

**Areas of Research and Preliminary Evidence on Microcephaly, Guillain-Barré Syndrome and Zika Virus Infection in the Western Hemisphere**

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## **Abstract**

Between January 2007 and February 2016, a total of 52 countries and territories reported autochthonous (local) transmission of Zika virus, this includes 40 countries that reported local transmission between 2015 and 2016. Six countries (Brazil, French Polynesia, El Salvador, Venezuela, Colombia and Suriname) have reported an increase in the incidence of cases of Guillain-Barré syndrome (GBS) following a Zika virus outbreak. Puerto Rico and Martinique have also reported cases of GBS associated with Zika virus infection, but without evidence of an overall increase in the incidence of GBS. An increase in microcephaly cases and other neonatal malformations have only been reported in Brazil and French Polynesia. A number of concurrent events have occurred in Brazil, each of which provides an alternative hypothesis for microcephaly worth consideration. We explore the preliminary evidence for each in a forthcoming publication. Hypotheses explored include:

- Direct Zika-related microcephaly through unspecified mechanisms;
- Molecular mimicry of Bordetella pertussis peptides in tetanus-diphtheria-acellular pertussis (TdaP) Vaccine and whole-cell Bordetella pertussis vaccine (wP);
- Pestivirus virus contamination in locally produced whole-cell Bordetella pertussis vaccine;
- Glyphosate toxicity in bovine products in TdaP or wP vaccine (via interactions w/aluminum in the vaccine);
- Zika p53-BAX induced apoptosis, as in Rubella virus;
- Use of paracetamol (Acetaminophen) to reduce fever in pregnancy and in newborns;
- Horizontal transfer of piggyBAC transposon from released GMO mosquitoes;
- Pre-natal vitamin folic acid toxicity in MTHFR mutation carriers
- Interactions among any of the above.

While we explore available preliminary data and plausibility and give detailed recommendations for specific research studies to test these hypotheses, we note especially that (1) no cases of microcephaly attributed to Zika have occurred in pregnancies where gestation did not occur in Brazil or French Polynesia; (2) the use of TdaP or wP in pregnant women occurs without specific CDC recommendation. Scant safety data exist for use of either during pregnancy; antibodies to wP or TdaP passed to the fetus may induce brain damage via molecular mimicry. In light of formal consideration and preliminary evidence, concerns over wP involvement, and Zika-induced p53-mediated apoptosis are especially high. Given the finding of Zika virus in the brain of a fetus, one or more unknown co-factors may be facilitating Zika's transfer across the placenta, and other factors may also be contributing to microcephaly in Brazil.

## **Introduction**

The Director-General of the World Health Organization (WHO) declared a Public Health Emergency of International Concern (PHEIC), in accordance with International Health Regulations (IHR), on February 1, 2016 based on information that clusters of microcephaly and Guillain-Barré Syndrome (GBS) in Brazil in 2015 and 2016, and similar reports of clusters in French Polynesia in 2014, have been temporally associated with Zika virus transmission.

WHO has recommended that “clinical, virologic and epidemiologic data related to the increased rates of microcephaly and/or GBS, and Zika virus transmission, should be rapidly shared with WHO to facilitate international understanding of these events, to guide international support for control efforts, and to prioritize further research and product development.”

Brazil reported an average of 163 microcephaly cases from 2001 to 2014, and 5,640 suspected cases from November, 2015 to February 2016, including 120 deaths (WHO Zika Situation Report 26 Feb 2016). The reported increase in microcephaly incidence in Brazil is concentrated in the Northeast Region. Investigations have been concluded for 1,533 cases of microcephaly in Brazil, and of these cases, 950 were discarded based on not fulfilling the operational case definition of microcephaly, 583 were confirmed and 4,107 remain under investigation. Among the 5,640 suspected cases of microcephaly, 120 child deaths occurred after birth or during pregnancy (miscarriage or stillbirth); 30 of these were confirmed as having microcephaly potentially linked to congenital Zika virus infection, 80 are still being investigated, and 10 were discarded (WHO Zika Situation Report 26 Feb 2016).

Public reports of Zika involvement in microcephaly in Brazil range from the recent introduction of involvement of certain vaccines (Tdap in pregnancy) to and a switch in the larvicide used to control mosquitoes (Pyriproxyfen). A role for the larvicide pyriproxyfen has been speculated.

Ocular disorders in newborns have also been linked to Zika virus infection. A case of microcephaly associated with Zika virus infection was reported by the Hawaii State Department of Health on 8 January 2016 and another case was reported by Slovenian public health professionals on 10 February 2016. Both mothers spent time in Brazil in early pregnancy and experienced symptoms compatible with Zika virus disease during that period. Neither mother had laboratory testing for Zika virus infection at that time. A Zika virus infection was laboratory confirmed in the baby born with microcephaly in Hawaii (Office of the Governor, 2016), and after autopsy of the fetus (mandatory in all cases of termination of pregnancy) in Slovenia (Ventura et al., 2016).

No carefully conducted studies have been conducted to determine whether Zika virus is associated with microcephaly in Brazil; therefore Zika virus has not been proven to be a cause of the reported increase of microcephaly in Brazil, and presently only anecdotal, temporal and geographical associations have been shown.

Testing hypotheses of causality requires first their enumeration, as well as consideration of synergy between factors via interactions. Brazil, Colombia, El Salvador, Suriname and Venezuela have reported an increase of GBS cases temporally associated with Zika virus outbreaks. In July 2015, Brazil reported 42 GBS cases in the state of Bahia, among them 26 (62%) with a history of symptoms consistent with Zika virus infection. In November 2015, seven patients presenting neurological syndromes including GBS were laboratory confirmed for Zika virus infection. In 2015, a total of 1,708 cases of GBS were reported in Brazil, representing a 19% average increase from the previous year (1,439 cases of GBS), though not all states reported an increase in incidence. From 2009 to 2015, Columbia reported an average of 223 GBS cases per year, and during the five weeks from mid- December 2015 to late January 2016, 86 GBS cases were reported. From 1 December 2015 to 9 January 2016, 118 GBS cases were recorded in El Salvador, including 5 deaths, while the average number of GBS cases per year prior to 2015 cases was 169. Suriname registered on average four cases of GBS per year prior to 2015, 10 GBS cases were

reported in 2015, and three in January 2016. Venezuela reported 252 GBS cases with a spatiotemporal association to Zika virus for January 2016, with 66 cases in the state of Zulia, and mainly in Maracaibo municipality. In Venezuela, Zika virus infection was confirmed by RT-PCR in three patients with GBS.

In French Polynesia, 42 GBS cases were identified during the 2013-2014 Zika outbreak, 88% of which presented an illness compatible with Zika virus infection. Retrospective sero-neutralisation tests revealed that all 42 cases suffered dengue and Zika virus infection. (World Health Organization Zika Situation Report 26 Feb 2016).

A small increase in the incidence of GBS in Zika-infected adults in French Polynesia in 2014 has raised concerns that Zika infection may induce GBS in infected adults as well.

Table 1. Countries reporting GBS cases potentially associated with Zika virus infection (<http://www.who.int/emergencies/zika-virus/situation-report-26-02-2016.pdf>)

Increased incidence of GBS cases (without biological confirmation of the association with Zika)	Increased GBS incidence and biological confirmation of Zika infection in some of the cases	Reporting GBS with laboratory confirmed Zika virus infection (without increase of GBS incidence)
Brazil	French Polynesia	Martinique
El Salvador	Suriname	Puerto Rico
Colombia	Venezuela	

We enumerate various hypotheses on putative etiologies of both microcephaly in Brazil and of the more geographically widespread incidence of GBS, and examine available evidence and present suggested research directions. We present new findings from a molecular standpoint, beginning with mode of transmission.

**Methods.** Standard bioinformatics approaches were used to generate alignments (PSI-Blast) and for visual inspection. Various iterations and forms of BLAST were explored, and we found remarkable stability due to very infrequent length differences among polyproteins from various Zika isolates. Epitopes selected for local mimicry studies were variously selected. Local matches were visually confirmed via global (gene) and pairwise Blast and PSI-Blast. None of the individual amino acid substitutions we have discussed are near gapped areas. Alignments, which were not visually edited, were inspected using Jalview and text inspection. Phylogenetic analysis was conducted using the NJ option at EMBL-EBI ClustalW2 Phylogeny web resources (distance correction on, gaps excluded, P.I.M, on).

***Phylogeography and Case Studies Implicate Human Travelers for Global Spread***

Phylogenetic analyses of available polyprotein sequences (Fig 1) indicate that Zika virus in South America likely arrived via airplane in infected travelers from Polynesia (roughly Africa->Malaysia->Micronesia->Cambodia->Thailand->Polynesia->Brazil), confirming Musso (2015).

Zika virus has now spread through Central American, the Caribbean, with evidence of sustained local mosquito transmission, and, by human travelers, in the United States, United Kingdom, Canada,

Australia and other countries with no evidence local transmission. It was suspected that Zika virus arrived in Brazil via travelers visiting during the World Cup Football Tournament. Our phylogeographic analysis using currently available polyprotein amino acid sequences (Fig 1) strongly supports a Pan-Pacific route to South America for Zika virus, and not a direct Africa to South American route. However, such re-introductions are nearly certain to occur in the future.

Figure. 1. NJ tree from Clustal O multiple sequence alignment of 23 polyprotein sequences (gi identifiers and location shown). First letter shown in the amino acid located at the 2295th position in the polyprotein (M2295I). The second letter shown is the amino acid at position 2633th from M2633V in the motif EEP(M/V)LVQ. Note the unique occurrence of V in the South American sequences, including Surinam.

### ***Microcephaly***

Zika virus infection was first reported in Brazil in May, 2015. The first reported cases of microcephaly began in October, 2015. Microcephaly in Brazil has been attributed to Zika virus infection since October, 2015. Public reports include:

- Two infected microcephalic infants found to be positive for Zika infection via RT-PCR detection (Oliveira et al., 2016);
- In Brazil, the amniotic fluid of two fetuses has been found to contain Zika virus, and both were diagnosed with microcephaly (Vogel, 2015);
- Zika virus is reported to have been found in the tissues of a baby who died shortly after birth (Vogel, 2015);
- A baby born with brain damage was born in Hawaii to a mother who had contracted Zika during the earliest months of pregnancy while traveling in Brazil (WHO, 2016);
- Dr. Clement Bailey, Medical Director of the Caravans St. Vincentius (Saint Vincentius Ziekenhuis), in Paramaribo reported to Dr. Lyons-Weiler (February 2015, *pers comm*). Zika has been present in Suriname since at least October, 2015, if not before. Dr. Clement Bailey reports a possible earlier case of undiagnosed Zika virus infection, who was preliminarily diagnosed with Chikungunya, but who had already previously had Chikungunya. The date was September 2015. A maculopapular rash began in September 15. Dr. Clement suspected Zika. The patient exhibited conjunctivitis, headache, slight fever and arthralgia in her joints. Dr. Clemente also disclosed that a local company had sprayed pesticide near the patient's house, and the patient had speculated reaction to the pesticide. This report has been confirmed by UNICEF Guyana & Suriname in a communication to Dr. Lyons-Weiler. The period of time has been sufficient for microcephaly to begin to be reported among premature births in Suriname.
- Of the original 4,180 cases of microcephaly, Brazilian health officials examined 732 cases very closely. Only 270 were confirmed as birth defects, ruling out 462. Of these, only six (2.2%) showed evidence of Zika infection.
- A report from a pediatric cardiology group in Brazil who incidentally recorded data on head circumference reported an increase in microcephaly in Brazil long before the arrival of Zika in the Western Hemisphere, placing the start as early as July 2012 (Soares de Araújo et al., 2016).

Table 2. Countries reporting microcephaly cases potentially associated with Zika virus infection (<http://www.who.int/emergencies/zika-virus/situation-report-26-02-2016.pdf>)

Reporting Country	Number of reported microcephaly cases potentially related to a Zika virus infection	Probable location of infected with Zika virus
French Polynesia	9	French Polynesia
Brazil	583	Brazil
United States of America	1	Brazil
Slovenia	1	Brazil

There have been reports that it is difficult to test for past exposure to Zika unless the infection was current or very recent. One factor for this could be that Zika can often be non-symptomatic. Another factor could be non-specificity of immunohistochemistry assays; while Zika-specific IgM assay exists, use of IgG can be complicated because natural antibodies are cross-reactive to both Zika and Dengue fever IgG (CDC Memorandum, 2016). A third factor could be low association between Zika infection and microcephaly. The high rate of infection can make observational correlations appear to be strong; however, with increased specificity in testing robust association studies should be possible (case/control or cohort studies). A fourth factor could be an as-yet unidentified co-factor that allows Zika virus to cross the placenta and the blood/brain barrier.

Past outbreaks of Zika have not reported occurrences of microcephaly associated with Zika infection. This includes an outbreak on Yap Islands in Micronesia (2007), thought to have affected 12,000 individuals, and an outbreak in French Polynesia (2013) infecting an estimated 28,000 of 270,000 residents. During the same outbreak, four women who gave birth to babies with central nervous system malformations in Polynesia also tested positive via IgG serological assay for Zika virus (PAHO/WHO, 2015). To our knowledge, those malformations have not been confirmed as microcephaly. While other microcephalic infants were also born during the outbreak, they were not considered to be associated with Zika. The absence of dramatic increase in congenital microcephaly outbreaks suggests that as-yet unidentified factor, or factors, are playing a role in the increased incidence of microcephaly in Brazilian births.

*Microcephaly: Suggested Areas of Research*

**Hypothesis 1:** Microcephaly is Zika virus Infection-Induced

**Preliminary Evidence** Microcephaly in Brazil in 2014 involved 150 cases, and 3,893 cases between October, 2015 and January, 2016. The lack of microcephaly reported outside of Brazil weakens the hypothesis that Zika infection alone is sufficient to induce microcephaly and support the null hypothesis (geographical, but not temporal coincidence w/microcephaly) and/or the ‘missing co-factor’ alternative.

**Mechanism:** A specific molecular mimicry mechanism for Zika-induced microcephaly has not been identified.

Molecular mimicry is a serious issue with vaccine adverse events. One of the best examples is the development of narcolepsy in only some families after Pandemrix vaccination for the H1N1 influenza (Saariaho et al., 2015).

**Figure. 2** Partial, local Zika virus polyprotein multiple sequence alignment showing the single amino acid change at position 2633 unique to the South American clade. French Polynesian isolates should be examined for this variant.

**Plausibility:** A lack of data on the co-occurrence of microcephaly with Zika infection due to issues with diagnosis specificity and/or sensitivity had made assessment of plausibility challenging. To date, only observational data are available, and causal claims are weakly supported. Modes of Zika transmission include insect vector (mosquito), blood transfusion (Musso et al., 2014) as well as documented instances of likely sexual transmission (Foy et al., 2011; Musso et al., 2015). Perinatal transmission has also been observed (Besnard et al., 2014). Zika virus in Surinam is derived from that from Brazil, not Africa or reintroduced from Asia (Fig 1). Suriname has seen over 1,000 Zika infections, however, microcephaly is not as yet reported to be on the rise in births in Suriname. Colombia has reported over 3,500 cases of Zika infection with no uptick in microcephaly. If eventually there is an increase, then microcephaly via Zika alone may well be confirmed, but the timing of such an increase to a second factor or factors should be considered. It has been reported that around 2.2% of confirmed cases of microcephaly (out of 700 examined closely) in Brazil show evidence of Zika virus infection. Importantly, all known cases of microcephaly attributed to Zika have been in pregnancies in women that have spent time in Brazil during their gestation. Importantly, microcephaly in Brazil predates the reports of Zika infection (Soares de Araújo et al., 2016).

A preliminary report (small cohort study) of fetal ultrasonography and clinical examination of 42 of 72 Zika virus positive and 16 Zika virus negative pregnant women in Brazil (Brasil et al., 2016) resulted in the finding of 2 fetal deaths (36 and 38 weeks gestation), growth restrictions (5 fetuses), various CNS lesions or calcifications (7 fetuses), and abnormal amniotic fluid volume, or cerebral or umbilical artery flow (7 fetuses). This study increases plausibility, however co-factors were not considered (prenatal folic acid, smoking etc.). The absence of microcephaly outside of Brazil points to the requirement of consideration of co-factors. Four fetuses from Zika positive pregnancies in the Brasil et al. (2016) had microcephaly either via ultrasound or at birth.

Our analysis of the Zika virus polyprotein sequences resulted in the discovery of a single amino acid change unique to the South American clade (M2633V; Fig. 1) in the motif EEP(M/V)LVQ. The rest of the local alignment is remarkably robust. Partial, local Zika virus alignment showing the single amino acid change at position 2,633 unique to the South American clade. While this shift is a candidate for causality for microcephaly, consideration of mechanism and potentially synergistic causes, including other amino

acids shifts that occurred during transition through Asia, cannot be ruled out, especially given the brain malformations reported in French Polynesia.

**Recommendations:** Studies of concurrent rates of microcephalic births in mothers confirmed Zika infection compared to well-matched controls of mothers without Zika infection are needed in countries with Zika infection. Studies proposed by CDC (Petersen et al., 2016) to examine rates of Zika infection in pregnancies with and without microcephaly will tend to result in findings of Zika infection in microcephalic pregnancies, but do not include consideration of other factors. Larger case/control studies are needed upon diagnosis, and larger cohort studies are underway (Brasil et al., 2016). Animal studies comparison neurological development with the 2633-V and 2633-M molecular subtypes of Zika should be conducted to determine sufficiency.

**Hypothesis 2:** Microcephaly is induced by Tdap or wP vaccination.

**Preliminary Evidence:** Tetanus, diphtheria and pertussis acellular (Tdap) and whole-cell (wP) vaccination was occurring in Brazil prior to 2014, but mandatory vaccination in pregnant women came into law in December, 2014, using a new, locally produced vaccine (Boostrix™). First reports of Zika virus infections in Brazil occurred in May, 2015, followed by reports of microcephalic births in Brazil in October, 2015. Communication with a Brazilian expert in local whole-cell pertussis vaccine development has revealed that wP tends to be used in Brazil in the general population to reduce cost, whereas use of the more expensive acellular pertussis is restricted to clinics (Dr. Dias, WO, Instituto Butantan, pers.comm.) By comparison, *Bordetella pertussis* vaccination in Surinam is conducted using ADACEL™, an acellular pertussis-containing vaccine, and women of child-bearing age are recommended to get ADACEL or any pertussis-containing vaccine prior to pregnancy to prevent transmission of pertussis to their fetus, following earlier established CDC recommendations. Brazil began their national “Stork” pre-natal care program in 2011, a year before the initial reports of microcephaly.

**Mechanism:** No specific mechanism for Tdap-induced microcephaly has been shown, however transmission of maternal vaccine-induced antibodies from mother to their fetus via the placenta is known to occur (CDC, 2008). We present three possible mechanisms for direct Tdap or wP vaccine-induced microcephaly (Hypotheses 3, 4 and 5):

**Hypothesis 3.** Molecular mimicry between *Bordetella pertussis* antigen(s) and one or more human antigen(s) involved in brain development (e.g., *Bordetella pertussis* Molecular Mimicry – Direct Action) Position-Specific Iterated protein BLAST (PSI-BLAST) search of *Bordetella pertussis* proteins against the NCBI’s nr Protein Data Base resulted in findings of amino acid sequence similarity and potential molecular mimicry between *Bordetella pertussis* Hemagglutinin protein and Toll-like receptor adaptor molecule 1 (TICAM1). Locally directed BLAST searches result in various findings of high amino acid sequence similarity, sufficiently high for concern over structural similarity and molecular mimicry.

**Preliminary evidence:** *Bordetella pertussis* filamentous hemagglutinin protein (Accession KCV20463.1) shows sufficiently high primary similarity and horizontal match, to hCG1811753, isoform CRA\_a

(Accession EAW69193.1). This gene is also known as TICAM1/TRIF (NP\_891549.1). A double iterated PSI-Blast of *Bordetella pertussis* filamentous hemagglutinin protein against TICAM1/TRIF resulted in local epitopic similarity in a surprisingly large number of local regions on isoform CRA\_a.

One match was considered significant (Max score 24.4, Total score 5206; 48% Query Coverage, 55% Identity, E-value 0.046). The significant match was:

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Bordetella pertussis hemagglutinin      2709  SWPSVLTVTSPPEAISAPPP  2728
                                         SWP    + SPPE  S PPP
Homo sapiens isoform CRA_a           254  SWPPSGEIASPPELPSSPPP  273
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**Mechanism:** Isoform CRA\_a is also known as TICAM1/TIF is an adapter protein involved in the Toll-like receptor and IL-1 receptor signaling pathway in the innate immune response. Its intracellular interactions include IRAK1, IRAK2, IRF7 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response. TICAM1/TRIF increases IL-8 transcription and is involved in IL-18-mediated signaling pathway. The activation of IRF1 results in its rapid migration into the nucleus where it mediates an efficient induction of IFN-beta, NOS2/INOS, and IL12A genes. All of these signals would directly result in activation of and failure to regulate microglial activation, leading to neuronal death via autophagy and apoptosis. MyD88-mediated signaling in intestinal epithelial cells maintains gut homeostasis and controls the expression of the antimicrobial lectin REG3G in the small intestine (GeneCards. 2015), and thus ASD-like gastrointestinal issues may be expected in the 2015/2106 Brazilian microcephalics.

Hosmane et al (2012) found that TICAM1/TRIF, a toll-like receptor adaptor protein, blocked induction of the interferon response and inhibited microglial phagocytosis of axon debris in vitro. An autoimmune destruction of TICAM1/TRIF would prevent mediation of microglial phagocytosis, allowing them to roam free in the brain, attacking neural precursor cells (Cunningham et al., 2013). Neurological development involves a balance between the neuronal growth (via cellular division) and death (via chronic microglial autophagy and apoptosis). At the end of the second trimester, the human brain is only 2/3 the size it will be at birth; cessation of cerebral growth at 28 weeks could lead to noticeable microcephaly.

Many additional significant matches exist in wP vaccine than in acellular. One especially notable match with high degrees of homology involves the orthologs of elongation factor Tu (found in both *Bordetella pertussis* and in *H. sapiens*; 97% coverage at 56% similarity, E-value 5e-172).

A second especially notable example is the non-orthologous tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG [*Bordetella pertussis*] and the protein MTO1 homolog, mitochondrial isoform a (*H. sapiens*), which has 45% identity at 91% similarity (E-value 9e-167), with much higher local similarity in long putative mimeotopes (Supplementary Material S1).

**Plausibility:** This preliminary evidence supports molecular mimicry between numerous mimeotopes *Bordetella pertussis* and putative self-antigens in humans, including a key gene involved in the brain development and maintenance.

TICAM1 mutations have been observed to cause encephalopathy from *Herpes simplex* infection more likely in some individuals (Sancho-Shimizu et al., 2011). Mutations in TICAM1 have resulted in acute, infection-induced encephalopathy 6 (aka Herpes Simplex Encephalitis 4), a rare autosomal genetic condition.

Homology between *Bordetella pertussis* hemagglutinin protein and human Toll-like receptor adaptor molecule 1 (TICAM1) could result in autoimmunity against TICAM1, resulting in a profound disruption of regulation of pathways critical for normal brain development.

Chronic microglial activation and mitochondrial dysfunction are leading candidate causal processes for autism. Autoimmune against the gene regulating microglia, and against mitochondrial genes could shut down essentially processes necessary for brain development, including cellular energy during gestation.

**Recommendations:** The observed similarities are sufficiently high to warrant further study of a role of *Bordetella pertussis* vaccination, and wP vaccination specifically, as a causal factor for microcephaly in Brazil, and other developmental abnormalities. Whole-cell pertussis vaccine was abandoned in the Northern Hemisphere due to reports of brain damage. Brazil may be inadvertently replicating the population-wide experiment. Epidemiological studies of vaccinated/unvaccinated (case/control) and studies of vaccination rates in microcephalic pregnancies vs. non-microcephalic pregnancies are warranted. Studies of similarities/differences between pertussis epitopes and other ingredients in BOOSTRIX™ and ADACEL™ are required. Association studies that test for individual and combined effects on the odds of microcephaly due to Tdap or wP vaccination alone and in combination with Zika virus exposure are needed. Use of IgM antibodies is recommended to insure specificity for Zika.

#### **Hypothesis 4. *Bordetella* Vaccination/Zika Virus Infection Interaction**

**Mechanism:** If *Bordetella pertussis* vaccination in Brazil makes infection-induced microcephaly involving Zika more likely due to autoimmunity, it could involve proteins at the placental barrier, or it could involve brain development proteins, or a combination of such factors.

**Plausibility:** Only 17 cases of infant central nervous system malformations were reported in French Polynesia in 2014-2015 (European Centre for Disease Prevention and Control). The outbreak in French Polynesia ranged from October 2013 to April 2014. Mandatory Tdap vaccinations began in 2014. Zika is thought to have also arrived in Brazil in 2014. A case study exists of atypical vanishing white matter disease with microcephaly and hepatosplenomegaly after wp (DPwT) diphtheria pertussis tetanus vaccination (Gowda et al, 2014). Microcephaly pre-dates mandatory Tdap vaccination, but not regional or even national Tdap vaccination efforts.

**Recommendations:** We consider the *Bordetella pertussis*- Zika hypothesis plausible and worthy of immediate follow-up inquiry. A regional experiment in the cessation of Tdap vaccination should result in a local drop in Tdap-susceptibility Zika-induced microcephaly. Epidemiological comparisons of rates of

microcephaly in Tdap- or wP- vaccinated vs. unvaccinated after January 2015, and studies of rate of microcephaly in Zika-infected women with, and without vaccination are also needed. Whatever the specific mechanism of Tdap x Zika interaction, individuals born to mothers with gestational Zika infection without Tdap vaccination should not suffer microcephaly, and neither should mothers with gestational Zika infection but who received Tdap. Thus, data collected must include consideration of vaccination schedule and evidence of Zika infection (IgM assay).

**Hypothesis 5.** Contamination of vaccine lots by Pestivirus. Contamination of biological products with fetal bovine serum containing the Bovine Viral Diarrhea virus 1 (BVD1), a Pestivirus, has been reported in Brazil, including contamination of vaccines for veterinary use (Giangaspero, 2013).

**Evidence:** Pestivirus has been associated with microcephaly (Giangaspero, 2013).

**Plausibility.** Moderate plausibility, given the past occurrence in veterinary medicine.

**Recommendations:** Examination of vaccine lots for contamination with Pestivirus is recommended.

**Hypothesis 6.** Glyphosate-contaminated bovine products and/or general dietary exposure to glyphosate may induce vaccine-based injuries.

**Preliminary Evidence:** It is unknown yet if glyphosate can substitute for conserved glycines in the proteins. Glyphosate has been recovered from the organs and tissue of animals (Krüger et al., 2014), and appears to indicate that Glyphosate may be incorporated directly into proteins during protein synthesis. Another possibility engendered by Dr. Stefanie Seneff and colleagues (*pers comm*) is glyphosate/glycine inactivation of protein DNA-PKcs, rendering it inactive. Defects as small as single point mutations (G4122) in DNA-PKcs have been observed to induce profound neurological deficiencies including microcephaly (Woodbine et al., 2013). Glyphosate is genotoxic in snails (Bakry et al., 2015). Locally produced vaccines could also contain glyphosate. The viruses for the vaccine are grown on an amino acid complex derived by proteolyzing cow casein. Casein is a protein in milk, contains a considerable amount of glycine. If the casein is derived from milk from cows fed “glyphosate-ready” corn and soy feed, glyphosate could substitute for glycine in the casein during *in vivo* protein expression. Glyphosate contamination in Tdap or wP, combined with generally wide exposure *in utero*, may contribute to microcephaly. Tdap also contains aluminum, and aluminum and glyphosate are synergistically toxic (Seneff et al., 2015).

Glufosinate is increasing in use due to appearance of weeds that are resistant to glyphosate. Glufosinate is a glutamate mimic, and glutamate and glycine analogues working together can include neuroexcitotoxicity. Impaired methylation may also be a route for glyphosate induced neural tube defects (Hartwell & Seneff, 2012).

**Plausibility :** Glyphosate and glufosinate are good candidates for co-factors, or even factors, given their widespread use. Exposure is commonplace. However, a specific mechanism for microcephaly has not been demonstrated.

**Recommendations:** Measurement of glyphosate levels in vaccine products and products used in the manufacture of vaccines are warranted. Comparisons of same across countries with Zika infection and with and without increased microcephaly would be informative. Substitution of glycine in living organisms by glyphosate has not yet been substantiated. Studies of organisms grown on glycine-poor, glyphosate-rich media are needed. Microcephaly rates in areas that farm glyphosate-free animals and that do not use glyphosate or Glufosinate on crops with or without Zika infections, with and without vaccination with suspected vaccines or adjuvants should be studied to implicate or exonerate glyphosate as relevant factor for microcephaly. Animal studies examining the effects of glyphosate on vaccinated and unvaccinated Zika-infected mice should reveal likely microcephaly. Direct Post-mortem examination of whole-tissue distribution of aluminum in microcephalic babies in Brazil from vaccinated mothers in Brazil should be compared to those in unvaccinated matched microcephalics in Brazil (if any exist). Attempts to induce microcephaly using animal models challenged with glyphosate and Glufosinate with vaccination-level doses of aluminum, with and without Bordetella pertussis virus, could isolate key factors that play a role in microcephaly in Brazil.

#### **Hypothesis 7: Zika virus/Rubella virus)/West Nile Virus Capsid and NS3 Viral Proteins Mediate Apoptosis/Necrosis via p53->BAX and Caspase Pathways in Association with Microcephaly**

Rubella Virus (RV), a member of the *Togaviridae* family, has been shown to cause microcephaly as a result of maternal infection during the first 12 weeks of gestation (Megyeri et al, 1999; Duncan et al., 2000). The pathogenesis has been attributed to a p53-Bax apoptosis mediated pathway. The p53 protein is a transcription factor that facilitates Bax-mediated apoptosis by stimulation of BAX or by repression of Bcl-2 expression (KEGG ENSP00000269305)). BAX accelerates programmed cell death by binding to, and antagonizing the apoptosis repressor BCL2. P53 induces a conformation change in BAX which results in translocation of BAX to the mitochondrion membrane, releasing cytochrome C (cytC) by permeabilization. The release of cytC subsequently activates the caspase apoptosis cascade (FIGURE ). Megyeri et al. (1999) reported the Rubella Virus [RV] capsid protein (1-300) was responsible for the p53 mediated apoptosis resulting in the teratogenic effects. Duncan et al in 2000 subsequently reported that because of the timing of apoptosis with the first 48 hours following infection, the structural proteins of RV were most likely involved in the initiation of apoptosis. They demonstrated that the N-terminal 170 residues of the Rubella Virus capsid protein were associated with a 46.3% cell survival as compared to the native capsid protein (92%). This indicated the apoptosis-inducing activity of capsid resided in the N-terminal 170 amino acids of its cytoplasmic domain.

The West Nile Virus (WNV), a Flavivirus associated with neuroinvasive disease, has also been demonstrated by Yang et al (2008) to cause p53 mediated apoptosis via its capsid protein domain. HDM2 binds p53 and targets it for degradation at the proteasome. The authors demonstrated inhibition

of the HDM2-p53 complex through sequestration of HDM2 to the nucleolus by the WNV capsid protein, leading to stabilization of p53 and apoptosis through the Bax-mitochondrial pathway.

**Caspase Apoptosis via Viral NS3**

Ramanathan et al demonstrated that the WNV viral NS3 protein alone was sufficient to induce caspase induced apoptosis. Expressions of the Peptidase 7 and Helicase domains in the West Nile Virus NS3 domain were sufficient to trigger caspase initiated apoptosis. Caspase-9 is activated by the mitochondrial release of cytC into the cytosol (Zou et al., 1999) and initiates the induction of Caspase-3. Caspase-3 induces cells to undergo characteristic morphological changes in caspase-independent apoptosis (Table 3):

1. Formation of apoptotic bodies consisting of cytoplasm with tightly packed organelles with or without a nuclear fragment
2. Organelle integrity is still maintained and cellular components remain enclosed within an intact plasma membrane.

**Table 3. Histologic Characteristics of Necrosis and Apoptosis**

<b>Apoptosis</b>	<b>Necrosis</b>
Single cells or small clusters of cells	Contiguous cells
Cell shrinkage and convolution	Cell swelling
Pyknosis and karyorrhexis	Karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane (permeabilization)
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation present

Chu et al in 2003 found that after cytC (cytC) was released during initial apoptosis, caspase-9 was activated increasing caspase-3, leading to further apoptosis (Figure 3). This would suggest the p53-BAX pathway leads to later caspase-initiated apoptosis via release of cytC from the mitochondria through membrane permeabilization and activation of caspase-9. This is required for the formation of the apoptosome. This would imply both the p53-BAX pathway via the viral capsid protein and caspase-9 activation pathways via the NS3 Peptidase 7 and Helicase domains are involved in necrosis and apoptosis.

**Figure 3. p53-BAX mediated Apoptosis Pathway**

The p53-BAX mediated apoptosis pathway was generated in STRING. The WNV-Cp sequesters HDM2 (yellow button) to the nucleolus and stabilizes p53 (green button). The resulting mitochondrial

membrane disruption (permeabilization) via BAX (red button) releases cytC into the cytosol, initiating the caspase apoptosis pathway.

### Neuropism in Zika Virus

Zika virus was previously found to be highly neurotropic in mice and was recovered from no tissues other than the brains of infected mice. Neuronal degeneration, cellular infiltration and areas of softening were present in infected mouse brains (Dick, 1952). The histomorphologic changes in the brain have recently been reported in a microcephalic child. Mlakar et al. (2016) reported on a case of an expectant mother who had a febrile illness with rash at the end of the first trimester of pregnancy while she was living in Brazil. Gross findings included intracranial cortical and subcortical calcifications, lissencephly, and autolysis. The found damaged fetal brain cells with a cluster of dense virions located in the disrupted endoplasmic reticulum.

Electron microscopy identified ruptured and lysed neuronal cells in association with numerous icosahedral virus- like particles. Zika virus was identified by PCR. The large number of viral particles associated with the necrosis and apoptosis is similar to the previously described association of primary necrosis at high m.o.i >10 and apoptosis when the multiplicity of infection is low (m.o.i. <1). Other known infectious causes of intracranial calcifications include toxoplasmosis, rubella, Cytomegalovirus (CMV) and Herpes simplex virus (HSV).

To date, no specific mechanism of the observed Zika virus neurotropism has been identified. However, *Microcephalin* (MCPH`1-IPRO22047- MCPH1) may play a role in neurogenesis and regulation of the size of the cerebral cortex. Additionally, MCPH1 has an important role in regulating cell growth through regulating the cell cycle and apoptosis (Figure 4). Mai et al (2014) reported that overexpression of MCPH1 activated mitochondrial apoptosis through regulating several apoptosis-related proteins such as p53, Bcl-2, Bax, cytC, caspase-3, and PARP-1 , similar pathways which are under consideration in this section (Mai et al., 2014). The authors found inhibition of cell growth due to overexpression of MCPH1.

### Figure 4. p53-Microcephalin Interaction

Microcephalin (MCPH1) activated mitochondrial apoptosis pathway through regulating several apoptosis-related pathways such as p53->Bax, and cytC -> Caspase induced apoptosis.

**Mechanism:** Microcephaly via both p53-Bax apoptosis and Caspase mediated apoptosis.

**Preliminary Evidence:** An initial multiple sequence alignment of the human cytomegalovirus UL133 protein, the Rubella capsid protein (Accession# AA134238.1), the sequence of the Zika virus capsid protein (1-122), Spondweni virus, and the Japanese encephalomyelitis virus extended to 300 residues was performed using the CLUSTAL Omega algorithm and visualized with Jalview (Waterhouse et al., 2009). The region from position 219-231 in the extended Zika virus capsid protein sequence demonstrated 54% identity and 79% positives with the capsid protein of Rubella virus (257-269).

```
RubV    257  LPPHTTERIETRS  269
        LP H+T ++ TRS
```

**Figure 5. The capsid protein of Rubella virus (residues 1-300) and West Nile Virus capsid protein domain have both been demonstrated to increase p53 resulting in Bax-associated apoptosis.**

To investigate additional homologies between the structural proteins of Zika Virus (1-700) and the Rubella virus capsid protein (1-300), multiple amino acid sequences were aligned using the CLUSTAL Omega algorithm within Jalview. Specific attention was directed at regions of homology with the N-terminal 170 residues of the RV capsid protein.

Zika virus was found to map to the N-terminal 170 residues of the RV capsid protein from position 325-595, and is identified to be located within the Flavivirus glycoprotein domain (291-592). Overall, there was only 21% identity between Zika virus and the RV capsid protein N-terminal 170 residues. However, several localized regions of >50% homologies were identified. The greatest homology (75% sequence identity), ASQSRPP, was located at position 31-37 of RV with Zika virus (Figure 6).

```

ZIKV_GP   ASQSRPPT
           ASQSR P
RV_CP     ASQSRRPR
    
```

**Figure 6. ZIKV Structural Protein Domain Alignment with Rubella Capsid Protein.** The ZIKV structural protein conserved domains (1-700) were aligned with multiple sequences of the Rubella virus capsid protein (1-300). The Rubella capsid protein N-terminal 170 residues demonstrated significant sequence homology with the Zika virus Glycoprotein domain (red rectangle enclosure). The N-terminus of the Rubella capsid domain has been demonstrated to responsible for the p53/Bax mediated apoptosis pathway. The conserved domains of ZIKV are displayed above the MSA. The default color scheme is percent identity and visualized in Jalview.

**NS3 DOMAIN: PEPTIDASE-7 AND HELICASE HOMOLOGY**

The Peptidase-7 and Helicase domains were evaluated for homology as these domains were previously discussed as being sufficient for caspase induced apoptosis. Between position 1620-1670 of the Peptidase 7 conserved domain in NS3 (Figure 7), the overall homology was found to have 56% identity, but also 78% positives suggesting the C-terminus may be the region of shared interaction with caspases.

**Figure 7. MSA of the Peptidase 7 conserved domain in the Flavivirus NS3 protein.**

The Helicase domain from position 1867-1976 (Figure 8) demonstrated 76% homology and 81% positives within the C-terminus. This may suggest a greater role of the Helicase domain in caspase induction and apoptosis.

**Figure 8. MSA of the Helicase domain in the Flavivirus NS3 Protein.** The Helicase domain is defined below the red bar. The GKT conserved motif is the start of the helicase domain. Coloring is percent identity in Jalview. The C-terminus homology was calculated to 76% identity with 81% positives.

**Plausibility.** Moderate to high plausibility. Zika virus may play a role in microcephaly due to the p53-Bax mediated apoptosis pathway via its capsid protein and ultimately the caspase apoptotic pathway via its NS3 protein, in a mechanism similar to neuroinvasive West Nile Virus. By homology, it appears that the C-terminus of the ZIKV Capsid Protein domain may be responsible for the BAX apoptosis/necrosis pathway, particularly when the histomorphologic findings are considered. However, without the phosphorylated serine residues at the 3' and 5' ends, ZIKV and DENV may not be able to bind HDM2 and may use another mechanism to induce p53, block anti-apoptotic Bcl-2, or bypass BAX and preferentially use caspase induced apoptosis. Additionally, with the significant homology that exists within the NS3 domain, specifically the C-terminus of both the Peptidase-7 and Helicase domains, evidence suggests a caspase induced apoptosis as a result of cytC release following BAX mitochondrial permeabilization. The histomorphologic evidence of both apoptotic bodies and necrosis in intracranial ZIKV infection would support both p53 and caspase pathways in ZIKV induced apoptosis/necrosis. The capsid protein of Rubella virus and West Nile domains have both been demonstrated to increase p53 expression, resulting in Bax-associated apoptosis. The caspase apoptotic pathway via the NS3 protein has also been reported in West Nile Virus, also a member of the *Flaviviridae* family. However, given the low concurrence of evidence of Zika virus infection in confirmed microcephalics, its activity is not likely singular nor universal and it should be studied for possible interaction with other factors, including MCPH1.

**Recommendations:**

Studies of the induction of p53 and caspase apoptosis pathways in vitro human cell lines due to Zika virus infection are recommended. These studies might also consider studies of synergistic roles with vaccine additives, and other variables reviewed in other hypotheses in this report. Additionally, in vitro and in vivo validation studies of the predicted immunological cross-reactivity identification of antibodies for these peptides are needed. Animal models employing these proteins should be explored to determine whether infection with Zika induces a P53 -> Caspase apoptotic cascade in the brain. Additionally, further *in vitro* and *in vivo* studies are required to evaluate a potential Zika virus interaction with MCPH1 in an effort to further explain the observed neurotropism.

**Hypothesis 8. OX513A *Aedes aegypti* Mosquito piggyBac Transposable Element Horizontal Transfer**

In 2011/2012, Oxitec released genetically modified OX513A *Aedes aegypti* mosquitoes in Juazeiro, Bahia, Northeast Brazil, 717 km from the 2015 outbreak of Zika. The release was conducted under a permit granted by the Brazilian National Biosafety Technical (CTNBio). Male mosquitoes were modified using the piggyBac transposon (Phuc et al., 2007) to sire offspring that could not thrive in the absence of tetracycline in their environment (sex-specific dominant lethal). It has been, however, reported that <5% of genetically modified mosquitos survive in the lab without tetracycline. The piggyBac transposon is prolific in its insertion around genomes, and perhaps across invertebrate species, likely mediated by a viral system (Handler et al., 2002).

In the OX513A mosquito, a synthetic sequence with a tetracycline binding domain and transactivator binds to its DNA binding site, tetO (tetracycline operator), which results in cytotoxic levels of tTAV (Curtis et al., 2015). Without tetracycline, tTAV is expressed causing death of the mosquito.

The piggyBac vector is isolated from the Cabbage Looper moth, *Trichoplusia ni* integrates DNA flanked by an open reading frame (ORF) within the element (Handler et al., 2002; Handler et al 1998). This ability is thought to exist only when a pair of inverted terminal repeats (ITRs) are intact, is capable of integrating DNA flanked by an open reading frame (ORF) within the element (Handler et al , 2002; Handler et al 1998). In the Oxitec mosquitos, the transposase gene of the piggyBac element was “irreversibly destroyed” by deletion of a section of that gene. A ‘helper plasmid’ is required that allows transposase activity. The helper plasmid is not present in the modified mosquitoes (GeneWatch UK. 2016).

A specific route to microcephaly via the piggyBac transposon is the vertical transfer of the piggyBac transposon from less fertile OX513A males to female mosquitos descendant, who could pick up Zika from humans. PiggyBac could then jump to Zika, and jump from Zika to humans. piggyBac not only causes insertion of itself and carrier genes randomly into and widely across genomes it infects (Fonager et al., 2011; Rad et al., 2010); it also causes increased rates of point mutation (Li et al., 2013).

**Preliminary Evidence:** Zika inserts mostly at TTAA/AATT, but also at non-canonical insertion sites CTAA/TTAG and ATAA/TTAT and others (<2%; Li et al., 2013). Examination of Zika sequences via BLAST reveals a number of near-perfect (7/8 bases) matches (Table 4). A single nucleotide mutation at any of these sites would result in an 8/8 match.

<b>Zika Strain</b>	<b>Gene</b>	<b>Insertion Type</b>	<b>Location</b>
Mr766	3'UTR	TTAA/AATT	8...14
Mr766	3'UTR	TTAA/AATT	10372...10378
ArD149810	NS5	TTAA/AATT	695...701
ArD149938	NS5	TTAA/AATT	695...701
ArA506	3'UTR	TTAA/AATT	285...291
ArA975	3'UTR	TTAA/AATT	285...291
ArA982	3'UTR	TTAA/AATT	285...291
ArA986	3'UTR	TTAA/AATT	285...291
ArD158084	3'UTR	TTAA/AATT	285...291
MR766-NIID	3'UTR	TTAA/AATT	8...14
ArD158095	polyprotein	CTAA/TTAG	5336...5342
ArD158084	polyprotein	CTAA/TTAG	5336...5342
ArD128000	polyprotein	CTAA/TTAG	5336...5342

MR766-NIID	genomic	CTAA/TTAG	5442...5448
CHI1058514	NS5	ATAA/TTAT	293...299

All matches involve 7/8 residues

While offspring survivorship without tetracycline is expected to be very low, Massonnet-Bruneel et al. (2013) noted 18% survival of sired offspring to adulthood, and attributed this to the type of food the larvae were raised on (cat food). Prior studies had used fish food. Tetracyclines are not absent from aqueous environments, and a very large proportion of ingested tetracycline is excreted in waste (Borghi et al., 2014).

*Aedes aegyptis* have been observed to breed to latrines, cesspools, and septic systems. Livestock are also heavily dosed with antibiotics, and of course mosquitos feed directly on livestock. Livestock waste ends up as liquid manure, which is found in run-off, surface water, soil, groundwater, and drinking water. Thus, tetracycline is likely locally abundant in certain areas, and plausibly available for local, large sustained populations of OX513A in most countries.

**Plausibility: Low, but non-Zero.** PiggyBac is lauded as being useful across the animal kingdom for moving genes into and around genomes (Wilson et al., 2007; Chen et al., 2010), and for mutagenesis in mouse and human stem cell lines (Gayle et al., 2015). Spread of piggyBac via horizontal transfer between eukaryotes is not unreasonable. The absence of both microcephaly and OX513A mosquitos in Suriname lends credibility to this hypothesis. The Zika virus genome, around 8.4Kb, would be nearly doubled by the piggyBac transposon, and would be detectable by PCR-based methods. Although transfer of piggyBac to a double-stranded RNA virus has never been observed, such as transfer would occur via a number of routes: transfer from mosquito to a human first; the mosquito (or human) would transfer piggyBac to the genome sites where Zika virus inserts in either (double-stranded DNA) human or mosquito. A small proportion of released mosquitos are female, and tetracycline is likely to be found in the environment. A specific and ubiquitous manifestation of microcephaly in humans is not necessarily expected from a jump of the transposon to humans, however.

The released mosquitos are reported to be devoid of the ability to transpose in the wild (GeneWatch, 2016). Studies of all GMO animals recaptured post-release to confirm loss of transposibility should be studied as a requirement for use in the wild.

**Recommendations:** This hypothesis is highly implausible, but imminently testable. Microcephalics should be examined for the presence of the piggyBac transposon, as should their mothers. Wild-caught Oxitec and non-Oxitec mosquitos should be examined as well and studied for presence of piggyBac and survivability without tetracycline to rule out this possible.

**Hypothesis 9: Zika Virus Infection Associated Guillan-Barré Syndrome (GBS)**

Guillan-Barré Syndrome (GBS) is an autoimmune disease of the peripheral nerves, involving both myelin sheaths and axons. Molecular mimicry is a known cause in some cases involving similarities between *Campylobacter jejuni* lipo-oligosaccharides and peripheral nerve gangliosides. Ang et al. (2004) review this example in detail and review numerous other examples of molecular mimicry-induced autoimmunity.

Numerous reports have suggested Zika virus-infection based causality GBS in regions in which Zika infections are prevalent. These reports include a case report (Oehler et al., 2015).

**Preliminary Evidence:** Reported cases of GBS associated with Zika infection have been sporadic; reporting to date cannot be used to reliably ascertain any increase in a manner consistent with attribution to Zika-related causality. Analysis of spatial correlation found no relationship between Zika virus infection and rates of GBS in the Pacific Islands (Craig et al., 2016). GBS is, however, on the rise in regions where Zika is now also found.

A PSI search for local peptide matches between GBS peptides and human proteins (nr database) resulted in a number of hits, including a hexamer motif WTR(Y/H)GE in NS5 (2083-2089) with 100% match with three polio virus receptors A, B and C.

The residue shift unique from Y->H (Tyr to His) unique to (Polynesia, S Am) clade in this very motif is likely a functionally important change as histidine is a charged, positive, amino acid while tyrosine is not.

**Mechanism:** This mutation may have resulted in a mimeotope that induces auto-immunity against the polio receptor as a self-antigen after Zika infection.

**Plausibility: High.** This one mutation in Zika leading to this one amino acid change could conceivably induce GBS via induced molecular mimicry. Others that show distributions that match GBS across countries and regions are equally good candidates. However, this variant has high biological plausibility given its target. A case (n=98) control (n=70) comparison found significant association of GBS with Zika virus infection (Cao-Lormeau et al., 2016), but no evidence of molecular mimicry for the molecules surveyed, which to our knowledge did not include polio virus receptors.

**Recommendations:** Further fine grained temporal and spatial correlation studies are needed to determine plausible causes of GBS increases. In vitro studies of cross-reactivity using antibodies to polio receptor and to NS5 peptides are needed. In vivo studies using animal models with Zika infection with human polio virus receptors may be a rapid route to determine plausibility of Zika-induced paralysis. Mouse:human homology in this area of the polio receptor protein is poor, therefore *Rhesus* macaque studies may be more informative. Examination of antibody profiles to polio receptor in suspected Zika-related GBS could be instructive. Any Zika vaccine developed should avoid Zika virus epitopes with high local homology to human proteins to avoid vaccine-induced GBS.

**Search details (Methods):** Query peptide: AEVWTRHGKRVLKPRWMDARV vs. non-redundant Protein database for *Homo sapiens*.

### **Hypothesis 10. Pre-Natal Vitamin Folic Acid Induced Microcephaly in MTHFR Mutation Carriers**

Mutations the MTHFR gene (1p36.3) can lead to 5,10-Methylene-tetrahydrofolate reductase deficiency (MTHFR) affects numerous enzyme systems. Abnormal intracellular folic acid metabolism results and prevents reduction of 5-10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for the remethylation of homocysteine into methionine. As a result, the disorder leads to methyltetrahydrofolate deficiency and consequently to homocystinuria and hypomethioninemia. Among the known symptoms include severe neurological signs including mental retardation, microcephaly, gait disturbance, recurrent apnea, convulsions, psychiatric disturbances and microcephaly. Homocystinuria occurs without megaloblastic anemia can occur due to methylene tetrahydrofolate reductase (MTHFR) deficiency. Numerous LOF and Change of Function (COF) mutations are known, some of which are relatively high frequency (e.g., 677C-T). A baby born to a consanguineous Turkish couple with a homozygous MTHFR, 1711C-T also have severe microcephaly (Kluijtmans et al. 1998). Other studies have found severe microcephaly in infants with MTHFR deficiencies (Balasubramaniam et al., 2013; Cappucino et al., 2014).

**Preliminary Evidence:** The timeline (Figure 5) clearly shows the advent of Brazil's national Stork program in 2011 as the only event preceding the massive increase in microcephaly in 2012. The spatial aggregation is consistent with population genetic structure of the distribution of such mutations. Frye et al. noted autoantibodies to cerebral folate receptors in children with autism, with correspondingly low cerebrospinal fluid 5-methyltetrahydrofolate concentrations.

**Plausibility:** Extremely High. The temporal association is correct. Given that no screening for MTHFR mutations in mothers is likely to be ongoing in Brazil, this hypothesis is extremely plausible, and nearly a certainty, that some Brazilian mothers will have babies with adverse outcomes due to high dietary intake of folic acid, a synthetic form of folate.

**Recommendations:** Mothers and babies with microcephaly should be screened for MTHFR mutations, and association of such mutations with microcephaly are predicted. A national screening program is recommended to help mothers with MTHFR LOF mutations and their fetuses avoid folic acid toxicity.

### **Discussion**

In examination of the available data and reports on Zika, microcephaly and GBS, we have identified hypotheses that require investigation. We suggest that ongoing considerations of Zika infection involved in microcephaly examine p53 apoptosis activation, and search for mechanisms of placental transfer of the virus and maternal antibodies. These studies should also include consideration of Tdap and wP vaccination, pesticides, and mosquito strains, if piggyBac is found in released mosquitos. Given the high plausibility that *Bordetella pertussis* vaccination may sensitize a fetus to Zika-based microcephaly via route that include disruption of normal microglial activation and mitochondrial dysfunction, we recommend reconsideration of WHO's recommendation for paracetamol and Acetaminophen for fever

control. Paracetamol/acetaminophen use is highly correlated with adverse neurological outcomes after use to control vaccination-induced fever (Schultz et al., 2008), and its use post-circumcision may be a contributing factor to the 4:1 (male: female) gender skew in the incidence of autism spectrum disorders (Frisch et al., 2015).

The timing of events leading up to and concurrent with the increased incidence of microcephaly is important (Figure 9). The report of microcephaly increase beginning in July, 2012 (Soares de Araújo et al., 2016) also noted that the increase in the severe cases of microcephaly began later (April, 2014), and that overall incidence included a degree of potential seasonality, potentially linking the incidence of microcephaly to mosquitos. We can say with certainty that all of the increase in microcephaly in Brazil cannot be attributed to Zika due to the timing of these events. Only carefully conducted cases/control, cohort, and vaccination/insecticide/glyphosate cessation programs in specific towns will reveal which if any of these factors are co-factors for microcephaly.

Genetic risk factors have not been explored. Genetic association studies for susceptibility to any of the viable factors that may contribute to microcephaly are warranted. The observations of Zika virus in amniotic fluid with, or without microcephaly would indicate placental transfer. A report of Zika virus in the brain of a microcephalic fetus (Mlakar et al., 2016) also points to a role for Zika virus. However, answers to the question of a causal link between Zika infection and microcephaly rigorously designed research studies that consider interactions among factors. We have suggested studies that would fill significant gaps.

**Figure 9.** A timeline of events preceding and concurrent with the emergence of microcephaly in Brazil. The CDC has concluded “strongest evidence yet” due to finding of Zika in the brain of a microcephalic fetus (Mlakar et al., 2016).

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