order to obtain ideas on relative importance of MERS-CoV risk factors and estimates of transmission risks from various types of contact between humans and dromedaries. Fourteen experts with diverse and extensive experience in MERS-CoV relevant fields were enrolled and completed an online questionnaire that examined pathways based on several scenarios, e.g., camels–camels, camels–human, bats/other species to camels/humans, and the role of diverse biological substances (milk, urine, etc.) and potential fomites. Experts believed that dromedary camels play the largest role in MERS-CoV infection of other dromedaries; however, they also indicated a significant influence of the season (i.e. calving or weaning periods) on transmission risk. All experts thought that MERS-CoV-infected dromedaries and asymptomatic humans play the most important role in infection of humans, with bats and other species presenting a possible, but yet undefined, risk. Direct and indirect contact of humans with dromedary camels were identified as the most risky types of contact, when compared to consumption of various camel products, with estimated “most likely” incidence risks of at least 22 and 13% for direct and indirect contact, respectively. The results of our study are consistent with available, yet very limited, published data regarding the potential pathways of transmission of MERS-CoV at the animal–human interface. These results identify key knowledge gaps and highlight the need for more comprehensive, yet focused research to be conducted to better understand transmission between dromedaries and humans.

Keywords: MERS-CoV, animal–human interface, transmission, epidemiology, infection, risk factors

INTRODUCTION

Nearly 4 years after the first report of the emergence of Middle-East respiratory syndrome Coronavirus (MERS-CoV) in humans and more than 1800 human cases later (1), mainly in Saudi Arabia (~75% of cases and almost all of the primary cases), the ecology of MERS-CoV and its epidemiology remain poorly understood (2). Human-to-human transmission of MERS-CoV accounts for approximately half of all the MERS-CoV cases reported to date (2). Inter-human transmission has been well documented in health care-associated outbreaks in the Middle East and Korea (3–5), and there appears to be limited inter-human transmission in household settings (6).

Many studies have now identified dromedary camels (*Camelus dromedarius*; dromedaries) as a natural host for MERS-CoV, and there appears to be ample evidence of widespread infection (either past or present) in dromedaries in the Middle East (7–10)

and in many parts of Africa (11–15). High levels of MERS-CoV specific seroprevalence have been observed in dromedaries, ranging from 0% in Central Asia to as much as 100% in Africa and the Arabian Peninsula (7–17) (see **Figure 1**). MERS-CoV strains isolated from dromedaries are genetically and phenotypically very similar or identical to those infecting humans (18, 19).

Since the beginning of the MERS-CoV outbreak, animals and specifically dromedaries, have been suspected of playing a role in transmission. The global camel population has more than doubled in the past 50 years, reaching ~30 million today, 95% of which are dromedaries. Approximately 60% of camels are found in East African countries, which are important exporters to the Arabian peninsula and Egypt (20). Camels play a major role in socio-cultural traditions in Saudi Arabia; a place where the camel population has increased from 80,000 to 200,000 heads over the last 50 years; a number which some experts estimate is actually closer to 800,000 heads (21). In parallel, a drastic decrease (from

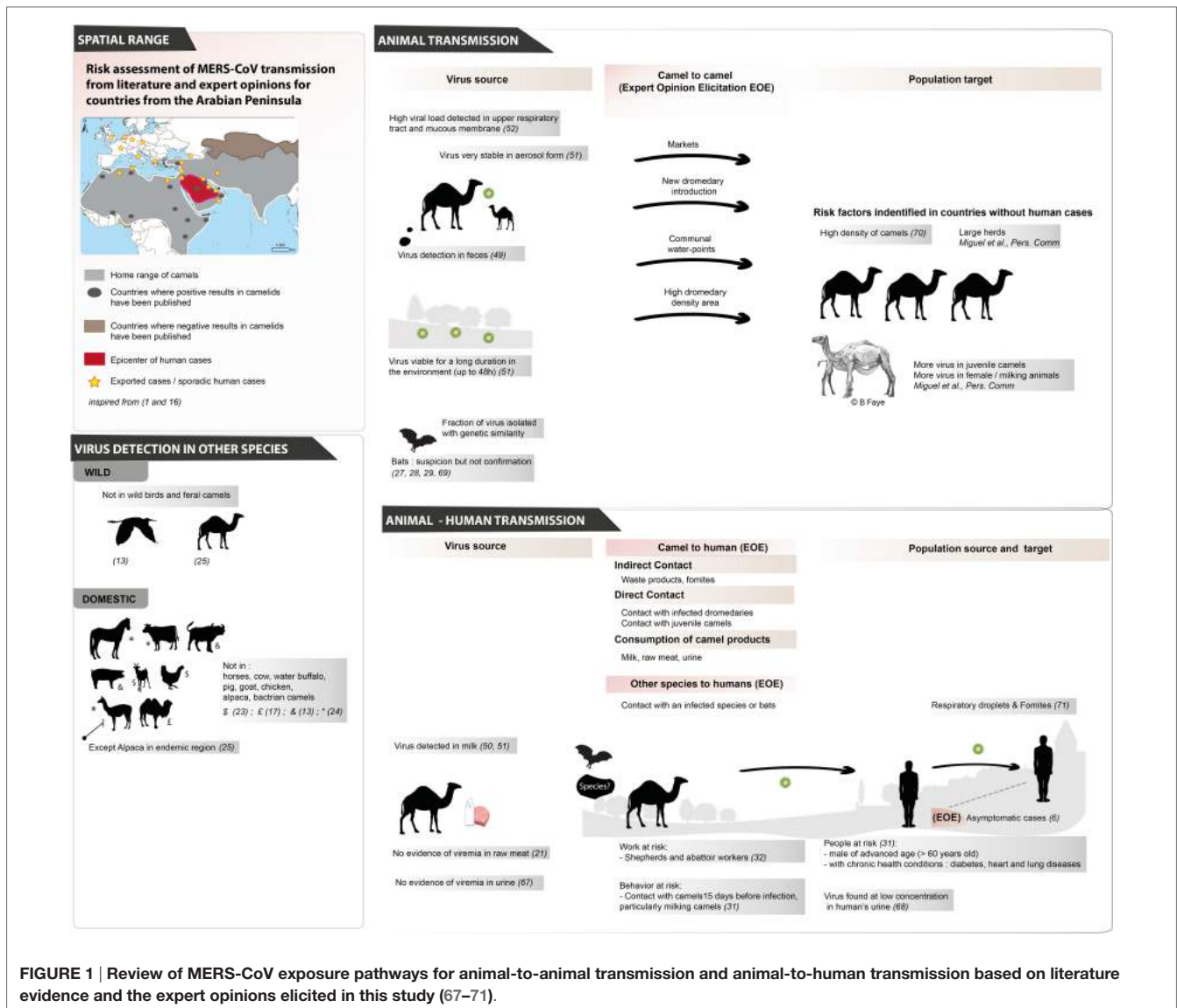


FIGURE 1 | Review of MERS-CoV exposure pathways for animal-to-animal transmission and animal-to-human transmission based on literature evidence and the expert opinions elicited in this study (67–71).

10 to 1.5%) of nomadic camel populations has been observed over this time period in favor of permanent (or semi-permanent) settlements often at the borders of cities (21). It is possible that the mentioned changes in global dromedary population dynamics have led to an increased spread and heightened detection of MERS-CoV in this species, both of which have made dromedaries the focus of most of the research conducted on MERS-CoV to date.

While coronaviruses are widespread in the animal kingdom (22), MERS-CoV seems to have a narrow host range. In the last few years, a large spectrum of domestic species have been negative after MERS-CoV serology tests, including horses, cattle, pig, water buffalo, chickens, goats, and Bactrian camels (13, 14, 17, 23, 24). An exception was published recently when antibodies were detected in Alpaca (*Vicugna pacos*) in Qatar; this is notably in a specific region where MERS-CoV is already endemic in dromedary camels (25) (**Figure 1**).

A number of studies on wild birds and swine in Hong Kong, feral camels in Australia and bats in several countries have not identified MERS-CoV in these species (13, 26) (**Figure 1**). Putative precursors of MERS-CoV have been detected in species of African bats (27), and Corman and colleagues raised hypotheses on the emergence of MERS-CoV from other animal species (28). They characterized the full genome of an African bat virus closely related to MERS-CoV and showed that human, camel, and bat viruses have phylogenetic relationships although these bat viruses are not closely similar to MERS-CoV. They suggest that, according to available serologic data on camels and humans since 2012 and molecular investigations of known cases, MERS-CoV moved from bats to camels in sub-Saharan Africa. They also suggest that camelids could be “mixing vessels for MERS-CoV and other mammalian CoVs” and that the virus can be transmitted between humans and camels (28). Up to now, MERS-CoV-like viruses have not been detected in any species other than camels, with the exception of an unconfirmed report of the detection of a very small fragment of MERS-CoV-like RNA in a specimen from a *Taphozous perforatus* bat collected in Saudi Arabia (29). *T. perforatus* and other bat species sampled in Egypt and Lebanon did not reveal MERS-CoV like viruses, although other coronaviruses were detected (30).

However, after more than 1800 reported cases over the past 4 years from 27 countries, only one case–control study evaluating non-human risk factors for infection has been performed and published (31). This study, which included 30 primary cases and 116 age, sex, and neighborhood-matched controls, confirmed suspicions that direct and indirect exposure to dromedary camels in the 14 days prior to symptom onset are risk factors for infection (31). This study also found that advanced age (>60 years old), being male, and having certain underlying chronic health conditions, such as diabetes, heart conditions, and chronic lung disease, were independent risk factors for disease (31). Several other seroprevalence studies evaluating the extent of MERS-CoV infection in occupationally exposed persons (e.g., farmers, herders, slaughterhouse workers) have identified that these populations have a higher levels of seroprevalence (32, 33) when compared to the general population (32) (see **Figure 1**).

What is currently unclear is why all primary human MERS-CoV cases have been reported from the Arabian Peninsula (2). Given that there is evidence of MERS-CoV circulation in dromedaries across large parts of Africa (11–15), it is likely that cases of MERS-CoV in humans have been missed. There are several potential hypotheses to explain this. First, surveillance for MERS-CoV in human populations focuses mostly on severe disease and on travelers returning from the Arabian Peninsula rather than on patients without a history of travel. Moreover, on-going surveillance in Saudi Arabia is now very intensive (34). Second, the prevalence of chronic underlying medical conditions in many countries in Africa is far lower than in the Middle East, with high rates of heart disease, diabetes, and obesity; third, it is likely that asymptomatic, mild or sub-clinical cases are missed with even the most robust surveillance systems. Fourth, the nature of contact with and the use of dromedary products differ between countries and cultures. Lastly, the viruses circulating in the Arabian Peninsula may be different. Although MERS-CoV in Africa are >99% identical at the nucleotide level with those in the Arabian Peninsula (35), it is conceivable that a few key amino-acid differences may make a major change in transmissibility and virulence.

In the case of MERS-CoV transmission, there is a large uncertainty about the various exposure pathways associated with new dromedary camel or human cases, and, although published research on MERS-CoV is actively increasing (36), few transmission risks have yet been quantified. There is an obvious need to collect more critical information from virological and eco-epidemiological studies, but also from social sciences (anthropology, sociology) studies about camel–human relationships, including behaviors at the interface. These studies can evaluate contact patterns, modes of transmission, viral shedding from animals, virus persistence in different environments, and biological samples. In view of all that remains to be done, we advocate a risk-ranking approach based on exposure pathways to guide allocation of resources for future data collection on the main sources of transmission of MERS-CoV. Risk assessment is a powerful modeling tool that enables decision-makers to determine the likelihood of disease occurrence and the magnitude of its consequences, which, in turn, allows identification of key steps and appropriate management measures to take. It is a structured and a systematic process that helps in the gathering of diverse and disparate information and data. However, when data are scarce and knowledge gaps are highly prevalent, such as with the recently identified MERS-CoV, too many transmission pathways would have been presented for the risk analysis. This is why we proposed, as a preliminary step, to call upon experts using expert-opinion elicitation (EOE), to explore scenarios and hypotheses of transmission among animal(s), fomites, and humans. From the EOE outputs, a qualitative and/or quantitative risk assessment model could then be implemented. Expert-opinion elicitation has proven to be useful in other zoonotic disease risk assessments, especially in cases where little quantitative information for the disease is already known (37, 38). The aim of this work is to allow for a triage of highly likely and unlikely pathways, and highlight areas that deserve increased attention for field surveys and studies.

MATERIALS AND METHODS

In our study, experts were defined as being persons with relevant experience on the topic, including having extensive technical experience in epidemiological or virological research through MERS-CoV or related animal and/or human studies. Considering the recent emergence of the virus as a cause for human disease, extensive experience in MERS-CoV research itself was not an inclusion criteria; however, all included experts needed to have some experience working on MERS-CoV and/or camel research topics within North Africa and the Arabian Peninsula, if not elsewhere. Furthermore, the experts’ publications and professional affiliations should have been significant enough to reflect this expertise. Recruitment was done first through relevance screening, where the researchers chose persons based on their own judgment. Following this original recruitment, “snowball” recruitment was used; experts who chose to participate were asked to recommend other experts to fill out the questionnaire. We aimed to enroll at least 10 experts, with extensive experience in relevant fields, for the exercise and, therefore, started by emailing invitations to 13 persons. All persons recommended by the first group of experts were invited to participate if their expertise was judged relevant for our study. All experts gave an informed consent before starting their participation in the survey. Written consent was not necessary for this type of study; experts could withdraw themselves from the study at any time and all opinion “results” would be presented in an anonymous fashion.

Searching into MERS-CoV literature (36, 39) and meeting reports (40) allowed us to identify potential pathways and risk factors needed for designing the EOE (see **Figure 1**).

The questionnaire was designed online using the tool Survey Monkey (www.surveymonkey.com). A pilot survey was sent to the team members in order to test the survey and optimize the consistency of the questions. A clear description of the study objectives and of what was expected for their participation was provided to experts in the invitation email. Following their acceptance to participate, the experts were emailed a link to the online survey. The beginning of the online survey included instructions, examples, and contact information of the administering researchers.

The questionnaire was designed to take about 30–40 min, and be filled in by experts individually using a link to online software (see Image S1 in Supplementary Material). It consisted of expertise questions, relative importance of risk factor questions,

transmission risk estimations, and open-ended responses, in that order, all of which will be described in more detail below.

The analytical hierarchy process (AHP) (41, 42) is widely used in marketing research (43) and has more recently been introduced as a tool in veterinary epidemiology (38, 44). AHP obtains opinions on the weight of the relative importance of one attribute of an object or event over another, through pair-wise comparisons. In our questionnaire, we used the technique to obtain experts opinions about the most relevant exposure pathways and their relative importance for five different animal–animal or animal–human transmission scenarios. Where appropriate, simple transmission diagrams were used to explain the potential exposure pathways in question. Experts were first asked to identify which exposure pathway they “believed in” out of a provided list, always with the opportunity to specify “other.” They were then asked to do pair-wise comparisons of each exposure pathway, comparing the risk factors of transmission, using the Saaty scale (41) (**Figure 2**).

In order to obtain quantitative estimates on the transmission risks from dromedaries to humans, we asked experts for their 3-point estimation (minimum, most likely, and maximum) considering different types of exposure between 10 susceptible camel workers and dromedaries. Exposures included consumption of camel products (e.g., milk, urine, meat), direct and indirect contact; separate estimations were asked for different scenarios of younger (≤ 50 years) or older (> 50 years) camel workers and adult or juvenile dromedaries. Using the same method, experts were also asked their estimates for transmission between potentially asymptomatic camel workers and family contacts.

Finally, a few open-ended questions on factors that may increase or decrease transmission and were posed to experts.

The survey was not anonymous in order to be able to come back to the experts in case of inconsistency in their answers. For every question, the experts were asked to respond not only expressing their opinion but also to assess their own confidence in their answers for each question, with a score from 1 to 5.

The analytical hierarchy process allowed us to weight each exposure pathway according to the level of importance given to it by each expert. Additional weight was attributed to each answer according to the level of confidence given by the expert. Then, for each pathway, a weighted aggregation of all expert answers was generated. An expert’s data were excluded from the combined estimates in case of any of the following criteria: $< 30\%$ consistency ratio, obvious erroneous entry, missing data for part of or the entire question. In this case, a 30% consistency ratio cut-off, taking into consideration that the historically recommended

<i>More important</i>					<i>Less important</i>			
Extremely	Very strongly	Strongly	Moderately	Equivalent	Moderately	Strongly	Very strongly	Extremely
9	7	5	3	1	1/3	1/5	1/7	1/9

FIGURE 2 | Simplified Saaty Scale used for comparing risk factors in the analytical hierarchy process.

10% cut-off is shown to be too severe for comparison matrices that have >3 variables and that the cut-off should increase with number of variables (45, 46); our questions introduced up to eight variables for each matrix. The overall level of agreement across experts in their ranking of the selected risk factors was calculated using the Kendall's W coefficient. The average weighted minimum, most likely and maximum transmission risk for each of the 3-point estimation questions was also generated using a similar weighting mechanism. An expert's data was excluded from the 3-point distribution combined estimates in case of consistently highly outlying estimates or missing data for part of or the entire distribution. Outliers were defined as estimated risks that were consistently greater than twice the estimates of all other experts. When possible, for missing or erroneous data, experts were re-contacted by email to clarify. The mean confidence level, across all included experts, was calculated for each pair-wise comparison and 3-point distribution question. Open-ended responses were summarized qualitatively.

RESULTS

Overall, 18 experts were contacted to take part in the questionnaire. Of these, 16 responded to the invitation, and 14 filled out the questionnaire in full, contributing data to this study. All respondents, except 1, had expertise in either MERS-CoV epidemiology and/or virology; the remaining expert had significant experience in camel production and husbandry and general epidemiology. Six and three respondents had experience in conducting studies of camels and bats, respectively. A detailed description of each participant's expertise can be found in **Table 1**.

MERS-CoV Infection of Dromedary Camels

On the topic of how dromedaries become infected with MERS-CoV, the following exposure pathways were presented to experts: infestation of infected bats in close proximity, daily close contact with infected camel workers (both ≤50 and >50 years old), short-term contact with an infected dromedary herd, short-term contact with a non-dromedary species infected with MERS-CoV, and

infection occurring during dromedary calving season. All of the above risk factors were selected by at least 5/14 experts. However, the most highly selected and importantly weighted exposures were “short-term contact with an infected dromedary herd” and “timing coinciding with dromedary calving season” (**Figure 3A**). Two experts selected the “other” option and specified the most risky season is dromedary-weaning season. Furthermore, one expert selected “other” and included the possible risk associated with contaminated camel feed.

MERS-CoV Infection between Dromedary Herds

Risk factors that were presented to experts, when asking about the possibility of dromedaries from different herds infecting each other, were: nomadic dromedary herds, introduction of a new dromedary into the herd, high dromedary density area, dromedaries taken to racetracks, dromedaries entered into/taken to beauty contests, dromedaries brought to communal water-points, dromedaries brought to/sold at markets, dromedaries pass through border points. Each factor was considered risky by at least 5 (36%) of the 14 experts, and no additional risk factors from experts were provided. The most highly selected (i.e., >70% of experts) risk factors, in order of weighted importance, were: bringing dromedaries to markets, introduction of a new dromedary into the herd, high dromedary density area, and bringing dromedaries to communal water-points. The first three of these risk factors were given similar weights by experts, while the last (communal water-points) was thought to only be half as important as them. Experts had sufficient agreement on their ranking of risk factors (Kendalls W = 0.25, p = 0.003), and had a mean response certainty of 3.9 and 3.8 (out of 5) for choosing risk factors and the subsequent rankings, respectively.

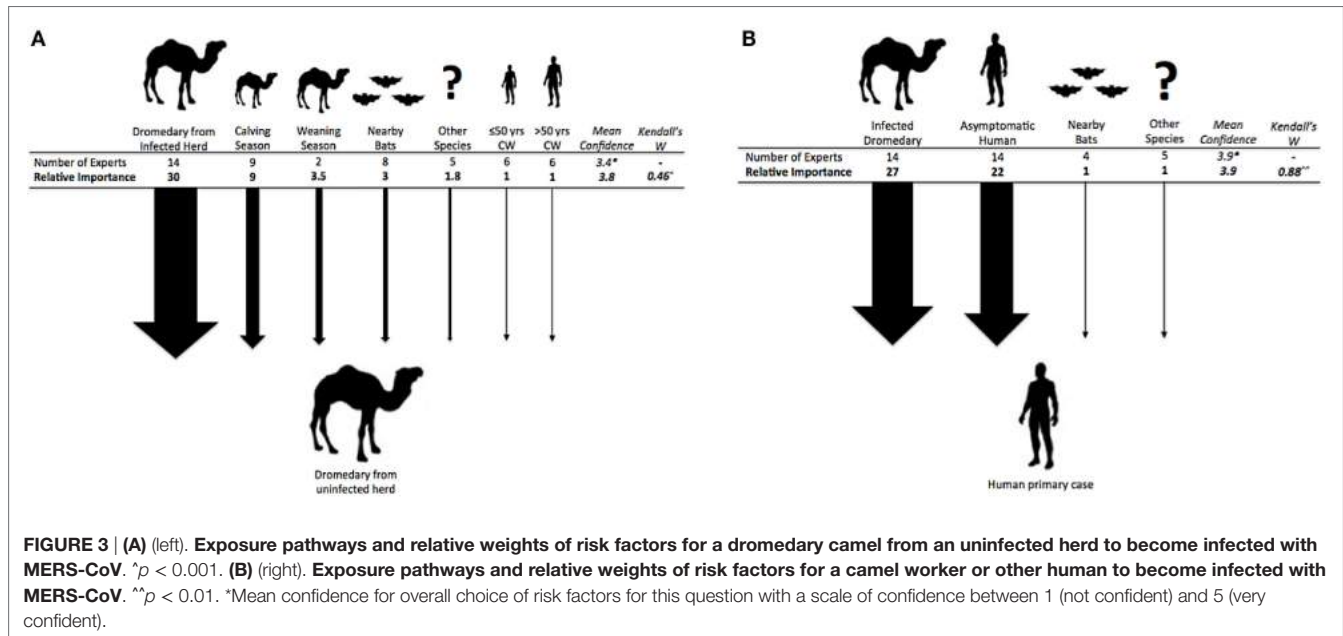
MERS-CoV Primary Infection in Humans

Exposure pathways for human primary case occurrence included: infestation of MERS-CoV infected bats in close proximity to human populations, contact with a MERS-CoV infected herd of dromedaries, contact with a non-dromedary MERS-CoV infected species, blood-biting pests (e.g., fleas, ticks) on an infected

TABLE 1 | Included Expert Profiles.

	Degree	Epidemiology	Virology	Camel studies	Risk analysis	Chiropterology (bats)
1	MD	✓ ^a 1–5 years	✓ ^a 1–5 years			
2	DVM	✓ ^a 10+ years	✓ ^a 10+ years	✓ 10+ years		
3	MD	✓ ^a 10+ years	✓ ^a 10+ years			
4	MPH	✓ ^a 6–10 years		✓ 1–5 years	✓ 1–5 years	
5	PhD	✓ ^a 10+ years	✓ 1–5 years		✓ 6–10 years	
6	DVM	✓ ^a 10+ years	✓ ^a 10+ years	✓ 10+ years	✓ 6–10 years	✓ 10+ years
7	DVM	✓ 10+ years	✓ ^a 10+ years			
8	DVM	✓ ^a 1–5 years				
9	PhD	✓ ^a 1–5 years	✓ ^a 6–10 years		✓ 10+ years	
10	DVM	✓ 10+ years		✓ 10+ years		
11	DVM	✓ ^a 10+ years			✓ 6–10 years	
12	PhD	✓ ^a 1–5 years		✓ 1–5 years	✓ 1–5 years	
13	MD	✓ ^a 6–10 years	✓ ^a 10+ years			✓ 6–10 years
14	DVM	✓ ^a 10+ years	✓ ^a 10+ years	✓ 1–5 years	✓ 6–10 years	✓ 1–5 years

^aIncluding MERS-CoV specific.



animal species or on humans, contact with another human who is asymptotically infected with MERS-CoV. All experts agreed that contact with infected dromedaries or asymptomatic humans were major risks for disease transmission, with the former being of higher risk (Figure 3B). About a third of experts (29–36%) thought that contact with MERS-CoV infected species other than dromedaries or bats may also play a role in human infection. Only one expert considered the possibility of blood-biting pests transmitting infection between dromedaries or other species and humans. Experts suggested no “other” risk factors.

Transmission from Infected Dromedaries to Camel Workers

The following possible exposure pathways from dromedaries to camel workers were presented to the experts: direct contact (e.g., face-to-face, touching, kissing), indirect contact (e.g., cleaning camel environment, contact with dromedary waste), consumption of unpasteurized milk, consumption or use of dromedary urine, and consumption of raw dromedary meat. All pathways were thought to be possible by the experts ($\geq 50\%$ each) and direct contact with dromedaries was thought to be a transmission risk factor by all experts (Figure 4). When asked to quantify these risks, by estimating the likely incidence of human cases, separately for adult and juvenile camels and older (>50 years) and younger (≤ 50 years) camel workers, experts estimated direct and then indirect contact with the highest risk; generally there was a slightly higher risk estimated when contact was with juvenile camels, and a clear trend for higher estimated risk when older vs. younger camel workers were exposed (see Table 2). Specifically, the risk of transmission was thought to be low ($\leq 5\%$) for camel workers consuming or using camel products, such as milk, urine, or raw meat. The estimation of the incidence was quantified as being between 13 and 24% for indirect contact with an infected

dromedary regardless of whether adult or juvenile, and between 22 and 33% with direct contact, varying by the age of both the camels and camel workers.

Transmission from Asymptomatic MERS-CoV Cases to Contacts

Experts were asked to estimate the risk of transmission from an asymptomatic infected individual to other individuals in close contact. The estimated “most likely” risk of transmission if the potentially asymptomatic camel workers were either ≤ 50 years or > 50 years old was 9%. The experts had a mean confidence of 2.8 (out of 5) for their answers for both age groups.

Risk Factors for Symptomatic MERS-CoV Infection in Humans

At least 10 of the 14 experts agreed that older age (>50 years), being immunocompromised, and the amount of viral dose transmitted, increases the chances that infected camel workers or other persons will become symptomatic after MERS-CoV infection. Being immunocompromised was given the highest overall comparative weight as a risk factor, followed by amount of viral dose transmitted. Also, genetic susceptibility and recent occurrence of an epidemic period for another disease (e.g., Influenza) were identified as risky by three and four experts, respectively. Experts had a good level of agreement on their ranking of the selected risk factors (Kendalls $W = 0.61$, $p < 0.01$), and had a mean response certainty of 3.4 and 3.3 (out of 5) for choosing risk factors and the subsequent rankings, respectively.

Responses to Open-Ended Questions on Transmission Dynamics

Experts were asked which factors led to increase viral shedding in MERS-CoV infected dromedaries. The most highly

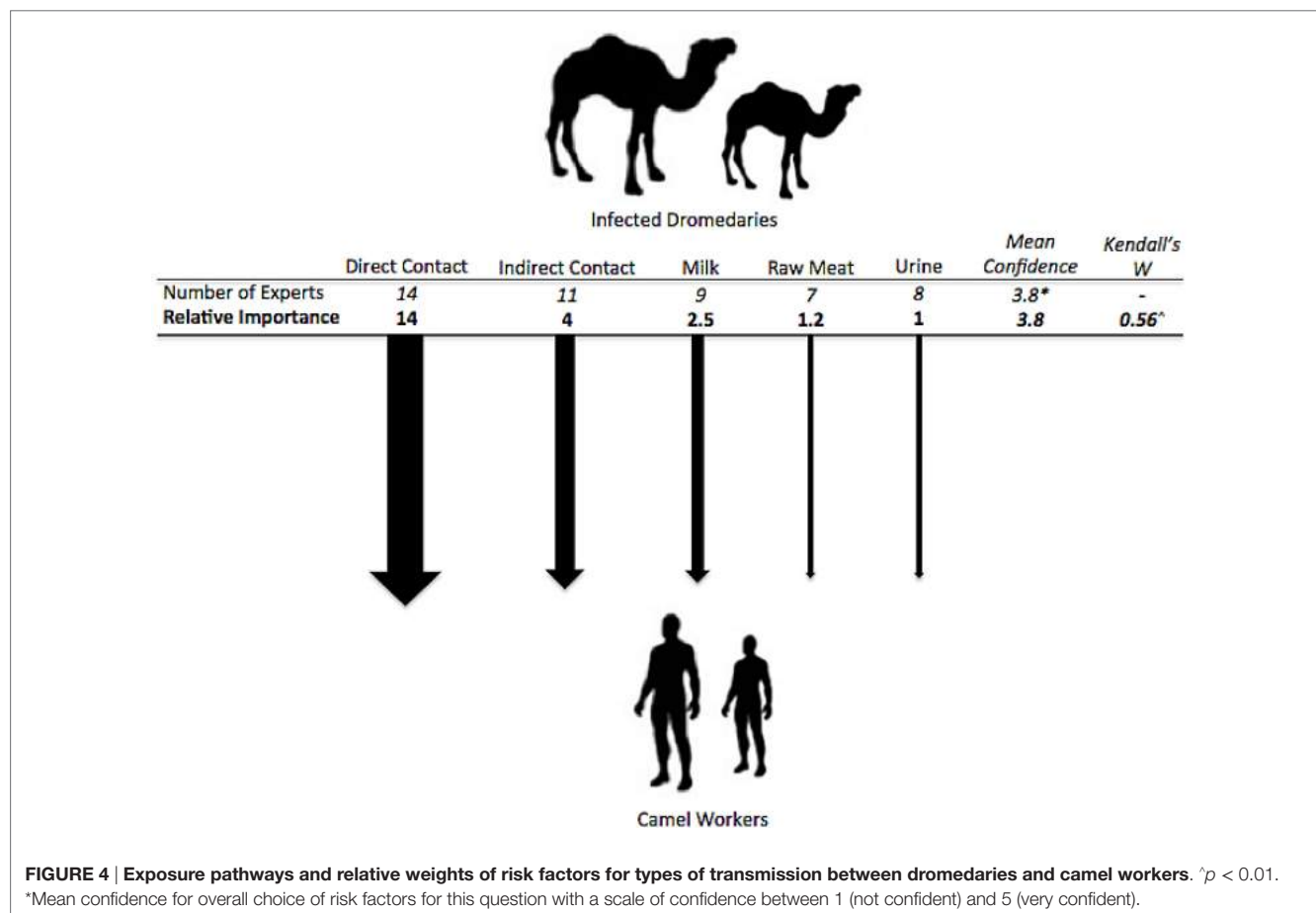


TABLE 2 | Estimated percentage transmission risk from adult and juvenile dromedaries to camel workers (CW).

	Adult dromedary						Juvenile dromedary					
	≤50-year-old CW			>50-year-old CW			≤50-year-old CW			>50-year-old CW		
	Most likely	Min/max	C ^a	Most likely	Min/max	C ^a	Most likely	Min/max	C ^a	Most likely	Min/max	C ^a
Milk	3	0/13	2.9	4	1/16	2.8	-	-	-	-	-	-
Urine	3	0/9	3.2	3	0/12	2.9	5	0/12	3	4	0/12	2.9
Raw meat	4	0/15	2.8	3	0/13	2.9	1	1/6	2.9	5	2/14	2.8
Direct contact	25	4/45	2.9	29	5/55	3	22	7/39	2.9	33	8/57	3.2
Indirect contact	13	1/33	2.9	18	4/36	2.8	19	4/34	3.1	24	6/48	3.1

^aC = Mean level of expert confidence for estimate with a scale of confidence between 1 (not confident) and 5 (very confident).

suggested items included: juvenile dromedaries lacking antibody immunity (*n* = 4), immunosuppressive conditions and secondary disease (*n* = 4), animal density (*n* = 2) and stressful environments for the animals (e.g., at slaughterhouses, markets) (*n* = 2). Experts believed factors that may lead to increased or more efficient transmission between MERS-CoV infected dromedaries and humans include repeated close contact with dromedaries with the chance of contact with respiratory secretions (*n* = 3), host susceptibility or immune

status (*n* = 2), increased virulence of the virus through genetic recombination or other (*n* = 2), and environmental contamination of camel-visited areas (*n* = 2). Experts were also asked whether or not they thought any other viruses might cross-immunize with MERS-CoV for either dromedaries or humans. Of the 12 experts who answered this question, 3 said “No,” while 6 were unsure or thought this was possible, and 3 experts believed that other coronaviruses might cross-immunize with MERS-CoV.

DISCUSSION

Our results use expert opinion to weigh the different transmission risks of MERS-CoV between animals and from animals to humans. Despite a lack of quantitative data, our results are supported by growing evidence from research published from MERS-CoV affected countries. Risk assessment is a tool that allows for the gathering of accessible data and information (e.g., expert opinion). The preliminary approach proposed in this paper synthesized available evidence regarding primary MERS-CoV transmission to humans. Our results highlight a general consensus in the order/rank of pathways, as well as for potential drivers and risk factors. According to the experts included in our study, dromedaries play a major role in transmission. However, the role of bats could not be ruled out and should be investigated further.

Despite the fact that new research reveals traces of antibodies against MERS-CoV in two livestock handlers in Kenya in 2013/14 (47), it is surprising that no locally acquired primary human cases have been reported where humans and infected dromedary camels are present outside the Arabian Peninsula. Recent workshops on MERS-CoV (Doha in April 2015 and Cairo in May 2015), organized by WHO, FAO, and the World Organisation for Animal Health (OIE), have produced numerous recommendations to improve surveillance and suggest research in animal and human populations (48). One of these recommendations is to investigate whether and why MERS-CoV infections of humans appear not to occur in Africa despite the high levels of infection in dromedaries, and why the virus is apparently absent in camels in Central Asia (dromedary and Bactrian camels).

The exact role of dromedary camels as a potential reservoir for MERS-CoV is also still unclear, and further investigations should be carried out to identify the mechanism of virus transmission and quantify the stability of the virus in various conditions more clearly. MERS-CoV has been detected in the oropharyngeal tract, feces, milk, and meat of dromedaries (21, 49–51). However, the modes of transmission are not clearly known. Our experts felt that the transmission risk from consumption of raw camel products, including milk, meat, and urine is low. It is assumed that the infection from dromedary camels to humans occurs through droplets or contact as high viral loads have been detected in the upper respiratory tract and nasal mucous membrane of dromedaries (52). However, milking activities and drinking unpasteurized milk, which is highly prevalent in Saudi Arabia (53), are considered as risky for the occurrence of primary cases in human populations. There is no evidence of MERS-CoV in camel meat, and it is known that cooking would kill the virus. One study from Qatar identified MERS-CoV in milk, but it was unclear whether the virus was excreted in the milk or if the milk had been contaminated by traditional milking techniques, which involves calves being used to initiate the milking process (50).

The role of and the extent to which infected asymptomatic human cases play a role in transmission is unknown. WHO estimates that ~20% of reported MERS cases are asymptomatic (54), but this estimate is likely underestimated given surveillance focuses on severe cases requiring hospitalization (55) and

evidence from serologic studies (32). One study documented prolonged shedding of MERS-CoV in an asymptomatic health care worker (56), which provides evidence that, if not properly isolated, asymptomatic cases in health care settings and in the community could lead to onward transmission. The experts included in this study believe that contact with asymptomatic cases is as important as that with infected dromedaries. Comprehensive testing of contacts of MERS-CoV patients, regardless of the presence of symptoms, is required to evaluate infection between known cases. The role of asymptomatic cases or carriers, if they are indeed infected, also needs careful consideration in the community setting. Not all reported primary cases can be traced back to contact with dromedaries, and it is likely, at least in some cases, that an asymptomatic or mildly symptomatic case may be an intermediary between dromedary contact and a symptomatic human case.

After 4 years, research on the role of camels and/or other sources of primary transmissions to human is inadequate. So far, most MERS-CoV studies have focused on virological or clinical aspects of the disease. No comprehensive analytical epidemiological studies have yet been carried out in MERS-CoV affected countries. With the exception of one case–control study (31) and individual case studies following investigation into single cases, transmission between dromedary herds and between dromedaries and humans has not been well studied. Even these detailed investigations are limited in terms of deciphering the cause–effect relationship. As human cases of MERS are relatively sporadic/rare, case control studies, especially matched case–control studies, can be well adapted during epidemics or outbreaks investigations and must be performed. Cohort studies are the best option in order to compare incidence among exposed (e.g., camel workers, immunocompromised people, etc.) and non-exposed populations. However, conducting cohort studies for rare diseases may be difficult.

In regions without reported human clinical cases of MERS, cross-sectional surveys based on serological investigation in humans and identification and quantification of potential risk factors for infection (behaviors, husbandry, contacts with camels and camel products, etc.) will assist in the suggestion of hypotheses, if human infection is prevalent and statistically exploitable for inference at the population level. Outside of the Middle East, these studies need to be undertaken, especially outside of the Arabian Peninsula and in African countries where MERS-CoV has been detected and/or isolated in dromedaries. Outside of the Arabian Peninsula, a number of joint human/camel serological studies are currently underway in North Africa (Pasteur Institut, Pers Comm) and planned in sub-Saharan African countries (HKU and Cirad, Pers Comm). For instance, in Ethiopia where MERS-CoV strains have been detected in camels (Peiris et al., Pers Comm), studies in at-risk human communities (e.g., nomadic people in close contact with camels, abattoir workers) have to be implemented: both analytical epidemiological studies and surveys on acute febrile illness (57), including respiratory and other signs could lead to clues about MERS-CoV infection and/or MERS-disease in humans.

At a more global scale, understanding differences in exposures and behaviors of individuals with dromedaries across the

Middle East and Africa is likely to explain some of the differences in potential infection risk. For that purpose, “ecological studies” could help to explore diverse drivers of transmission among different environments and societies. However, studies based on aggregated data are prone to many biases (58) making it difficult to know if individuals have really been exposed to the risk factor in question. Furthermore, social sciences have to be enlisted in order to puzzle out the relationships between camels and humans. Outputs can serve for epidemiological studies and modeling (e.g., multi-agents systems, see hereunder). Additionally, improved surveillance systems in humans and animals in rural and nomadic areas are required for MERS-CoV considering possible changes of the public health situation due to virus evolution (e.g., toward more pathogenic strains or diffusion of strains from areas with human disease, etc.) over time, modification of camel husbandry, etc. For population-based studies, epidemiology and surveillance, we need to have species-adapted and validated serological tools. Indeed, performances of tests are often lacking and should be assessed using frequentist or Bayesian approaches.

In addition to epidemiological studies, additional data from viral ecology studies among camels and other species, including bats, are required; phylogeography studies of MERS-CoV, and ecological studies on bat species living in the proximity of camels and suspected to play a role in the circulation of the virus, including a better understanding of their home ranges, migration patterns, biology (especially reproduction), roosting sites, and mechanisms of contact with camels are needed. Studies of viral shedding in animals, of virus persistence in different biological specimens of humans and animals, and in the environment under different conditions would help to quantify, or at least help to characterize, potential transmission risks.

The effect of MERS-CoV on camel health is not well documented; is the camel an asymptomatic carrier (reservoir/vector) or can MERS-CoV infection induce mild symptoms and/or pave the way for secondary infections? To address this question, camel studies should focus not only on MERS but also on the diverse etiologies of respiratory syndromes (59). This could be significant because, first, if MERS is recognized as a camel disease, more research resources could be allocated, second, super-infections could play a role in MERS-CoV transmission traits. Finally, multi-pathogens studies and multi-disease surveillance in camel populations can improve, through an economy of scale, MERS-CoV detection and the collection of data and metadata. Similarly, health conditions and infectious and parasitic diseases of camels may have an impact on MERS-CoV ecology and/or MERS epidemiological features. Indeed, immunosuppressive effects of several origins (husbandry and farming conditions, under-nutrition, deficiencies, parasites, co-infections, etc.) could enhance the infectivity of the MERS-CoV.

Considering the recent emergence of MERS-CoV as a zoonotic threat, and the lack of information already quantified on it, we appropriately included a small number of experts in this EOE exercise, but those who had diverse and extensive experience in relevant fields. The questions included in the

exercise were feasible for persons who are not accustomed to formal prioritization methods; AHP is known to be adapted for complex information situations, to be intuitively understandable and to allow scientists to score the attributes with minimal confusion. Our study has several limitations. First, it is commonly considered best practice to give a training exercise on EOE methods as well as provide a multi-page literature review on the topic in question to participating experts, prior to administering the questionnaire, however, this was not done here. In this case, experts were provided with a detailed document describing how to fill out the questionnaire, with examples, and were invited to contact the authors if they had any questions or confusion (see Image S1 in Supplementary Material). It is possible to “calibrate” experts, by including some items in the questionnaire for which a general scientific consensus or quantification already exists; the expert response to these questions can then be matched to the real answer in order to see how close that expert arrives. There was no calibration done in this study, largely due to the fact that there are almost no solidly quantified risks associated with MERS-CoV at present. Experts were weighted instead only on their confidence level for each question answered; however, it is always possible that experts are overconfident, giving scores that do not reflect their real uncertainty on their knowledge of a variable. Linguistic uncertainty in the questionnaire could have led to some bias; experts with varied origins and experience can interpret questions and imagine contexts differently, and this can be exacerbated by ambiguity or lack of specificity in questions. For the aggregation of our results, we used a mathematical approach by combining the weighted estimates of all experts. Another option would have been to use a more inclusive and participatory behavioral approach that would allow experts to revise their answers after seeing those of others and eventually come to a consensus together. However, empirical results have suggested that mathematical methods can outperform behavioral techniques in certain circumstances (60, 61), and it is also possible that group dynamics could bias estimations of risks toward a more extreme consensus (62). Overall, the experts were more certain in answering the AHP questions, which involved choosing and ranking risk factors. For all animal–animal or animal–human AHP questions, the experts consistently had mean confidence levels of close to 4 (out of 5), however, when asked to rank risk factors for asymptomatic human–human transmission, the mean confidence level was lower (closer to 3). When estimating minimum, most likely, and maximum transmission risks based on scenarios, the experts had lower overall mean confidence in their answers, with scores of between 2.8 and 3.2 for all estimations. This lower certainty is likely related to the fact that so few transmission risks for MERS have yet been quantified, whereas in choosing and ranking risk factors, there are already strong trends as presented in the literature.

Apart from virological, ecological, and epidemiological approaches, simulation models will allow for the testing of different scenarios of transmission, and this can be compared with reported cases. However, the scarcity of the data at the animal–human interface impedes the use of data-driven models like the stratified (animal–human) SEIR models, contact networks

TABLE 3 | Recommended MERS-CoV studies at the animal–human interface.

Studies	Main outputs	Key strengths	Shortcomings and constraints
1. Experimental studies			
1.1 Virology	Virus strains comparisons among animals and humans. Phylogeography	Deciphering of pathways between mammals species	Statistical power: require sufficient and representative strains to be analyzed
1.2 Experimental infections in bats and camels (and other livestock species)	Pathophysiology and clinical outcomes. Immunological response. Virus ecology; virus shedding in animals	Epidemiological parameters for modeling, e.g., shedding, viral excretion	Bioethics. Biosecurity. Costly
2. (Empirical) Observational studies			
2.1 Ecological studies on bats and camels	Roles as reservoirs and/or vectors of MERS-CoV	Identification of drivers of MERS-CoV ecology	Authorization to work on endangered bats. Need efficient non-invasive methods. Devices to follow livestock movements and bats migrations
2.2 Epidemiological studies	Prevalence and incidence in camels/humans. Serological test performance in humans/animals. At-risk behaviors and risk factors for MERS-disease in humans	Cross-sectional and ecological studies, which are relatively simple to be carried out	Costly for case–control and cohort studies
2.3 Sociology and anthropology studies	At-risk human behaviors at individual and community levels	Will feed epidemiological studies and models	Implementation of participatory approaches in pastoral and challenging territories (e.g., low-income countries, remote areas)
2.4 One health surveillance systems	Follow-up of virus, antibodies, clinical signs in humans and animals	Detection of emergence in humans; collection of viruses. infection timeline	Complex (need agreement among public health and vet services) and costly (need sustainability)
3. Modeling			
3.1 Probabilistic models (e.g., QRA)	At-risk pathways of transmission	Useful for disease management even if all mechanisms are not known	Long and iterative process for QRA. Data and information needed, including experiment data
3.2 Dynamic models (e.g., SIR, IBM, SNA)	Testing hypotheses (simulation) of MERS-CoV transmission. Drawing up the levels of vaccination needed	Deciphering of transmission ways between mammals species	Need data. Complex models required (SIR stratification animal/human, joint models, e.g., SIR and SNA, etc.)
3.3 Multiple-criteria decision-making or MCDA	Decision process. Risk mapping for spatialized MCDA	Straightforward to be implemented (literature review and expert opinions)	Model validation (but could be done with Human cases in Arabian peninsula)

SIR, compartmental models; IBM, individual-based modeling or multi-agent systems; SNA, social network analysis or contact network analysis; MCDA, multi-criteria decision analysis; QRA, quantitative risk assessment.

models, etc. Moreover, in order to analyze and simulate the complete pattern of the disease, there is also a need to capture the behaviors of animals and people (63). The individual-based model built on multi-agents systems is a computerized system combining multiple interacting agents (e.g., humans, animals) within a given environment (64). Such a model could be built in close interaction with stakeholders (farmers, camel workers, etc. (65)) and could drive toward more precise hypotheses about initial transmissions to humans (66).

This EOE study has several limitations but it is a preliminary step for implementing a more comprehensive risk assessment. Risk assessment is a time-consuming and iterative process that needs to be fed by several sources of data, lab experiments and field observations (see **Table 3** for a summary of recommended studies). Risk communication, which is part of the risk analysis and closely linked to the risk assessment, is essential, especially considering that MERS is a major public health issue and could have indirect economic and social impacts on the “dromedary world.” The questionable responsibility of dromedaries regarding human MERS-CoV cases could indeed spur inappropriate and overdone control measures. More broadly, this EOE can help in identifying gaps and needs in terms of experimental, field and modeling studies that will give a better understanding of the zoonotic transmission pathways of MERS.

AUTHOR CONTRIBUTIONS

MVK and FR designed the study, contributed to the analyses, and drafted the manuscript. AF and FG designed the data collection instrument, analyzed the data, and drafted the manuscript. MP, EM, MB, VC, and BF reviewed the results and drafted the manuscript. All authors agree with the conclusions of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fvets.2016.00088>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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