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Novel Genomics Strategies for Clinical Prognosis in Early-Stages of ZIKV Infections: The Search for New ZIKV-Associated Disease Biomarkers

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BACKGROUND INFORMATION

Zika virus (ZIKV) is a virus belonging to the *Flavivirus* genus and *Flaviviridae* family. Its genome consists of a single-stranded positive-sense RNA, coding for a single polyprotein. Following post-translational processing of the polyprotein it yields capsid (C), membrane precursor (prM), envelope (E) structural proteins as well as the non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [6]. ZIKV is an arthropod-borne virus (arbovirus) primarily spread by the *Aedes sp.* mosquito [1]. Of note are *Aedes aegypti* and *Aedes albopictus* which are highly associated with habitation in urban environments [1]. Although the *Aedes* mosquito has been shown to be the primary mosquito vector, other mosquito genera such as *Culex sp.* mosquitoes have been shown to be able to transmit ZIKV, albeit not to the same extent. [7]. In addition to mosquito transmission, recent evidence has shown that ZIKV can also be transmitted sexually [8], vertically [9], and through blood transfusion [10]. ZIKV was first isolated from a sentinel rhesus monkey in Uganda, in 1947. In 1948, it was isolated from *Aedes africanus* at the same site [11]. The first human isolation occurred in Nigeria in 1954. From then, the incidence of the infection in humans was sporadic and the virus was believed to be maintained primarily in a sylvatic cycle between monkeys and mosquitoes. It began with sporadic cases in Africa, however cases began to appear on the Asian continent with cases being reported in Malaysia and Indonesia [1]. In its first deviation from low-level endemicity, in 2007 an outbreak of ZIKV occurred in Yap State, Federated States of Micronesia. This marked the beginning of a

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series of outbreaks along an eastward migration path with further outbreaks in French Polynesia in 2013, Brazil and the Americas in 2015-16, and a re-emergence in Africa. It is expected to continue this eastward path returning to Asia. Along this eastward migration, ZIKV has accrued multiple mutations, delineating two major strains: the African strain and the Asian strain [1,12,13]. When first characterized, the symptoms of ZIKV infection seemed non-specific showing signs of rash and fever, symptoms similar to other *Flavivirus* infections such as Dengue (DENV). Furthermore, 80% of ZIKV cases are asymptomatic. It wasn't until the French Polynesia outbreak that the evidence of more serious clinical outcomes presented itself, with the outbreak in Brazil following suit [14]. The outbreak in French Polynesia saw an increased incidence of the autoimmune disease Guillain-Barré Syndrome (GBS) [14,15]. Brazil also saw a marked increase in the incidence of GBS [16]. However, another novel clinical manifestation was characterized in Brazil, an increased incidence of microcephaly [1, 2]. This prompted a retrospective study to be done in French Polynesia which also showed an increase in the incidence of microcephaly [17]. Although the incidence of GBS in the two outbreaks was relatively similar [14], Brazil showed markedly higher incidence of microcephaly, particularly in the Northeastern region [14, 18,19]. This pathogeographical difference raises questions as to its possible causes. One possibility is that key mutations have occurred along the ZIKV migration path that have enabled these new pathogenic outcomes. Research has begun to emerge showing possible links between ZIKV genome mutations and disease incidence, with variations found in coding regions [20], non-coding regions [13], and various places throughout the genome [21] as candidates. A second possibility in explaining the pathogeographical trend could be host genomic differences in the various populations. There is currently no literature exploring a causative link between host gene polymorphisms and ZIKV clinical outcomes, largely due to the lack of data available. However, there are few known ZIKV-associated host factors such as the AXL cell surface receptor that could pose as possible candidates of interest down the line [22, 23]. A final factor to consider is the interplay between other endemic *Flaviviruses* and ZIKV. DENV is a *Flavivirus* that is also spread by the *Aedes aegypti* mosquito [1]. DENV has four main serotypes that circulate, and may co-circulate. In South America, for example, the seroprevalence of DENV in ZIKV affected areas is greater than 90% [24]. The issue arises in the high degree of similarity of DENV and ZIKV. Research has shown a high degree of antibody cross-reactivity between the two viruses. Furthermore, there is now evidence suggesting that DENV antibodies might drive the antibody-dependent enhancement (ADE) of ZIKV [24]. As previous infection with a heterologous serotype of DENV increases risk of Severe Dengue clinical manifestations [25], so might previous infection with DENV affect the pathophysiological outcomes of ZIKV infection. Another issue that arises from the antibody cross-reactivity between DENV and ZIKV is the high incidence of false positives during immunological assays [3]. The lack of a uniform, accurate, and sensitive ZIKV diagnostic is worrying as it leaves much of the data from places using sub-par diagnostics subject to skepticism. Even the current Center for Disease Control and Prevention (CDC) standard diagnostics of reverse-transcriptase polymerase chain reaction (RT-PCR) are not free from issues as their relatively low sensitivity causes a high risk of false negatives [4]. To try and address these issues a promising candidate ZIKV diagnostic was developed by Pardee *et. al*, a team based out of the University of Toronto. They have developed a cell-free, paper-based sensor to detect the ZIKV RNA genomes at low femtomolar range from plasma. In addition, using CRISPR-based technology, the sensor is able to discriminate between ZIKV strains at a single-base resolution. All of this is done easily, portably, and at very low cost [5]. The accuracy of this diagnostic, paired with its low cost makes it a prime candidate for global adoption. With a uniform, sensitive, and accurate ZIKV diagnostic in place, further data

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collection from ZIKV infected patients can be pursued with confidence.

RESEARCH QUESTIONS

Little is known about ZIKV pathogenesis and host-interaction. This lack of knowledge paired with the rapid appearance of serious ZIKV clinical manifestations on a global scale highlights the need for resources to be directed towards ZIKV research. A global effort to establish a uniform systematic framework of ZIKV patient and viral diagnostics must be pursued. The first step is the implementation of a uniform and effective ZIKV diagnostic such as the paper-based sensor developed by Pardee *et. al* [5]. Once areas of *Flavivirus* endemicity are able to efficiently diagnose ZIKV infections in their populations, further thorough diagnostics need to be performed. Subsequent deep sequencing of all *Flavivirus* RNA in the patient as well as deep sequencing of their genome should then be done on the ZIKV infected patient. This data, in conjunction with longitudinal monitoring of patient ZIKV-associated disease manifestations is key in answering two specific research questions. Firstly, this data can be used to see if the difference in ZIKV disease manifestations seen in various populations are due to mutations in the ZIKV genome. Secondly, it may also shine light on host gene polymorphisms which may affect varying susceptibilities to the different ZIKV-associated clinical outcomes. Not only can this data be used to answer the aforementioned two research questions, possibly serving as a prognostic tool, but may also contribute to elucidating ZIKV molecular mechanisms by providing insight into key host or viral factors, as well as possible connections to other *Flavivirus* infections. This may have implications not only on furthering knowledge of ZIKV, but also in the application of anti-virals, both in the broad sense and in the avenue of precision medicine. In addition to the deep sequence diagnostic approach, serological probing of population at risk of or currently experiencing endemic *Flavivirus* circulation is required. The patients can then be longitudinally monitored for any ZIKV or DENV-associated disease manifestations. This data primarily can answer the research questions of whether antibodies to a previous *Flavivirus* infection can have an effect on the clinical manifestations of a heterologous *Flavivirus* infection. This information can possibly lead to the use of serological profiles as a prognostic or as vital data for efficient vaccine development and use.

PROJECT NARRATIVE

Are mutations in the ZIKV genome creating new pathogenic implications such as microcephaly and GBS?

The idea that mutations in a viral genome can create new pathogenic implications is not a new one. Perhaps the best-known example of this was demonstrated in another *Flavivirus*: West Nile Virus (WNV). One lineage of WNV seemed to have markedly higher rates of neurological disease and death in humans. The two lineages were sequenced and compared, and found to be only 5.1% different at the nucleotide level and only 0.6% different at the amino acid level. However, they found that a key amino acid substitution in the envelope glycoprotein of the more pathogenic strain resulted in the addition of a glycosylation site [26]. Further research supported the evidence that this glycosylation state is important in the pathogenicity and neuroinvasiveness of WNV [27]. Although the most significant, the envelope glycoprotein mutation is only one of many WNV mutations that have effects on virulence and pathogenicity with examples of mutations in both structural and non-structural proteins having an impact [28, 29, 30]. In fact, a recent paper showed that single mutation in the envelope protein of both WNV and DENV, both *Flaviviruses*, affected their stability and antigenicity. Interestingly, this specific mutation did not have similar results in ZIKV [31]. These examples highlight the importance of collecting similar data for ZIKV. However, to thoroughly assess the global variation of ZIKV, a uniform and systematic framework must be set in place. Areas experiencing or at risk of endemic *Flavivirus* circulation should be closely monitored for ZIKV infection. Firstly, accurate diagnosis of ZIKV infection in patients presenting themselves with fever or other known ZIKV-associated symptoms is necessary. This should be done with a combination of methods, such as the paper-based sensor of Pardee *et. al* along with thorough serological testing to allow diagnosis through the multiple time frames of possible ZIKV infection. Once a patient has been confirmed with ZIKV infection, next generation sequencing (NGS), for example using the methods outlined by Marston *et. al,* of any *Flavivirus* RNA in the patient should be performed [32]. Research has shown that both serum and urine in combination to identify ZIKV infection should be used [33] and similar results have been shown for DENV as well [34]. Patients should be monitored longitudinally for the appearance of any ZIKV-associated clinical manifestations. All this

information is to be kept updated on a secure global database. As data begins to be collected this database will allow the analysis of ZIKV strains and single nucleotide polymorphisms (SNPs) against subsequent ZIKV clinical manifestations. Although not on such a large scale, similar studies have already begun to emerge, providing a snapshot into the expected findings such a database could provide. A recent study found differences in six amino acids at the interface between pr domains between pre-epidemic and post-epidemic ZIKV strains. The researchers hypothesize that the amino acids found in the post-epidemic strain allow more stable interactions in the immature virion form of ZIKV. This would increase the structural heterogeneity of the virion and possibly have antibody-dependent enhancement-like ramifications on ZIKV infections [20]. Another study found seven critical mutations in the 3' untranslated (UTR) region of the ZIKV genome. They suggest that these mutations that occurred in the Asian strain may be the cause of currently known ZIKV-associated medical problems. These mutations may not only confer stability to the ZIKV genome through secondary structures, but may also be examples of subgenomic *Flavivirus* RNA (sfRNA) [13]. sfRNAs have been shown to be important factors in *Flavivirus* pathogenicity, with roles in inhibiting immune pathways and acting in RNA interference (RNAi) pathways [13, 35]. These findings are prime examples of the connections such a database would allow us to make. If there are other key mutations in ZIKV strains affecting relative pathogenicity, this database would quickly allow us to find them. This information can allow us to zoom in our areas of research, expediting the understanding of ZIKV molecular mechanisms and pathogenesis. Similarly, down the line, this information will be key in the application of anti-viral treatments and precision medicine treatments based on individual ZIKV strain infections, as well as serving as a prognostic to assess risk of particular clinical manifestation based on the ZIKV strain. However, looking at the viral genome in isolation only gives part of the story. The interaction between virus and host is complex and the variations in host factors play a critical role in mediating viral outcomes. To gain a more complete picture as to the underlying mechanisms and pathogenesis of ZIKV, host genomics must also be considered.

Are host gene polymorphisms contributing to the differential ZIKV-associated clinical outcomes?

Host gene polymorphisms are known to be crucial in determining viral susceptibility and pathogenic outcomes. Although there are many cases of host genetics playing a role with viruses such as HIV [36], examples of such cases with *Flaviviruses* allows better comparisons to ZIKV. There have been multiple cases of host gene polymorphisms affecting the outcome of WNV infection. Research showed that a single nucleotide polymorphism in equine and murine models increased susceptibility to WNV encephalitis [37, 38]. Other WNV studies showed that a deficiency in chemokine receptor 5 (CCR5) can increase risk of both early and late WNV-related clinical manifestations [38, 39]. DENV is also an example with host polymorphisms in various receptors modulating risk of developing Severe Dengue [38]. With these examples, it is not hard to believe that similar factors may be contributing to the differential disease manifestations displayed by ZIKV. To explore this possibility, the proposed framework of ZIKV diagnosis and genome sequencing must be built upon. In addition to sequencing viral RNA in every ZIKV confirmed case, complete genome sequencing of the patient must also be performed. With technological advancements in NGS, such as the Illumina platform, this type of data collection is no longer out of reach [40]. Adding this information to the global ZIKV database is crucial in finding causative links between host polymorphisms and ensuing clinical manifestations. Although at the time of writing there have been no such studies, based on the current molecular knowledge of ZIKV, possible host gene candidates can be proposed. A possible area of interest is in the interaction of ZIKV with the host AXL receptor. AXL has been shown to mediate the entry of ZIKV into human glial cells and neural progenitor cells, possibly playing a large role in the development of microcephaly. The interaction occurs through ZIKV binding Growth arrest-specific 6 (Gas6) protein, the natural ligand of AXL, which then binds AXL and mediates ZIKV entry. In addition to entry, there is also evidence that this interaction is involved in dampening host interferon responses and increasing infectivity of the virus [22]. Polymorphisms in Gas6 have already been linked to increased risk of clinical issues such as stroke and type 2 diabetes [23, 41] and have been suggested to have an effect on the biology of the Gas6 protein itself [41]. It may be possible that polymorphisms affecting the relative affinities or levels of the Gas9 and AXL proteins may increase susceptibility to neurological ZIKV complications. Comparing incidence of microcephaly with polymorphisms in the *AXL* and *Gas6* genes using information collected in the database could provide a

possible explanation for increased microcephaly susceptibility. Another possible initial gene of interest is the tumor suppressor 53 gene (*TP53*) encoding for the P53 protein. Research has shown that ZIKV strongly activates the P53 pathway, leading to genotoxic stress and apoptosis in human neural progenitor cells. It is suggested that this could be a probable cause of ZIKV-induced microcephaly [42]. The consideration of *p53* as a candidate gene stems from previous research of a single codon polymorphism having effects on pathogenicity of Hepatitis C Virus, a member of the *Flaviviridae* family [43]. As our molecular knowledge of ZIKV increases, more candidate genes will be discovered. Conversely, as the information in the proposed ZIKV database begins to find correlations between host genes and disease manifestations, the speed at which our knowledge of molecular factors involved in ZIKV pathogenesis will increase rapidly. The impacts of finding host genetic variants causing varying susceptibility to ZIKV-associated diseases are tremendous. Aside from accelerating the rate and focus of research to specific molecular targets, these links are key in the development of both prognostics and antiviral treatment. If a gene variant is shown to pose increased risk of susceptibility to ZIKV, single gene assays may be implemented as patient prognostics. Also, if a host gene is found to be key in increased or decreased susceptibility to ZIKV infection or particular disease manifestation, this can possibly provide a streamlined focus to an antiviral target. These outcomes illustrate the importance of including host genomic information in the ZIKV global database, providing a more through and complete databank. However, a database with a focus on a *Flavivirus* would not be complete without serological data. The need for serological probing of populations moves towards providing a thorough understanding of ZIKV, it's pathogenicity, and its interaction with previous *Flavivirus* infections.

Can population serological testing elucidate the role of antibodies in differential ZIKV disease manifestations?

With recent evidence of antibody-mediated enhancement of ZIKV by DENV antibodies, the role that antibodies play in ZIKV pathogenesis presents itself as a key area of interest [24]. The importance of antibodies in DENV infection has been well documented. It has been shown that secondary infection with a heterologous DENV serotype increases the risk of developing Severe Dengue, shifting clinical outcomes to more severe manifestations [25]. The leading hypothesis to explain this occurrence is the concept of antibody-dependent enhancement. The hypothesis primarily suggests that existing heterologous DENV antibodies are not able to neutralize the secondary DENV infection due to the 30-35% amino acid difference in the envelope (E) glycoprotein. However, the antibodies may bind with sufficient avidity to act as an opsonin, increasing DENV infection of Fc receptor (FcR)-bearing cells such as monocytes and macrophages, both cell types in which DENV is able to replicate [25]. Adding to the structural heterogeneity of *Flaviviruses* is the interaction between the membrane precursor (prM) protein and the E glycoprotein. The prM protein must be cleaved by furin-like proteases in order for the virus to be infectious. However, the extent of uncleaved prM retained upon release of the virion varies depending on the *Flavivirus* and the cell line in which the virus replicates. DENV in particular is generally deemed as "partially mature" as a large portion of prM remains uncleaved upon virion release [44]. Antibodies specific for prM may therefore also contribute to ADE as they are shown to have poor neutralizing capabilities even at high concentrations. In addition, at levels too low to neutralize the virions, almost all antibodies to both the E glycoprotein and the prM protein can promote ADE [25]. Similar results have even been shown in WNV [45]. With examples in two other *Flaviviruses*, it is not unlikely that infection with a related *Flavivirus* such as DENV, or previous ZIKV infection may have a role in the clinical outcomes of a subsequent ZIKV infection. It is interesting to note that the area in which there was the highest incidence of ZIKV-associated microcephaly, found in Northeastern Brazil [18,19], was found to have high DENV seroprevalence ranging from 74% to 91% [46]. Does this suggest a causative relationship? In order to investigate this and more, a longitudinal seroprevalence study must be undertaken. Areas of focus should be those at risk of or currently experiencing endemic *Flavivirus* circulation. For every patient, a serological profile of antibodies to known *Flaviviruses* must be compiled using a series of serological assays. There are already multiple serological assays, such as the multiplex immunoassay outline by Beck *et. al*, which can be implemented in characterization of such viruses [47, 48] and research has shown that a combination of multiple assays searching for multiple arboviruses can aid in minimizing identification error [49]. After using multiple assays to develop a serological profile for the patient, the information will be uploaded to the global

database and the patient will once again be monitored longitudinally for development and severity of any ZIKV or DENV-associated clinical manifestations. With time this information could elucidate the relationship between previous *Flavivirus* infections and subsequent ZIKV clinical manifestations. For example, if a high incidence of severe ZIKV clinical manifestations occur in patients with previous DENV infections, perhaps DENV antibodies are a risk factor or causative agent to various ZIKV pathogenic outcomes. Conversely, research has shown that ZIKV antibodies may increase the severity of subsequent ZIKV infection [50, 51]. For this reason, it is important to also include the monitoring of DENV-associated clinical manifestation that may arise in the patient. Similarly, one could hypothesize declining antibody titer from a previous ZIKV infection having impact on subsequent ZIKV infection. Research has shown that mutations in pathogenic strains of ZIKV may provide structural heterogeneity, limiting the availability of neutralizing epitopes on the virion. This may cause ADE or similar effects for subsequent ZIKV infections [20], leading to more severe clinical manifestations. This longitudinal serological study can allow us to observe these relationships if they do indeed exist. The implications of findings from this serological study are many. Firstly, if previous infection with a certain *Flavivirus* increases risk of a certain disease manifestation, such as microcephaly, then this can begin to elucidate the molecular mechanisms by which this may happen. Secondly, an individual's serological profile may be used as a prognostic tool to evaluate risk of various clinical outcomes. This could pave the way to precision medicine approaches in which patient is treated based on their relative risk to a certain ZIKV disease. Finally, this data would be invaluable in the future development of vaccinations. Knowing the possible cross-reactivity risks can start to lead us in the right direction towards a safe and effective vaccine for both DENV and ZIKV [52].

SUMMARY AND CONCLUSION

The field of precision medicine represents a paradigm shift to the traditional, generalized treatments of today. It allows an individualized approach to minimize the risk and maximize the benefit of treatment. The area of infectious disease and virology represents an enormous field for precision medicine to break into. Although a fairly new field, its applications are already being considered in areas such as vaccination [53] and tailoring anti-viral treatments to host genetic polymorphisms [54]. While the potential of precision medicine is great [55], it can only go as far as the understanding of the issues it is treating. To apply precision medicine approaches, solid knowledge of the pathophysiology of the illness is needed. In the case of viral-associated diseases this would include things such as the viral infection and replication mechanisms, the viral-host interactions and pathogenesis, and the various host factors relevant in mediating the clinical manifestation. The issue in the case of ZIKV is that our knowledge of it is still very limited. We are just beginning to explore the molecular mechanisms of ZIKV infection and its pathogenesis is still largely a mystery. Without even having a solid generalized antiviral or prophylactic treatment for ZIKV, the area of ZIKV precision medicine seems still years away. However, the purpose of implementing this global database framework is to increase our knowledge of ZIKV in hopes of paving the way towards precision medicine approaches (summarized in Fig. 1). This

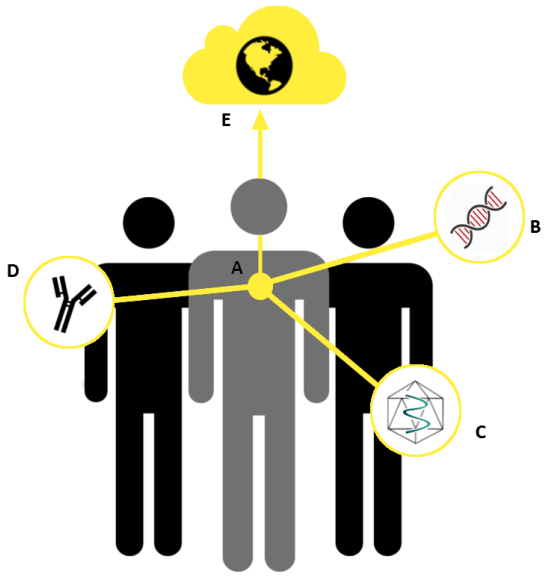

FIG. 1 A three-pronged approach at elucidating the risk factors for various ZIKV-associated clinical manifestations
A: Individual in area at risk of or currently experiencing *Flavivirus* endemicity. They will be monitored longitudinally for ZIKV or DENV associated clinical manifestations.
B: The patient's genome is sequenced and uploaded to a global database.
C: Any *Flavivirus* RNA found in the patient is sequenced and uploaded to the global database.
D: A patient's *Flavivirus* serological profile is uploaded to the global database.
E: The global database will allow these 3 factors to be correlated to subsequent clinical manifesations.

database will hopefully allow us to find key mutations in ZIKV strains causing various severe pathogenic

outcomes. This may elucidate the role of these mutations in the ZIKV lifecycle and further our molecular knowledge of ZIKV infection and pathogenesis. This information is also useful as it will allow risk assessment and prognosis based on the particular ZIKV strain circulating. In addition, this database might reveal key interactions between host genetic polymorphisms and ZIKV-associated clinical manifestations. These correlations again will shine light on various host factors of interest, allowing a better understanding of ZIKV infection, and providing future areas of research. In the area of precision medicine, an individual may have certain "risk" genes sequenced as prognosis for developing various ZIKV-associated diseases. This information will prove invaluable when the first ZIKV anti-virals begin to be developed and used in treatments or prophylaxis. Treatment may be tailored to the individual. Finally, the database might show key correlations between previous *Flavivirus* infections and a secondary *Flavivirus* infection. These might show increased risk of ZIKV-associated diseases upon secondary infection. This means that an individual's serological profile may be used a prognostic tool. It may also allow a better understanding of the immunogenic responses to *Flaviviruses*, providing data to use towards the development of a safe and effective ZIKV vaccine. However, one must consider the difficulties and logistics of implementing such a massive framework. Firstly, this endeavor would require massive funding everywhere that it is implemented. Although the funding required is large, the increasing prevalence of ZIKV as a global health issue should prove to be a motivating factor to provide funding. Secondly are the issues of security and bioethics of such a global health database. In order for a database of genomic and health-related data to be implemented properly, guidelines should be adhered to. Guidelines such as those outlined by Knoppers provide a solid foundation for an ethical, secure, and logical global database [56]. The implementation of this database seems like a monumental undertaking, but similar frameworks have been established and shown to be a massive success [57]. In conclusion, this large-scale framework of ZIKV and *Flavivirus* diagnostics will allow us to advance our knowledge of ZIKV infection and pathogenesis, paving the way for precision medicine applications in the future.

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