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ORIGINAL ARTICLE

First report of collapsing variant of focal segmental glomerulosclerosis triggered by arbovirus: dengue and Zika virus infection

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ABSTRACT

Background. The collapsing variant of focal segmental glomerulosclerosis (FSGS) is the most aggressive form of FSGS and is characterized by at least one glomerulus with segmental or global collapse and overlying podocyte hypertrophy and hyperplasia. Viruses can act as aetiological agents of secondary FSGS. This study aims to establish an aetiological link between dengue virus (DENV) infection and the collapsing variant of FSGS and to analyse possible influences of the *apolipoprotein 1 (APOL1)* gene risk alleles on the disease.

Methods. Biopsies and medical records were gathered from 700 patients of the Instituto de Nefropatologia, Belo Horizonte, Brazil. Screening for the collapsing variant of FSGS was performed and serological, immunohistochemical, tissue polymerase chain reaction (PCR) and genetic analysis were conducted.

Results. Eight patients were identified with positive DENV serology and negative serological and/or tissue markers for hepatitis B virus, hepatitis C virus, Epstein–Barr virus, human immunodeficiency virus, cytomegalovirus and parvovirus B19. In PCR analysis, six patients had positive markers for DENV strain genetic material, one patient had positive markers for co-infection of Zika virus (ZIKV) and DENV and one patient had positive markers only for ZIKV infection. Six of the eight patients did not show risk alleles of the *APOL1* gene. One patient had only one risk allele (G1) and the sample from another did not contain enough DNA for genetic analysis to be performed.

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Conclusions. This study provided strong evidence that DENV can infect renal tissue and possibly functions as a second hit to the development of the collapsing variant of FSGS. Nonetheless, this study also highlights the possible implication of ZIKV infection in FSGS and supports the argument that risk alleles of the *APOL1* gene may not be implicated in the susceptibility to FSGS in these patients.

Keywords: arbovirus, chronic kidney disease, dengue infection, focal segmental glomerulosclerosis, renal histopahology

INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is defined by a morphological pattern of injury in one or more glomeruli that consists of the occlusion of glomerular capillary loops by sclerotic material [1]. FSGS is mainly associated with a healing process that directly or indirectly involves podocyte injury [1]. The collapsing variant of FSGS is the most aggressive form of the disease and is characterized by at least one glomerulus with segmental or global collapse and overlying podocyte hypertrophy and hyperplasia [2]. It is known that viruses can act as aetiological agents of secondary FSGS. Examples of viruses that cause this disease are hepatitis B (HBV) [3] and C (HCV) [4] viruses, cytomegalovirus (CMV) [5], human immunodeficiency virus (HIV) [6], Epstein–Barr virus (EBV) [7] and parvovirus B19 (PVB19) [6].

Dengue virus (DENV) and Zika virus (ZIKV) are positive-sense RNA viruses of the *Flavivirus* genus and Flaviviridae family that are transmitted by the *Aedes aegypti* mosquito vector. Infection with these viruses produces a wide spectrum of acute or subacute manifestations, ranging from an asymptomatic or self-limited non-severe clinical course to severe symptoms. In the case of dengue, the disease can be subdivided into three syndromes: dengue fever, dengue haemorrhagic fever and dengue shock syndrome [8].

Despite the abundance of acute or subacute clinical cases reporting kidney injury and acute glomerulopathies [9, 10], most of them involving interstitial and tubular damage, data are scarce on chronic pathological findings of glomerular, epithelial and podocyte damage as a consequence of dengue infection. To the best of our knowledge, no previous study has associated DENV with FSGS. Herein we present for the first time, a series of eight cases of collapsing FSGS clearly associated with renal tissue positivity to *Flavivirus* genus: six positive to DENV, one to ZIKV and one to both DENV and ZIKV. In addition, we investigate whether polymorphisms of the *apolipoprotein 1 (APOL1)* gene may have a role in collapsing FSGS triggered by DENV and ZIKV.

MATERIALS AND METHODS

Biopsy specimens were gathered from a total of 700 renal biopsies conducted in the Renal Pathology Institute during the first semester of 2016. Of these, 68 biopsies were diagnosed as FSGS and were analysed further, as described in the 'Results' section. All patients had been investigated for recent [immunoglobulin M (IgM) antibodies] DENV, HBV, HCV, CMV, PVB19 and HIV using commercial enzyme–linked immunosorbent assay–based techniques. Serology for ZIKV was not routinely available in 2016. In patients in whom CMV serology was not carried out, biopsies were subjected to immunohistochemistry for the detection of CMV antigens to rule out this infection. The ethics committee of the Universidade Federal de Minas Gerais approved the study, which adhered to the Declaration of Helsinki. Informed consent was obtained from all included patients.

Formalin-fixed, paraffin-embedded tissue was sectioned at 2 μm and stained with rabbit monoclonal anti-CMV (clones DDG9 and CCH2, Diagnostic BioSystems, Pleasanton, CA, USA) as the primary antibody. All cases were stained manually using routine protocols, including deparaffinization, followed by antigen retrieval (tissue section was boiled in 1 mM ethylenediaminetetraacetic acid pH 8.0 for 10 min followed by cooling at room temperature for 20 min), protein blocking (DPB-125S; Spring Biosciences, Pleasanton, CA, USA) and incubation of the primary antibody at room temperature for 30 min (1:40) and the secondary goat anti-rabbit IgG (DHRR-999; Spring Biosystems) at 1:360. Detection was performed with streptavidin/horseradish peroxidase (SPB-125; Spring Biosystems) and developed with Stable DAB (Spring Biosystems).

The renal biopsy specimens were collected and macerated with a Beadbeater 16 homogenizer in phosphate-buffered saline with glass beads. Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Nearly 1 μg of total RNA was reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. cDNA was used in a reverse transcription polymerase chain reaction (PCR) protocol previously described by Bronzoni *et al.* [11], which consisted of PCR (detection of viruses from the *Flavivirus* genus) followed by a semi-nested PCR targeting the nonstructural protein 5 (NS5) region (differential detection of DENV serotypes). ZIKV was detected by real-time PCR, according to the method previously described by Lanciotti *et al.* [12].

For *APOL1* genetic characterization, PCR using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and appropriate primers (forward: 5′-TGAGGGAGTTTTTGGGTGAGA-3′; reverse: 5′-AGAGCTTGCAGTGAGCTGAGA-3′; annealing temperature: 60°C) was performed to amplify 580 bp of the *APOL1* gene containing the rs73885319, rs60910145 and rs71785313 variants. The amplicon was purified using NucleoSpin Gel and a PCR Clean-up Kit (Macherey-Nagel, Düren, Nordrhein-Westfalen, Germany) and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies, Carlsbad, CA, USA). The sequencing reaction was performed using the BigDye Terminator v3.1 mix (Applied Biosystems, Foster City, CA, USA) according to the instructions in the kit. Sequence data were generated with an ABI3730 capillary sequencer (Applied Biosystems) using standard protocols and analysed by Sequence Scanner software version 2.0 (Applied Biosystems).

RESULTS

In 2016, Belo Horizonte experienced the largest epidemic of dengue in its history and there was also co-circulation of ZIKV towards the end of the epidemic period [13]. Initially 68 biopsies were identified as FSGS. These patients were evaluated further for dengue positivity. Thirteen patients were found to be IgM positive for DENV and negative for all of the other viral infections tested (Figure 1). Among these 13 patients who were IgM positive for DENV with FSGS, 1 patient had the Tip variant of

FSGS, 1 showed FSGS not otherwise specified (NOS) variant and 11 showed collapsing FSGS (Figure 2).

Eleven renal tissue specimens with the collapsing variant of FSGS were tested further to detect viruses of the *Flavivirus* genus by a semi-nested PCR targeting the NS5 region for differential detection of DENV types [11]. The presence of ZIKV in renal tissue was also investigated by real-time PCR [12]. Among 11 renal tissue specimens, 8 were positive for viruses of the *Flavivirus* genus. Of these, six were positive for only DENV, one was positive for only ZIKV and one was positive for both DENV and ZIKV (Figure 1).

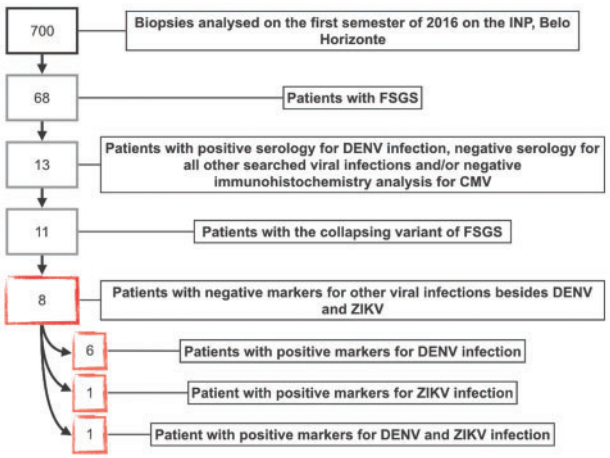

FIGURE 1: Illustrative schema of patients and biopsies selection of the study. Among 13 patients with focal segmental glomerulosclerosis (FSGS) and positive serology only for Dengue virus (DENV) infection, 11 showed collapsing FSGS. Eight among 11 cases of collapsing variant of FSGS had renal tissue positivity for virus of Flavivirus genus, being six positive for DENV, one for Zika virus (ZIKV) and one for both DENV and ZIKV.

Table 1 shows the molecular and serological diagnoses of the eight cases, with renal tissue positivity for viruses of the *Flavivirus* genus and the collapsing variant of FSGS. Renal tissue PCR analysis for DENV showed three cases of DENV-1, three cases of DENV-2 and one case of DENV-3 (Table 1). The seventh case was positive for DENV-2 and also for ZIKV (Table 1). The eighth case was positive for a *Flavivirus* genus virus, but was negative for DENV and positive for only ZIKV (Table 1). Serological tests (IgM) were negative for HBV, HCV, PVB19 and HIV (Table 1). Infection of CMV was also excluded by the negativity of anti-CMV IgM antibody in three cases and by the absence of immunohistochemistry for CMV in renal tissue in all eight patients (Table 1).

As shown in Figures 2 and 3, histological findings of the collapsing variant of FSGS associated with renal tissue positivity for viruses of the *Flavivirus* genus were similar to those observed in other forms of this variant of FSGS. There was dilatation of Bowman's space with glomerular collapse and podocyte hyperplasia and several glomeruli showing collapsing glomerulopathy and tubular dilatation. Immunofluorescence showed trapping of C3 in the mesangial spaces (Figure 2). There was diffuse effacement of podocytes on electron microscopy (Figure 3).

The wild-type allele for *APOL1* has no correlation with renal disease, but studies suggest a strong relationship between the G1 and G2 variant alleles and the development of renal diseases [14], especially FSGS [15]. Among the studied patients, one (14%) had *APOL1* risk alleles and six (86%) had no risk alleles (Table 2). In one patient, *APOL1* risk allele genotyping was not performed due to DNA quality. Figure 4 shows a representative DNA sequence chromatogram of the *APOL1* gene for a homozygous individual with the wild-type for the rs73885319, rs60910145 and rs71785313 variants.

The outcome data of these eight patients were also evaluated. Seven patients evolved to advanced stages of chronic kidney disease (CKD) about 2 years after their renal biopsies. Six patients were at CKD Stage 4 (creatinine clearance < 30 mL/min)

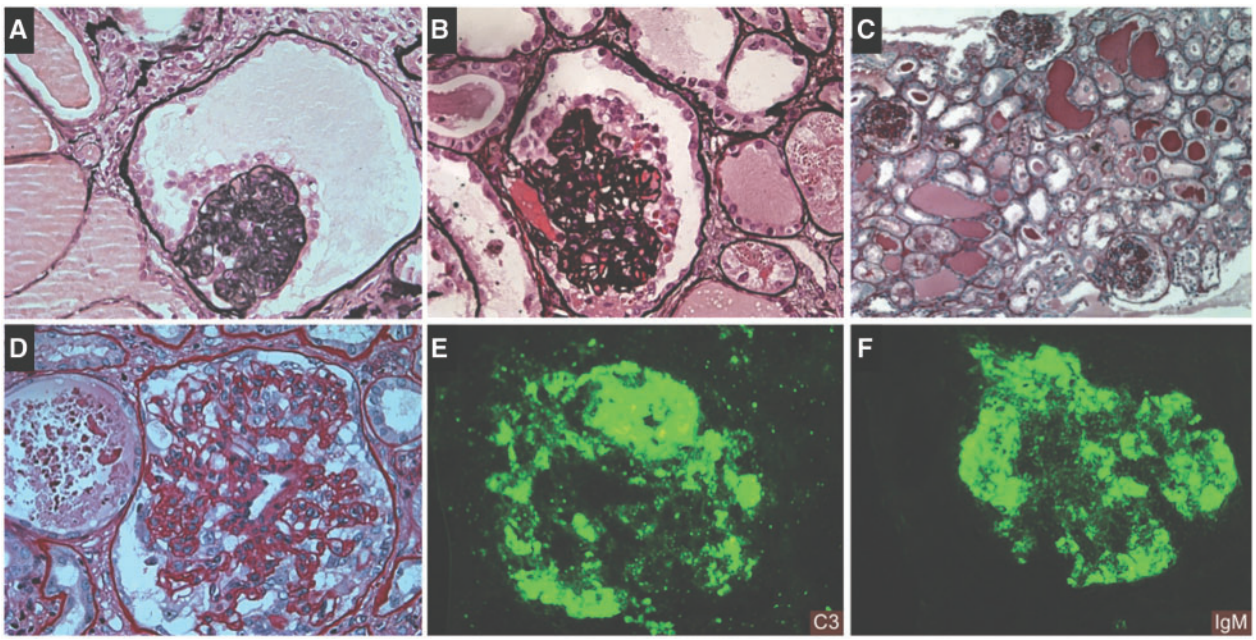

FIGURE 2: (**A**) Bowman Space dilatation with glomerular collapsed and Podocyte hyperplasia. (**B**) Rim of crowed and reactive podocytes resembling a celullar crescent with collapsed capilar loops (Jone's stain 40X). (**C**) Several glomeruli showing collapsing glomerulopathy and tubular dilatation (PAS stain 10X). (**D**) Sloughed cells intubules. (**E** and **F**) Immunofluorescence showing focal trapping of mesangial C3 and IgM (40X).

Table 1. Molecular and serological diagnosis of viral agents

Patient	PCR dengue	PCR ZIKV	HBsAg	Anti-HBs	Anti-HBC	Anti-HVC	Anti-HIV-1 and -2	CMV (IgM)	CMV tissue PCR	PVB19
1	Positive DENV-2	Negative	Negative	Negative	Negative	Negative	Negative	–	Negative	Negative
2	Positive DENV-1	Negative	Negative	Negative	Negative	Negative	Negative	–	Negative	Negative
3	Positive DENV-3	Negative	Negative	Negative	Negative	Negative	Negative	–	Negative	Negative
4	Positive DENV-1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
5	Positive DENV-2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
6	Positive DENV-1	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7	Positive DENV-2	Positive	Negative	Negative	Negative	Negative	Negative	–	Negative	Negative
8	Negative	Positive	Negative	Negative	Negative	Negative	Negative	–	Negative	Negative

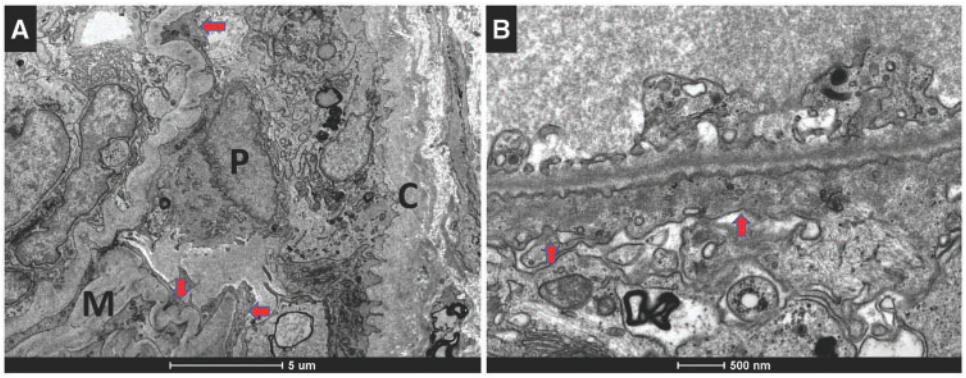

FIGURE 3: Transmission Electron Microscopy. (**A**) Diffuse wrinkling of the glomerular basement membrane (**M**) with electron-dense protein reabsorption droplets and widespread effacement of the podocyte (**P**) foot process (see arrows). Bowman's capsule (**C**). (**B**) Detail of the foot process effacement (see red arrows).

Table 2. Molecular characteristics of patients and *APOL1* risk status

Patient	rs73885319 *APOL1*	rs60910145 *APOL1*	G1[a]	rs71785313 *APOL1*	G2[b]	Risk alleles
1	AA	TT	0	TTATAA/TTATAA	0	0
2	AA	TT	0	TTATAA/TTATAA	0	0
3	AA	TT	0	TTATAA/TTATAA	0	0
4	AA	TT	0	TTATAA/TTATAA	0	0
6	AG	TG	1	TTATAA/TTATAA	0	1
8	AA	TT	0	TTATAA/TTATAA	0	0

[a]Non-synonymous amino acid substitutions S342G (rs73885319) and I384M (rs60910145).
[b]Deletion of amino acid residues N388 and Y389 (rs71785313).

with significant proteinuria and one patient had already been submitted for renal transplantation. Five among the six patients at CKD Stage 4 are currently being prepared for haemodialysis. Only one among the eight patients still has preserved renal function, with an average proteinuria of 500 mg/24 h. No association with any specific virus and prognosis was found. The six patients at CKD Stage 4 included two cases of DENV2 infection, one of DENV1, one of DENV3, one of ZIKV and one of co-infection with ZIKV and DENV2. The patient submitted for renal transplantation had previous infection with DENV1, whereas the case that still had preserved renal function and mild proteinuria was also infected with DENV1.

DISCUSSION

Our findings were gathered when Brazil was going through one of the most intense epidemics of dengue, particularly in Minas Gerais (MG) state [13]. While there was a total of 1 487 673 probable cases of Dengue in Brazil from March to October 2016, ~35% of this total (527 022 cases) took place in MG. The incidence in Belo Horizonte was extremely high, with >6% incidence in the year 2016. There was evidence of ZIKV infection towards the end of the transmission period in that year [13]. Because of the very large number of cases (>150 000 reported cases in 2016), it is reasonable that atypical or unpredictable manifestations of the disease, such as the collapsing variant of FSGS triggered by DENV, could be seen.

Although there are many possible causes of this type of FSGS, the collapsing variant is highly suggestive of viral aetiology and is most frequently associated with HIV and PVB19 infection [2]. The link between a specific form of glomerulonephritis and an occult viral aetiology can be made by the presence of viral nucleic acids in renal tissue and in peripheral blood mononuclear cells, despite the complete absence of detectable systemic viraemia by standard PCR amplification techniques [16]. Dengue infection is classically described as an acute infection, with a short period of viraemia during the febrile phase that ends by the third day after the beginning of symptoms [17]. Several clinical manifestations occur when viral levels are undetectable in peripheral blood [17]. In addition, DENV nucleic acid in peripheral blood mononuclear cells is undetectable during the convalescence period [18]. Among the eight cases presented here, three patients had no history of symptomatic dengue and four developed kidney injury symptoms 2, 4, 5 and 12 weeks following complete resolution of DENV infection symptoms. In the case of Patient 8, in which there was positivity for ZIKV and negativity for DENV in tissue PCR, no history of symptomatic viral infection was reported. These chronological findings suggest that the antigenic material

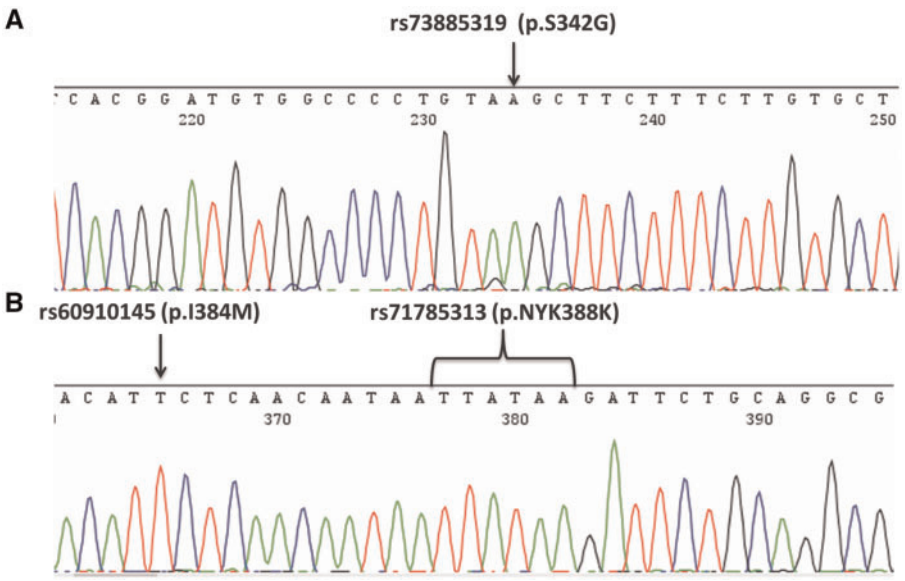

FIGURE 4: DNA sequence chromatograms of the APOL1 gene showing a homozygous individual with the wild type for (**A**) rs73885319 (p.S342G), (**B**) rs60910145 (p.I384M), and rs71785313 (p.NYK388K) variants.

of the viruses, such as the viral RNA found in renal tissue, might be able to trigger FSGS.

In an attempt to exclude other possible viral aetiologies, we gathered medical data reporting blood and serum analyses from the time when the biopsies happened. Patients were HBV, HCV and HIV negative. Three patients were tested for CMV serology, with negative results, and all biopsy specimens were tested with immunohistochemistry for CMV, also showing negative results. PVB19 infection was investigated by renal tissue PCR analysis that indicated no presence of the virus in the specimens. We found no data regarding serology testing for EBV, but, as discussed by Chandra *et al.* [19], evidence linking EBV infection and the collapsing form of FSGS is very scarce.

Very few data are available regarding an effect of ZIKV on kidney cells. To date, only two studies have reported that ZIKV is able to infect tubular, podocyte, epithelial and mesangial human cells *in vitro*, supporting the notion that the kidney may serve as an amplification reservoir [20, 21]. These findings are in accordance with the prolonged period during which ZIKV can be detected in the urine compared with serum [22]. The positivity for ZIKV in renal tissue occurring at the same time as the detection of DENV in Patient 7 raises the question of whether ZIKV also plays a role in the collapsing variant of FSGS. However, in Patient 8, despite positive serology for DENV, only positivity for ZIKV was found in renal tissue PCR. It is well known that cross-reaction in serological tests between DENV and ZIKV is not uncommon. Therefore we believe that the possibility of ZIKV infection should be investigated further in renal tissues of patients with unexplained FSGS, mostly in epidemic scenarios.

The analysis of patient outcomes indicates a bad prognosis for the collapsing variant of FSGS associated with DENV and ZIKV infection. The prognosis seems to be similar to that of previous reports of the collapsing form of FSGS [23, 24]. Despite the small number of patients, we did not find any association between previous infection with DENV strains and ZIKV and disease outcome. When compared with HIV-associated collapsing FSGS, which is probably the best studied virally induced disease, the prognosis seems to be almost the same. Carbone *et al.* [24] previously reported that only 4 among 26 patients (15.4%) with HIV-induced collapsing FSGS evolved preserved renal function during follow-up, while we found only 1 of 8 patients (12.5%) in the same condition.

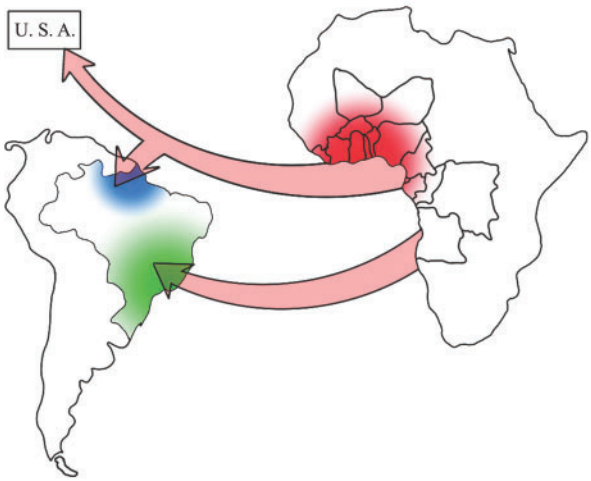

FIGURE 5: The scheme illustrates the immigration flux from Africa to America. The region of biggest prevalence of the APOL1 gene risk variant is evidenced in red, along the territories of Sierra Leone and the previously called Golden Coast. In blue and green are indicated, respectively, the Brazilian regions of biggest prevalence of African immigrants that came from the same African region as the USA (blue) and from the previously called Angola Coast.

APOL1 is one of the six members of the *APOL* gene family and plays a role in the lysis of trypanosomes [14]. APOL1 is the only secreted member of the family and is produced systemically and locally in the kidney [15]. The wild-type allele for *APOL1* has no correlation with kidney disease, but studies have pointed to a strong relationship between the G1 and G2 variant alleles and the development of kidney diseases [25], especially FSGS [26]. Furthermore, studies showing the collapsing variant of FSGS as being associated with HIV and PVB19 have proposed

a link between the *APOL1* risk variants and infection with these viruses. The presence of risk variants of *APOL1* is considered the 'first hit' and the virus infection functions as the 'second hit' that triggers the development of renal disease [27, 28]. Therefore we analysed a possible link between dengue infection and the genetic predisposition of our patients. There was no association between risk variants and infection, as none of our patients were homozygous for any of the risk variant alleles. However, some epidemiological and historical aspects must be taken into account. These risk variants of *APOL1* are frequent among African populations [23] and, considering the genetic origins of the Brazilian population, it is intuitive to hypothesize about the presence of these alleles among these individuals. According to the colonization process of the American continent, it is possible to determine that Brazilians and Americans exhibit, as the constituent bases of their populations, three main ethnicities: Europeans, Indians and Africans. However, it is important to highlight that there are genotype and phenotype differences between the black populations of Brazil and the USA that date back to the colonization process of both regions. Genetic and historical documents indicate that more African-descendent Americans' ancestors came from different geographical locations within the African continent than African-descendent Brazilians [29]. While most African Americans are descended from ancestors from the northwestern region of Africa, in Brazil, especially in the southeast region where our study took place, African-descendents' ancestors primarily came from the African territory previously called Angola Coast, located on the central western side of Africa, as illustrated in Figure 5. This historical difference between Brazil and the USA probably interfered with the genetic pool of both countries, which in turn might have contributed to differences in FSGS. In this regard, a cross-sectional study conducted in Africa identified that *APOL1* risk variants are very frequent among the population that resides in the region of ancestral origin of American African descendants. In contrast, the frequency of risk variants is very low in the ancestral region of Brazilian African descendants [30]. Indeed, previous reports in Brazil did not find an association between risk variants of *APOL1* and the collapsing variant of FSGS [31]. Further studies are necessary to investigate genetic risk factors in the Brazilian population.

The mechanisms by which DENV may cause FSGS are unclear, since there are no data in the literature. HIV is currently the best studied virus with regard to renal diseases. Studies have shown the expression of viral genes in kidney cells, inducing podocyte proliferation and dedifferentiation, apoptosis and fibrosis [32]. However, HIV has a very different structure than DENV, which makes their comparison very difficult. From the viral agents that have already been linked with the development of FSGS, the most structurally similar to DENV is HCV, a *Flavivirus* composed of a 10-kb single positive RNA strand [33]. The mechanisms by which HCV infection produces kidney tissue damage are still unknown [33]. A possible link between viral infection and the collapsing variant of FSGS is activation of the complement cascade, resulting in deposition in renal tissue. Indeed, the presence of mesangial deposition of the C3 fraction of complement is characteristic of the collapsing variant of FSGS [17], and was also detected in our cases.

Podocyte injury is the major pathogenetic factor of FSGS [17]. Bariety *et al*. [34] previously showed that podocytes in collapsing glomerulopathy express macrophagic-associated markers of the CD68 cluster, suggesting that these cells are metaplastic podocytes that may have acquired the ability to process and present antigens. In this context, DENV might enter into podocytes, as all DENVs are capable of infecting host cells that express antigen-presenting cell surface proteins like dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN) and mannose receptors [35]. DC-SIGN is a receptor for DENV and the expression of this receptor can be induced in podocytes during DENV infection [36, 37].

Finally, this study provided strong evidence that DENV can infect renal tissue and possibly functions as a second hit to the development of the collapsing variant of FSGS. Nonetheless, this study highlights the possible implication of ZIKV infection in one of our cases. Also, we believe that the role of *APOL1* risk variants as markers of predisposition for the occurrence of FSGS may have less importance in the Brazilian population. This issue must be further investigated. The pathophysiological mechanisms of arbovirus infections and their organ-specific complications have not yet been fully elucidated. As neglected diseases, research on these infections has received little economic and political support. However, arboviruses represent an important socio-economic problem in developing and underdeveloped countries, and many of their consequences are still unknown. DENV infection should be considered in cases of the collapsing variant of FSGS that occurr in countries with a high prevalence of dengue.

FUNDING

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AUTHORS' CONTRIBUTIONS

S.d.A.A. and A.C.S.e.S. were responsible for the research idea and study design. T.M.e.C. and R.F.d.A.A. were responsible for data acquisition. S.d.A.A. was responsible for histological analysis. P.E.S.M. and D.B.d.O. were responsible for extraction of genetic material. A.R.B. was responsible for genetic analysis. T.M.e.C., A.R.B., R.F.d.A.A. and D.B.d.O. were responsible for data analysis and interpretation. E.G.K., M.M.T. and A.C.S.e.S. were responsible for supervision or mentorship.

CONFLICT OF INTEREST STATEMENT

None declared.

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