1. Introduction

Acute viral bronchiolitis is the leading cause of hospitalization among infants aged < 12 months. Approximately 100,000 bronchiolitis admissions occur annually in the United States, at an estimated cost of $1.73 billion (Meissner, 2016; Ralston et al., 2014; Hall et al., 2009).

Respiratory syncytial virus (RSV) is the causative agent in approximately 70% of bronchiolitis cases, and nearly all children become infected with this virus during the first two years of life (Meissner, 2016; Ralston et al., 2014; Hall et al., 2009). RSV infection ranges from a mild upper respiratory illness to severe bronchiolitis, which may require admission to the intensive care unit (ICU), mechanical ventilation, and possibly lead to death. Treatment is supportive (Meissner, 2016; Ralston et al., 2014; National Collaborating Centre for Women's and Children's Health (UK), 2015). Globally, RSV is estimated to cause 66,000 to 199,000 deaths per year among children younger than five years of age (Meissner, 2016). The second most frequent virus in bronchiolitis is Rhinovirus, which has been implicated in approximately 20% of cases, and occurs in up to 39% of cases (Meissner, 2016; Ralston et al., 2014).

Because bronchiolitis can progress from mild to severe disease it is important to recognize risk factors predisposing to severe disease. We recently published a review of these topics, which include prematurity, passive smoking, young age, absence of breastfeeding, chronic lung disease, and congenital heart disease; some of these risk factors are controversial in the literature (Alvarez et al., 2013). Some controversy also exists regarding the influence of the type of virus and the presence of codetection in the severity of bronchiolitis (Hervás et al., 2012; Weigl et al., 2004; da Silva et al., 2013a; Brand et al., 2012a).

Most infants hospitalized with bronchiolitis present with no risk factors and are otherwise healthy. This led researchers to believe that epidemiological factors are not solely responsible for determining the prevalence and severity of bronchiolitis, and that these might be influenced by genetic variability. Indeed, one study including 12,346 pairs of twins, of whom a fraction was hospitalized for RSV bronchiolitis, found a correlation of 0.66 in homozygous twins and 0.53 in dizygotic twins, estimating a genetic contribution from 16% to 20% for RSV severity (Thomsen et al., 2008).

Bronchiolitis infection is restricted to the superficial cells of the respiratory epithelium. These epithelial cells recognize RSV through specialized pattern recognition receptors known as Toll Receptors or Tool Like Receptors (TLR). The human Toll-like family of proteins consists of at least 10 members of pattern recognition receptors present in macrophages and dendritic cells that represent a critical link between immune stimulants produced by microorganisms and the initiation of host defense (Tal et al., 2004; Löfgren et al., 2010; Goutaki et al., 2014; Murawski et al., 2009; Mailaparambil et al., 2008).

Toll-like receptor (TLR)4 is principally expressed in macrophages, dendritic cells, and in the other cell types. It serves as a transmembrane signaling receptor of lipopolysaccharide (LPS) from Gram-negative bacteria. TLR4 is also involved in an acute innate immune response to RSV. Previous studies have shown evidence that TLR4 is engaged in pattern recognition of RSV F glycoprotein, that TLR4 expression is activated in RSV bronchiolitis, and that genetic variation of *TLR4* represents a risk factor of RSV infection (Tal et al., 2004; Löfgren et al., 2010; Goutaki et al., 2014). *TLR2*, *TLR9* and *TLR10* also carry polymorphisms that have been associated with bronchiolitis (Murawski et al., 2009; Mailaparambil et al., 2008), as well as other genes such as C-C motif chemokine ligand 5 (*CCL5*) also known as regulated on activation, normal T cell expressed and secreted (*RANTES*) (Amanatidou et al., 2008), vitamin D receptor (*VDR*) (Kresfelder et al., 2011; Janssen et al., 2007), inducible nitric oxide synthase (*NOS2*), interferon alpha 5 (*IFNA5*), and Jun proto-oncogene, AP-1 transcription factor subunit (*JUN*) (Janssen et al., 2007).

The aim of this study was to determine the genetic features associated with a severe course and risk of bronchiolitis.

2. Materials and methods

2.1. Patients and control group

We prospectively evaluated all severe acute viral bronchiolitis patients aged < 2 years admitted at three hospitals, in the region of the city of Campinas, São Paulo State, in Brazil, in a 2-year period (Jan/2013 to Dec/2014), who required oxygen therapy. This was the patient group, with 181 cases. One hundred thirty-one of these patients had no comorbidities (premature birth, chronic respiratory disease, and congenital heart disease). The diagnosis of bronchiolitis was based on clinical data, using the most widely accepted definition, which considers it to be the first episode of acute respiratory distress with wheezing, preceded by upper airway symptoms such as rhinorrhea and cough, with or without fever, in children under 2 years of age (Ralston et al., 2014). The severe bronchiolitis criterion was oxygen saturation < 92%. Patients with this condition were admitted for oxygen therapy (Ralston et al., 2014). Patients with previous wheezing were excluded. Patients were admitted in ICU when oxygen saturation remains < 92% even with the patient getting inspired oxygen fraction > 60%. Patients were submitted to mechanical ventilation if arterial partial pressure of oxygen were < 60 mm Hg or arterial partial pressure of carbon dioxide were > 50 mm Hg in arterial blood gas analysis. The oxygen therapy was suspended when oxygen saturation remains > 92% in room air. The patient was discharged 24 h after suspending the oxygen therapy.

Patients were evaluated for the presence of comorbidities (premature birth, chronic respiratory disease, and congenital heart disease) and for other epidemiological variables: birth weight, gender, cesarean delivery, gestational age, breastfeeding, maternal smoking during pregnancy, passive smoke exposure, allergies in parents and siblings, number of siblings, numbers of persons in the house, mold exposure, pets in the house, down syndrome, day care attendance and mother's years of education. Parents or guardians answered a questionnaire about epidemiological factors. Outcome of disease was studied performing a longitudinal follow-up of these patients until the time of discharge, evaluating the length of hospital stay, length of oxygen use, need and length of ICU stay, need and length of mechanical ventilation, and progression to death.

Patients underwent nasopharyngeal aspirate for the detection of viruses, and blood collection for the identification of polymorphisms.

The control group consisted of 536 healthy controls (aged 19 to 25 years), randomly invited to participate in the study, with no personal or family history of lung or other chronic disease for two generations, and was from the same geographic region as the patients group. Participants in the control group were all interviewed, and it was ruled out that they had been hospitalized in childhood for respiratory problems. In this way, we eliminated the possibility that they have presented severe acute viral bronchiolitis. In our study, no healthy controls were included from preexisting cohort, blood bank, patient population and/or patient's parents. The study of ancestry was not performed due to the high cost of analysis. Using a control group with healthy adults is a useful and accepted tool that has been applied in a large number of genetic association studies (Tal et al., 2004; Mailaparambil et al., 2008; Amanatidou et al., 2008; Janssen et al., 2007; Arruvito et al., 2015; Ricciardolo et al., 2004).

2.2. Virus screening

RNAprotect® Cell Reagent (Qiagen, Valencia, CA, USA) was added to nasopharyngeal aspirates in a 1:5 ratio, and stored at − 80 °C. Stored material was centrifuged and the supernatant discarded. The cell pellet was then resuspended in buffer RLT Plus and DNA and RNA isolation was performed using the AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

cDNA was synthesized using the High Capacity cDNA Reverse

Transcription kit (Applied Biosystems/Thermo Fisher Scientific, São Paulo, Brazil) according to the manufacturer's protocol. Samples were then tested using the Seeplex® RV15 ACE detection kit (Seegene, Concord, CA, USA) for 13 types of RNA viruses and two types of DNA viruses according to the manufacturer's instructions. PCR was conducted in a thermocycler Mastercycler Eppendorf® with vapo.protect™ (São Paulo, Brazil) based on the manufacturer's protocol in a final volume of 20 μL containing 3 μL of cDNA (or 1.5 μL cDNA and 1.5 μL DNA; concentration included was 100 ng/μL), 4 μL of 5 × RV15 ACE primer (A, B, or C), 3 μL of 8-MOP (8-Methoxypsoralen) solution, and 10 μL of 2 × master mix (DNA polymerase and buffer containing dNTPs and dye). Amplified PCR products were analyzed in 2% agarose gels stained with GelRed (Biotium Inc., Hayward, CA). We chose to study the most frequently virus cited in the literature as causing bronchiolitis (Meissner, 2016; Ralston et al., 2014). The viruses tested were: RSV subtypes A and B; rhinovirus A/B/C; parainfluenza virus 1, 2, 3, and 4; adenovirus; coronaviruses 229E/NL63 and OC43; influenza A virus and influenza B virus; bocavirus 1/2/3/4; metapneumovirus; and enterovirus.

2.3. Polymorphism screening

The SNPs enrolled in our data were selected based on previous published studies (Tal et al., 2004; Löfgren et al., 2010; Goutaki et al., 2014; Murawski et al., 2009; Mailaparambil et al., 2008; Amanatidou et al., 2008; Kresfelder et al., 2011; Janssen et al., 2007; Arruvito et al., 2015; Puthothu et al., 2006; Lee et al., 2015; Fan et al., 2003; Ricciardolo et al., 2004). All SNPs studied are bi-allelic. Genomic DNA was extracted from blood samples using the QIAamp® DNA Blood Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The OpenArray® Real-Time PCR Platform and AccuFill™ System (Thermo Fisher Scientific, São Paulo, Brazil) were used to screen the following 16 polymorphisms with TaqMan® OpenArray® Genotyping Plates, format 16 (PN: 4413546): rs4986790 (*TLR4*), rs4986791 (*TLR4*), rs1927911 (*TLR4*), rs1898830 (*TLR2*), rs7656411 (*TLR2*), rs352162 (*TLR9*), rs187084 (*TLR9*), rs1065341 (*CCL5*), rs2107538 (*CCL5*), rs2280788 (*CCL5*), rs2280789 (*CCL5*), rs10735810 (*VDR*), rs2228570 (*VDR*), rs1060826 (*NOS2*), rs10757212 (*IFNA5*), and rs11688 (*JUN*).

2.4. Statistical analysis

Epidemiological and laboratory data were described by frequency (percentage) for categorical variables, and means ± standard deviation or medians (minimum and maximum) for quantitative variable data. Disease outcomes were compared between patients, and polymorphism frequency was compared between patients and controls. We also analyzed polymorphism frequencies in patients for each type of virus (RSV, specific RSV subtype A or RSV subtype B, rhinovirus, and virus codetection) comparing each subgroup with controls. A second analysis, for all previously mentioned comparisons, was performed excluding 50 patients with comorbidities (premature birth, chronic respiratory disease, and congenital heart disease), this group remained with 131 patients. This analysis was performed to exclude the possible influence of those comorbidities in patient's outcomes.

Fisher's exact test and the χ^2 test were applied to compare categorical data. We reported odds ratios (OR) and 95% confidence intervals; two-sided $P < 0.05$ was considered statistically significant. Data were analyzed using Statistical Package for the Social Sciences 21.0 software (SPSS Inc., Chicago, IL).

We evaluated minor allele frequency (MAF) and Hardy-Weinberg equilibrium (HWE) using OEGE software (Rodriguez et al., 2009). In order to evaluate genetic interaction among the polymorphisms and clinical data in our sample, we used the Multifactor Dimensionality Reduction (MDR) model, which is a nonparametric, genetic, and environmental model-free data mining for nonlinear interaction identification among genetic and environmental attributes (Gola et al., 2016). To adjust results for multiple comparisons, we performed a MDR permutation test in our sample, totalizing 100,000 permutations.

Details about statistical analysis:

(a) Association study models: For single nucleotide polymorphisms (SNPs) evaluation we performed comparison considering all major possibilities of genetic association, including the allelic analysis. For example, regarding one hypothetic SNP with the allele 1 (A1) and allele 2 (A2), we performed the allelic analysis + all genotypes comparisons [(i) A1A1 versus A1A2 versus A2A2; (ii) A1A1 versus A1A2 + A2A2; (iii) A1A1 + A1A2 versus A2A2; (iv) A1A2 versus A1A1 + A2A2]. We chose to present only the associations with P value < 0.05.
(b) Models for calculation of OR in the presence of codominance: OR was calculated for the three possibilities when P value were < 0.05 for co-dominant model: (i) A1A1 versus A1A2 + A2A2; (ii) A1A1 + A1A2 versus A2A2; (iii) A1A2 versus A1A1 + A2A2.
(c) Overdominance analysis: The overdominance analysis was made considering the important contribution of heterozygous genotype in maintaining the prevalence of several diseases studied in the literature, including cancer and asthma. Although many studies have reported the importance of the heterozygous genotype prevalence of different diseases, the role of heterozygosis is not well known in many cases and needs to be better understood by genetic association studies in different populations.
(d) Bonferroni correction: Association studies, including genetic variables, environmental variables (such as viral identification) and clinical data of patients and healthy controls allow the possibility of performing multiple comparisons. Bonferroni correction is a method employed to minimize the effect of multiple comparisons and the presence of false positives. We tested 16 SNPs but two showed no amplification, so Bonferroni correction was applied and correction of the P value was carried out by multiplying by 14 ($\alpha = 0.05/14$). The Bonferroni correction method for multiple testing was published in 1935. Even with wide applicability and use, some authors consider unnecessary to carry out this correction, so we decided to present both data (corrected and uncorrected).

Additional references for the statistical models applied in the study was set at online supplemental material 1.

2.5. Ethics statement

This study was approved by the National Research Ethics Commission and was conducted according to the Declaration of Helsinki principles. Legal representatives of patients, and participants of the control group, received explanations about the research and gave written informed consent.

3. Results

Table 1 shows patient demographics and clinical characteristics. Table 2 shows details of the viruses identified. One hundred and eighty-one patients were included in the study, 173 of these patients underwent nasopharyngeal aspirate. Viral identification was positive in 87.3% of cases; the most frequently detected virus was RSV in 121 samples (69.9%), followed by rhinovirus, which was present in 46 samples (26.6%). One hundred and thirty-one patients had no comorbidities, 126 of these patients underwent nasopharyngeal aspirate. Viral identification was positive in 87% of cases; the most frequently detected virus in this group was RSV in 89 samples (70.6%), followed by rhinovirus, which was present in 31 samples (24.6%).

The control group was used to achieve the SNPs genotype frequency and to associate with severe acute viral bronchiolitis group.

Table 1
Demographic and clinical characteristics in 181 infants hospitalized with bronchiolitis (173 underwent nasopharyngeal aspirate). Data of the 131 infants without comorbidities is shown in a separated column (126 underwent nasopharyngeal aspirate).

Item	All patients (181)	Patients without comorbidities (131)
Demographic and clinical characteristics in infants hospitalized with bronchiolitis		
Birth weight (gr)	3032.47 ± 671.79; 3102 (565 to 4850)	3264.10 ± 474.32; 3240 (2050 to 4850)
Male gender	105 (58%)	72 (55%)
Cesarean delivery	103 (58.2%)	72 (55.8%)
Gestational age (weeks)	37.42 ± 2.39; 38 (28 to 42)	38.43 ± 1.34; 38 (37 to 42)
Premature birth (< 37 weeks)	38 (21%)	–
Age (days)	140.61 ± 118.13; 104 (16 to 622)	137.16 ± 101.18; 111 (16 to 469)
Infants breastfed since birth	64 (36.6%)	25 (19.1%)
Family and environmental data in infants hospitalized with bronchiolitis		
Maternal smoking during pregnancy	18 (10.2%)	12 (9.4%)
Passive smoke exposure	50 (28.4%)	37 (28.9%)
Mother with asthma	22 (12.4%)	13 (10.1%)
Father with asthma	21 (11.9%)	15 (11.6%)
Siblings with asthma	38 (21.5%)	19 (14.7%)
Mother with allergic rhinitis	45 (25.6%)	33 (25.6%)
Father with allergic rhinitis	27 (15.3%)	20 (15.5%)
Siblings with allergic rhinitis	33 (18.6%)	23 (17.8%)
Mother with atopic dermatitis	8 (4.5%)	6 (4.7%)
Father with atopic dermatitis	3 (1.7%)	2 (1.6%)
Siblings with atopic dermatitis	17 (9.6%)	11 (8.5%)
Number of siblings	1.34 ± 1.31; 1 (0 to 7)	1.19 ± 1.15; 1 (0 to 5)
Numbers of persons in the house	4.52 ± 1.43; 4 (2 to 13)	4.36 ± 1.34; 4 (2 to 13)
Mold exposure	37 (21%)	27 (21.1%)
Pets in the house	79 (44.9%)	62 (48.4%)
Day care attendance	25 (14.1%)	17 (13.2%)
Mothers with ≤ 9 years of education	83 (48%)	60 (47.2%)
Mothers with 10 to 16 years of education	62 (35.8%)	49 (38.6%)
Mothers with ≥ 17 years of education	28 (16.2%)	18 (14.2%)
Comorbidities in infants hospitalized with bronchiolitis		
Chronic respiratory disease	3 (1.7%)	–
Congenital heart disease	13 (7.3%)	–
Down syndrome	4 (2.2%)	–
Outcome in infants hospitalized with bronchiolitis		
Length of hospital stay (days)	6.5 (1 to 64)	6 (1 to 26)
Length of oxygen therapy (days)	5 (1 to 63)	5 (1 to 24)
Intensive care unit (ICU) admission	61 (34%)	41 (32%)
Length of ICU stay (days)	9 (2 to 37)	8 (2 to 20)
Need of mechanical ventilation	38 (21%)	25 (19.5%)
Length of mechanical ventilation	8.5 (2 to 37)	8 (2 to 18)
Death	5 (2.8%)	4 (3.1%)

Data are expressed as mean ± standard deviation; medians (range) or frequencies (percentage).

Table 2
Virus identified in 181 infants hospitalized with bronchiolitis (173 underwent nasopharyngeal aspirate). Data of the 131 infants without comorbidities is shown in a separated column (126 underwent nasopharyngeal aspirate).

Item	All patients (181)	Patients without comorbidities (131)
Respiratory syncytial virus	121 (69.9%)	89 (70.6%)
Respiratory syncytial virus A	91 (52.6%)	64 (50.8%)
Respiratory syncytial virus B	31 (17.9%)	25 (19.8%)
Rhinovirus	46 (26.6%)	31 (24.6%)
Parainfluenza virus	6 (3.5%)	4 (3.2%)
Adenovirus	8 (4.6%)	5 (4%)
Coronavirus	3 (1.7%)	2 (1.6%)
Influenza virus	2 (1.2%)	–
Bocavirus	1 (0.6%)	–
Metapneumovirus	3 (1.7%)	3 (2.4%)
Enterovirus	2 (1.2%)	2 (1.6%)
Negative	22 (12.7%)	17 (13%)
Codetection	37 (21.4%)	26 (20.6%)

Data are expressed as frequencies (percentage).

3.1. Association between polymorphisms and bronchiolitis presence and severity

Table 3 shows SNPs description including MAF and HWE. SNPs rs10735810 (*VDR*) and rs11688 (*JUN*) assays failed in our test. SNPs rs1065341 and rs2280788 (*CCL5*) are not in HWE for patients and controls subjects. Moreover, SNP rs1060826 (*NOS2*) is not in HWE only for controls subjects.

Fig. 1 and Table 4 show association between polymorphisms and the presence of bronchiolitis for different types of virus. The evaluation of the presence of bronchiolitis for different types of virus revealed an association between SNP rs2107538*CT (*CCL5*) and bronchiolitis caused by RSV (OR = 1.646; 95%CI = 1.054 to 2.57) and RSV subtype A (OR = 1.754; 95%CI = 1.06 to 2.902) specifically, and between SNP rs1060826*GG (*NOS2*) and bronchiolitis caused by rhinovirus (OR = 2.649; 95%CI = 1.309 to 5.362). In patients without comorbidities, we observed an association between SNP rs1060826*GG (*NOS2*) and bronchiolitis caused by rhinovirus (OR = 3.369; 95%CI = 1.435 to 7.908).

Fig. 2 and Table 5 show association between polymorphisms and bronchiolitis severity. SNPs rs4986790*AG (*TLR4*) (OR = 8.025; 95%CI = 1.26 to 51.12) and rs2228570*CC (*VDR*) (OR = 8.889; 95%CI = 1.312 to 60.21) were associated with progression to death, rs352162*TT (*TLR9*) (OR = 5.207; 95%CI = 2.118 to 12.8), and rs187084*TC (OR = 0.455; 95%CI = 0.222 to 0.935) (*TLR9*) with ICU admission, and rs352162*CC + CT (*TLR9*) (OR = 2.718; 95%CI = 1.103 to 6.695) and rs2107538*TT (*CCL5*) (OR = 4.974; 95%CI = 1.047 to 23.63) with need for mechanical ventilation.

Table 3
SNPs description including minor allele frequency and Hardy-Weinberg equilibrium.

Gene	SNPs	Ancestral allele (AA)	Rare allele (RA)	Functional consequence	Amino acid AA[a]	Amino acid RA[a]	Group	MAF	HW
TLR4	rs4986790	A	G	Missense	Asp	Gly	SAVB	0.09 (G)	> 0.05
							Control	0.07 (G)	> 0.05
	rs4986791	C	T	Missense	Thr	Ile	SAVB	0.02 (T)	> 0.05
							Control	0.05 (T)	> 0.05
	rs1927911	T	C	Intron variant			SAVB	0.34 (T)	> 0.05
							Control	0.33 (T)	> 0.05
TLR2	rs1898830	C	A	Intron variant			SAVB	0.34 (C)	> 0.05
							Control	0.34 (C)	> 0.05
	rs7656411	G	T	Downstream variant 500B			SAVB	0.34 (G)	> 0.05
							Control	0.37 (G)	> 0.05
TLR9	rs352162	C	T				SAVB	0.47 (T)	> 0.05
							Control	0.46 (T)	> 0.05
	rs187084	C	T	Upstream variant 2 KB			SAVB	0.39 (C)	> 0.05
							Control	0.42 (C)	> 0.05
CCL5	rs1065341	A	G	Intron variant, UTR 3 prime			SAVB	**0.49 (G)**	**< 0.05**
							Control	**0.48 (G)**	**< 0.05**
	rs2107538	T	C	Upstream variant 2 KB			SAVB	0.28 (T)	> 0.05
							Control	0.27 (T)	> 0.05
	rs2280788	C	G	Intron variant, Upstream variant 2 KB			SAVB	**0.43 (G)**	**< 0.05**
							Control	**0.38 (G)**	**< 0.05**
	rs2280789	C	T	Intron variant			SAVB	0.2 (C)	> 0.05
							Control	0.18 (C)	> 0.05
VDR	rs10735810	T	C	Missense	Met	Thr	SAVB	–	–
							Control	–	–
	rs2228570	T	C	Missense	Met	Thr	SAVB	0.33 (T)	> 0.05
							Control	**0.33 (T)**	**< 0.05**
NOS2	rs1060826	G	A	Synonymous codon	Thr	Thr	SAVB	0.33 (A)	> 0.05
							Control	0.35 (A)	> 0.05
IFNA5	rs10757212	A	G	Synonymous codon	Thr	Thr	SAVB	0.3 (A)	> 0.05
							Control	0.3 (A)	> 0.05
JUN	rs11688	G	A	Synonymous codon	Gln	Gln	SAVB	–	–
							Control	–	–

AA, ancestral allele; RA, rare allele; SNP, single nucleotide polymorphism; MAF, minor allele frequency; HW, Hardy-Weinberg Equilibrium; *TLR4*, Toll-like receptor 4; *TLR2*, Toll-like receptor 2; *TLR9*, Toll-like receptor 9; *CCL5*, C-C motif chemokine ligand 5; *VDR*, Vitamin D Receptor; *NOS2*, Inducible nitric oxide synthase; *IFNA5*, interferon alpha 5; *JUN*, Jun proto-oncogene, AP-1 transcription factor subunit; SAVB, severe acute viral bronchiolitis; UTR, untranslated region; (–), assays that failed in our test.
In bold type shown the SNPs that are not in Hardy-Weinberg equilibrium.
[a] Data obtained from NCBI including the information about ancestral and rare alleles.

In patients without comorbidities, SNP rs1898830 (*TLR2*) was associated with progression to death because the four patients who died during the study were heterozygous for this SNP (P = 0.036). Additionally, rs1927911*CC (*TLR4*) (OR = 3.578; 95%CI = 1.238 to 10.34) and rs2107538*TT (*CCL5*) (OR = 6; 95%CI = 1.221 to 29.48) were associated with need for mechanical ventilation, rs7656411*GT (*TLR2*) (OR = 2.5; 95%CI = 1.093 to 5.718) with length of oxygen use, and rs352162*TT (*TLR9*) (OR = 4.886; 95%CI = 1.689 to 14.13) and rs2280788 (*CCL5*) with ICU admission. The rs2280788 (*CCL5*) heterozygous genotype was only present in patients who required ICU admission (P = 0.036).

After Bonferroni correction, the following associations maintained P < 0.05: rs1060826*CC + TT (*NOS2*) and patients with Rhinovirus infection (Fig. 1, Table 4); rs352162*TT (*TLR9*) and ICU admission (in all patients group) (Fig. 2, Table 5).

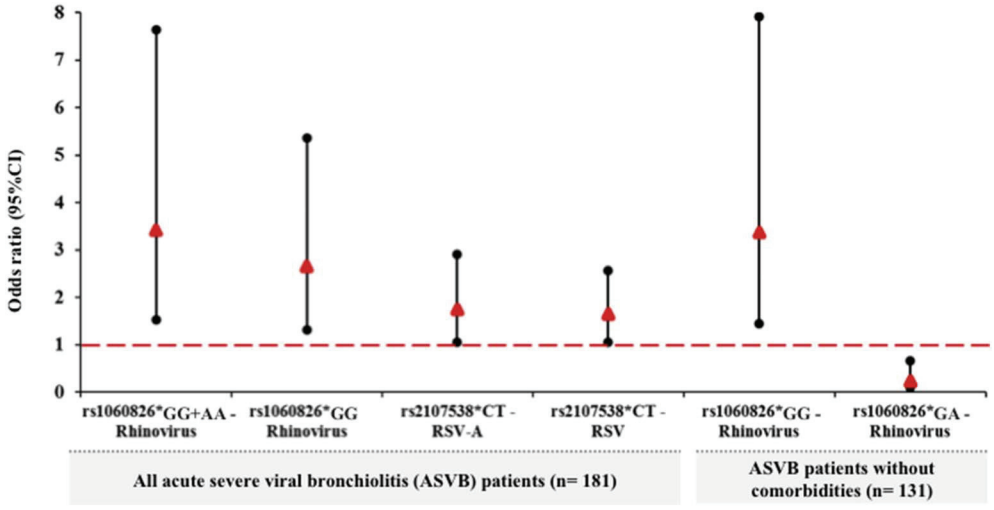

Fig. 1. Association between polymorphisms and presence of bronchiolitis for different types of virus, data for all patients and patients without comorbidities. RSV, respiratory syncytial virus; OR, odds ratio; CI, confidence interval. All parameters show significant differences between or among groups before Bonferroni correction (P < 0.05). The SNP rs1060826*CC + TT shows significant difference between groups after Bonferroni correction (P < 0.05) in patients with Rhinovirus infection. Fisher's exact test and the χ^2 test were applied considering data distribution. The statistical model applied in genetics analysis, considering the SNP genotype used for odds ratio calculation, is presented in parentheses. The MDR analysis showed no evidence of interaction of genetic data with acute severe viral bronchiolitis for the positive association.

Table 4
Associations between polymorphisms and presence of bronchiolitis for different types of virus[a], data for all patients and patients without comorbidities.

All patients						
Polymorphism		Yes	Control group	Total	Odds ratio	95%CI
RSV						
rs2107538 (*CCL5*)	CT	48	170	218	1.646	1.054 to 2.57
	CC + TT	47	274	321	1	–
RSV subtype A						
rs2107538 (*CCL5*)	CT	37	170	207	1.754	1.06 to 2.902
	CC + TT	34	274	308	1	–
Rhinovirus						
rs1060826 (*NOS2*)	GG	23	185	208	2.649	1.309 to 5.362
	GA + AA	13	277	290	1	–
Rhinovirus						
rs1060826 (*NOS2*)[b]	GG + AA	28	234	262	3.41	1.522 to 7.64
	GA	8	228	236	1	–

Patients without comorbidities						
Polymorphism		Rhinovirus		Total	Odds ratio	95%CI
		Yes	Control group			
rs1060826 (*NOS2*)	GG	18	185	203	3.369	1.435 to 7.908
	GA	5	228	233	0.244	0.091 to 0.659
	AA	3	49	52	1.099	0.318 to 3.795

RSV, respiratory syncytial virus; CI, confidence interval; *CCL5*, C-C motif chemokine ligand 5; *NOS2*, Inducible nitric oxide synthase. All parameters show significant differences between or among groups before Bonferroni correction ($P < 0.05$).

[a] The types of virus were screened only in the patients with bronchiolitis.

[b] Parameter shows significant difference between groups after Bonferroni correction ($P < 0.05$). Fisher's exact test and the χ^2 test were applied considering the data distribution. The MDR analysis showed no evidence of interaction of genetic and viral data with acute severe viral bronchiolitis for the positive association (data not showed).

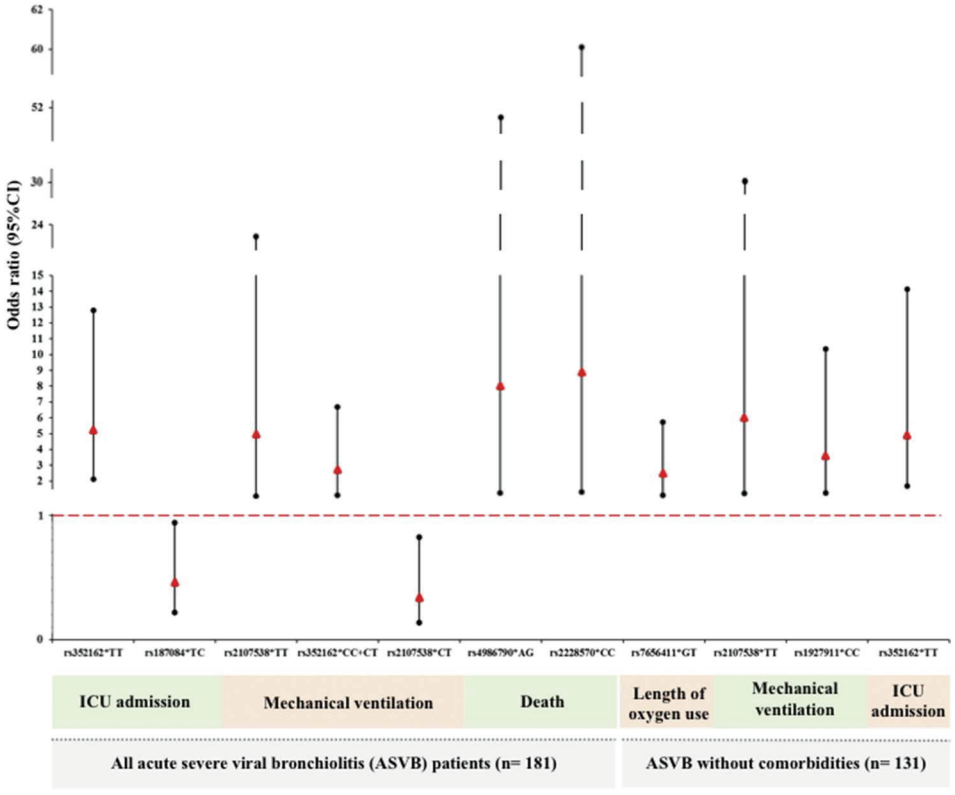

Fig. 2. Association between polymorphisms and bronchiolitis severity, data for all patients and patients without comorbidities. OR, odds ratio; CI, confidence interval; ICU, intensive care unit. All parameters show significant differences between or among groups before Bonferroni correction ($P < 0.05$). The SNP rs352162*TT shows significant difference between groups for ICU admission (in all patients group) after Bonferroni correction ($P < 0.05$). Fisher's exact test and the χ^2 test were applied considering data distribution. The statistical model applied in genetics analysis, considering the SNP genotype used for odds ratio calculation, is presented in parentheses. The MDR analysis showed no evidence of interaction of clinical and genetic data with acute severe viral bronchiolitis for the positive association. The OR axis is out of scale.

Table 5
Association between polymorphisms and bronchiolitis severity, data for all patients and patients without comorbidities.

All patients						
Polymorphism		Yes	No	Total	Odds ratio	95%CI
Death						
rs4986790 (*TLR4*)	AG	3	20	23	8.025	1.26 to 51.12
	AA	2	107	109	1	–
ICU admission						
rs352162 (*TLR9*)[a]	CC	9	27	36	0.467	0.198 to 1.099
	CT	21	47	68	0.575	0.282 to 1.172
	TT	19	9	28	5.207	2.118 to 12.8
Mechanical ventilation						
rs352162 (*TLR9*)	CC + CT	11	17	28	2.718	1.103 to 6.695
	TT	20	84	104	1	–
ICU admission						
rs187084 (*TLR9*)	TC	20	50	70	0.455	0.222 to 0.935
	TT + CC	29	33	62	1	–
Mechanical ventilation						
rs2107538 (*CCL5*)	CC	18	45	63	1.833	0.799 to 4.204
	CT	8	52	60	0.336	0.137 to 0.825
	TT	4	3	7	4.974	1.047 to 23.63
Death						
rs2228570 (*VDR*)	CC	2	9	11	8.889	1.312 to 60.21
	CT	3	63	66	1.571	0.254 to 9.719
	TT	0	57	57	–	–

Patients without comorbidities						
Polymorphism		Mechanical ventilation		Total	Odds ratio	95%CI
		Yes	No			
rs1927911 (*TLR4*)	TT	1	12	13	0.281	0.034 to 2.299
	TC	5	34	39	0.412	0.136 to 1.248
	CC	14	30	44	3.578	1.238 to 10.34
Polymorphism		Length of oxygen use		Total	Odds ratio	95%CI
		> median	< median			
rs7656411 (*TLR2*)	GG	1	6	36	0.195	0.022 to 1.688
	GT	25	20	68	2.5	1.093 to 5.718
	TT	16	28	28	0.571	0.252 to 1.298
Polymorphism		ICU admission		Total	Odds ratio	95%CI
		Yes	No			
rs352162 (*TLR9*)	CC	6	21	27	0.473	0.169 to 1.323
	CT	14	36	50	0.605	0.257 to 1.423
	TT	12	7	19	4.886	1.689 to 14.13
Polymorphism		Mechanical ventilation		Total	Odds ratio	95%CI
		Yes	No			
rs2107538 (*CCL5*)	CC	10	35	45	1.143	0.426 to 3.066
	CT	6	37	43	0.44	0.153 to 1.268
	TT	4	3	7	6	1.221 to 29.48

CI, confidence interval; ICU, intensive care unit; *TLR4*, Toll-like receptor 4; *TLR9*, Toll-like receptor 9; *CCL5*, C-C motif chemokine ligand 5; *VDR*, Vitamin D Receptor. All parameters show significant differences between or among groups before Bonferroni correction ($P < 0.05$).

[a] Parameter shows significant difference among groups after Bonferroni correction ($P < 0.05$). Fisher's exact test and the χ^2 test were applied considering the data distribution. The MDR analysis showed no evidence of interaction of genetic and clinical data with acute severe viral bronchiolitis for the positive previous association.

3.2. Interaction analysis

The MDR analysis showed no evidence of interaction of genetic variants enrolled between severe acute viral bronchiolitis and healthy control. For patient's outcomes, no positive interaction was observed among the clinical data.

3.3. Allelic analysis

No significant association was find between the SNPs alleles and the presence of bronchiolitis considering or not the presence of comorbidities (Tables 6, 7).

4. Discussion

To the best of our knowledge, this is the first study into the association of SNPs in genes involved in the immune response with the severity of bronchiolitis that compared the outcomes of patients that have been admitted to a hospital. Until now, studies analyzing the prevalence of bronchiolitis have compared patients with controls, while those examining the severity of bronchiolitis have compared inpatients with patients seen in the emergency room and then discharged.

Table 6
Associations between polymorphisms and presence of bronchiolitis for the allelic model.

Gene	SNPs	Allele	SAVB	Control	Total	P-value	OR	95%CI
TLR4	rs4986790	A	241	811	1052	0.491	0.84	0.511 to 1.38
		G	23	65	88		1	–
	rs4986791	C	236	819	1055	0.152	1.873	0.784 to 4.478
		T	6	39	45		1	–
	rs1927911	T	89	306	395	0.874	1.024	0.766 to 1.368
		C	175	616	791		1	–
TLR2	rs1898830	C	91	300	391	0.854	1.027	0.769 to 1.372
		A	173	586	759		1	–
	rs7656411	G	91	339	430	0.357	0.874	0.657 to 1.163
		T	179	583	762		1	–
TLR9	rs352162	C	143	509	652	0.767	0.96	0.731 to 1.26
		T	125	427	552		1	–
	rs187084	C	105	391	496	0.432	0.895	0.678 to 1.181
		T	163	543	706		1	–
CCL5	rs1065341	A	132	456	588	0.717	1.051	0.802 to 1.379
		G	136	494	630		1	–
	rs2107538	T	75	242	317	0.712	1.059	0.78 to 1.438
		C	189	646	835		1	–
	rs2280788	C	4	24	28	0.264	0.548	0.189 to 1.594
		G	256	842	1126		1	–
	rs2280789	C	54	170	224	0.426	1.149	0.817 to 1.617
		T	214	774	988		1	–
VDR	rs2228570	T	88	315	403	0.938	0.989	0.741 to 1.319
		C	180	637	817		1	–
NOS2	rs1060826	G	86	326	412	0.415	0.886	0.663 to 1.185
		A	178	598	776		1	–
IFNA5	rs10757212	A	78	265	343	0.793	0.961	0.711 to 1.298
		G	186	607	793		1	–

OR, odds ratio; CI, confidential interval; *TLR4*, Toll-like receptor 4; *TLR2*, Toll-like receptor 2; *TLR9*, Toll-like receptor 9; *CCL5*, C-C motif chemokine ligand 5; *VDR*, Vitamin D Receptor; *NOS2*, Inducible nitric oxide synthase; *IFNA5,* interferon alpha 5; *JUN*, Jun proto-oncogene, AP-1 transcription factor subunit; SAVB, severe acute viral bronchiolitis; UTR, untranslated region; (–), reference. The χ^2 test was applied considering the data distribution.

Table 7
Associations between polymorphisms and presence of bronchiolitis for the allelic model excluding patients with comorbidities.

Gene	SNPs	Allele	SAVB	Control	Total	P-value	OR	95%CI
TLR4	rs4986790	A	160	811	971	0.323	0.754	0.431 to 1.321
		G	17	65	82		1	–
	rs4986791	C	174	819	993	0.162	2.071	0.731 to 5.872
		T	4	39	43		1	–
	rs1927911	T	65	306	371	0.995	0.999	0.72 to 1.386
		C	131	616	747		1	–
TLR2	rs1898830	C	67	300	367	0.857	1.03	0.743 to 1.429
		A	127	586	713		1	–
	rs7656411	G	62	339	401	0.24	0.82	0.589 to 1.142
		T	130	583	713		1	–
TLR9	rs352162	C	107	509	616	0.957	1.009	0.74 to 1.374
		T	89	427	516		1	–
	rs187084	C	76	391	467	0.425	0.88	0.642 to 1.206
		T	120	543	663		1	–
CCL5	rs1065341	A	98	456	456	0.61	1.061	0.78 to 1.444
		G	98	484	484		1	–
	rs2107538	T	58	242	300	0.456	1.138	0.81 to 1.601
		C	136	646	782		1	–
	rs2280788	C	3	24	27	0.328	0.551	0.164 to 1.849
		G	191	842	1033		1	–
	rs2280789	C	39	170	209	0.534	1.131	0.767 to 1.667
		T	157	774	931		1	–
VDR	rs2228570	T	59	315	374	0.417	0.871	0.624 to 1.216
		C	137	637	774		1	–
NOS2	rs1060826	G	64	326	390	0.543	0.903	0.65 to 1.254
		A	130	598	728		1	–
IFNA5	rs10757212	A	60	265	325	0.883	1.026	0.732 to 1.436
		G	134	607	741		1	–

OR, odds ratio; CI, confidential interval; *TLR4*, Toll-like receptor 4; *TLR2*, Toll-like receptor 2; *TLR9*, Toll-like receptor 9; *CCL5*, C-C motif chemokine ligand 5; *VDR*, Vitamin D Receptor; *NOS2*, Inducible nitric oxide synthase; *IFNA5,* interferon alpha 5; *JUN*, Jun proto-oncogene, AP-1 transcription factor subunit; SAVB, severe acute viral bronchiolitis; UTR, untranslated region; (–), reference. The χ^2 test was applied considering the data distribution.

4.1. Virus

RSV was present in 69.9% of patients; 52.6 of patients had RSV-A and 17.9% had RSV-B. The second most common virus was rhinovirus, which was present in 26.6% of patients. Viral codetection was present in 21.4% of the patients. Codetection has been reported in up to 65% of patients with bronchiolitis (Rodríguez et al., 2014). Some controversy exists regarding the influence of the presence of codetection in the severity of bronchiolitis. Some studies suggest that codetection increases the severity of bronchiolitis (Rodríguez et al., 2014; da Silva et al., 2013b), while other studies have shown that patients with viral codetection do not present a more serious disease than patients infected with a single virus (Ricart et al., 2013; Brand et al., 2012b). In our study codetection was associated with patient age and frequency of nursery attendance but was not associated with bronchiolitis severity. The influence of codetection in bronchiolitis severity was not an objective of our study so we will not make an extensive discussion of this topic.

4.2. Toll like receptors

4.2.1. Toll like receptor 4 – TLR4

The interaction between TLR4 and the RSV fusion protein leads to the production of pro-inflammatory cytokines (interleukins 6, 8, 10, and 13, tumor necrosis factor, CCL5, and CX3CK1) and surfactant proteins. Some of these factors have direct antiviral properties, while others stimulate the activation of natural killer cells, granulocytes, monocytes, and macrophages, thus initiating the adaptive immune response (Farrag and Almajhdi, 2016; Arruvito et al., 2015; Lambert et al., 2014; Choi et al., 2013). A TLR deficiency may lead to the absence of Th1 polarizing signals, and change T cell responses from protective Th1 and cytotoxic T cell immunity toward dysregulated Th2 and Th17 polarization, causing bronchiolitis in susceptible infants (Farrag and Almajhdi, 2016; Arruvito et al., 2015). A recent study demonstrated that the RSV fusion protein was capable of inducing the formation of neutrophil extracellular traps (NETs), which immobilize and kill pathogens, through TLR4 activation. The excessive production of NETs contributes to the pathology of respiratory viral infections (Funchal et al., 2015).

Previous studies found that severe RSV bronchiolitis is associated with SNPs in *TLR4* (rs4986790 and rs4986791) (Tal et al., 2004; Puthothu et al., 2006). Moreover, peripheral blood mononuclear cells from children expressing exonic *TLR4* variants were shown to have blunted responses to RSV (Ricart et al., 2013). However, other studies found no association between these SNPs and bronchiolitis (Löfgren et al., 2010; Goutaki et al., 2014). The present study found that death from bronchiolitis is associated with SNP rs4986790 (*TLR4*), but no association was detected between bronchiolitis severity and SNP rs1927911 (*TLR4*). SNP rs1927911 (*TLR4*) was recently associated with an increased risk of asthma development in children with a history of bronchiolitis (Lee et al., 2015), so we are following up our patients to verify any association between bronchiolitis severity, the virus type, genetic polymorphisms, and asthma development. Environmental factors have also been shown to interact with the *TLR4* genotype to modulate the RSV infection severity (Caballero et al., 2015).

4.2.2. Toll like receptor 2 – TLR2

TLR2 is expressed on the surface of immune cells and tissues as a heterodimer complex with either TLR1 or TLR6. Using knockout mice, TLR2 and TLR6 signaling in leukocytes was shown to activate innate immunity against RSV by promoting tumor necrosis factor alpha, interleukin-6, chemokine (C-C motif) ligand (CCL)2, and CCL5, and was important for controlling viral replication and promoting neutrophil migration and dendritic cell activation in vivo (Murawski et al., 2009). Additionally, TLR4 signaling was reported to influence TLR2 expression following certain stimuli, suggesting a role for both TLR4 and TLR2 in the response to RSV (Fan et al., 2003). One study found no association between *TLR1*, *TLR2*, and *TLR6* polymorphisms and the bronchiolitis severity (Nuolivirta et al., 2013). We found that death in bronchiolitis is associated with SNP rs1898830 (*TLR2*) in patients without comorbidities.

4.2.3. Toll like receptor 9 – TLR9

TLR9 has previously been associated with different diseases, such as bronchial asthma (Lazarus et al., 2003). Additionally, RSV was shown to inhibit the production of interferon-γ in human plasmacytoid dendritic cells by TLR9 signaling (Schlender et al., 2005), and TH2 response upregulation, which is characteristically seen in severe RSV-associated diseases. Thus, an involvement of TLR9 in the genetics of bronchiolitis seems reasonable. An association of SNP rs5743836 (*TLR9*) with RSV infection was documented in an earlier study (Mailaparambil et al., 2008), and we found that SNPs rs352162 (*TLR9*) and rs187084 (*TLR9*) were associated with a requirement for ICU admission, and that SNP rs352162 (*TLR9*) was also associated with the need for mechanical ventilation.

4.3. C-C motif chemokine ligand 5 – CCL5

In the course of RSV infection, enhanced chemokine activity modulates cell recruitment and infiltration to the inflammation site. CCL5 is a chemokine produced by CD8 + T-lymphocytes, macrophages, platelets, and epithelial cells that attracts monocytes, eosinophils, basophils, and memory T-lymphocytes to the area of infection. It is highly expressed in respiratory epithelial cell lines, nasal secretions, and broncho-alveolar lavages of RSV-infected subjects. Moreover, evidence supports an association between CCL5 activity and RSV infection (Hattori et al., 2011).

A previous study reported an association between SNPs rs2107538 and rs2280788 in the promoter region and SNP rs2280789 in intron 1 of *CCL5* with RSV bronchiolitis (Caballero et al., 2015). However, another study found no association between RSV bronchiolitis and these SNPs when tested separately, but observed a significantly more common combined SNP genotype in patients than in controls (Amanatidou et al., 2008). We found an association between SNP rs2107538*CT (*CCL5*) and bronchiolitis caused by RSV and RSV subtype A specifically. We also found that SNP rs2107538 (*CCL5*) was associated with the need for mechanical ventilation in bronchiolitis patients.

4.4. Inducible nitric oxide synthase – NOS2

In the respiratory tract, nitrite oxide (NO) is produced by a wide variety of cell types and is generated via oxidation of L-arginine that is catalyzed by the enzyme NO synthase (NOS). NOS exists in three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). NO derived from iNOS seems to be a proinflammatory mediator with immunomodulatory effects. In the respiratory tract alone, expression of iNOS has been reported in alveolar type II epithelial cells, lung fibroblasts, airway and vascular smooth muscle cells, airway respiratory epithelial cells, mast cells, endothelial cells, neutrophils, and chondrocytes. The stimuli that cause transcriptional activation of iNOS in these cells varied widely and included endogenous mediators (such as chemokines and cytokines) as well as exogenous factors such as bacterial toxins, virus infection, allergens, environmental pollutants, hypoxia, tumors, etc. The high level of NO released by iNOS has an effect as immune effector molecule in halting viral replication, and in eliminating various pathogens. This mechanism may involve, at least in part, inhibition of DNA synthesis by inactivation of ribonucleotide reductase and by direct deamination of DNA. Finally, NO appears to signal through its reactivity with cysteine groups, particularly those located at consensus motifs for *S*-nitrosylation with primary sequence or tertiary structure of a protein. One of the general mechanisms of antimicrobial defenses involving NO is *S*-

nitrosylation by NO of cysteine proteases, which are critical for virulence, or replication of many viruses, bacteria, and parasites (Ricciardolo et al., 2004). A previous study demonstrated an association between SNP rs1060826 (*NOS2*) and RSV bronchiolitis (Janssen et al., 2007). We found an association between SNP rs1060826 (*NOS2*) and bronchiolitis caused by rhinovirus.

4.5. Vitamin D receptor – VDR

Vitamin D modulates white blood cell proliferation, maturation, and cytokine expression through the VDR on lymphocytes and macrophages. VDR signaling also contributes to the expression of antimicrobial peptides, which are important for the innate defense against viruses and bacteria (Liu et al., 2007). Lower vitamin D levels have been postulated as a risk factor for respiratory illness based on the well-established seasonality of respiratory infections that occur during winter when UV-B production of vitamin D is low. Subsequent bronchiolitis research in developed countries investigating subclinical vitamin D deficiency supports this hypothesis (Roth et al., 2010). Additionally, a prospective newborn cohort study identified low cord blood vitamin D levels as an independent predictor of RSV infection during the first year of life (Belderbos et al., 2011).

Genetic alterations of *VDR* have the potential to affect vitamin D signaling through impaired gene transcription, mRNA stability and translation, protein activity, and protein stability. A common *VDR* SNP, rs2228570, has previously been associated with moderately lower *VDR* transcriptional activity and a recent meta-analysis concluded that presence of the SNP rs2228570 (*VDR*) T allele significantly increased the risk of RSV bronchiolitis (McNally et al., 2014). Similarly, we found that SNP rs2228570 (*VDR*) was associated with death from bronchiolitis in the current study.

In airway epithelial cells, vitamin D controls the expression of signal transducer and activator of transcription (STAT)1. A recent study demonstrated that the predisposition of SNP rs2228570 (*VDR*) to severe RSV bronchiolitis may involve the impaired ability of vitamin D to restrain antiviral signaling in airway epithelia, and that vitamin D fails to regulate STAT1 phosphorylation and downstream gene expression in cells expressing this *VDR* variant. Strong activation of the STAT1 pathway in RSV-infected cells may therefore contribute to RSV immunopathogenesis (Stoppelenburg et al., 2014). Two studies found that another *VDR* SNP, rs10735810, was associated with an increased likelihood of bronchiolitis and that carriers of the T allele were more likely to develop this disease (Kresfelder et al., 2011; Janssen et al., 2007).

Also regarding genes in the immune system a recent study reported that SNPs rs2227543 (IL-8) and rs2275913 (IL-17) showed significant associations with the severity of acute viral bronchiolitis (Pinto et al., 2017).

Development of an RSV vaccine is a high priority for public health, but attempts to date have been frustrated. Resolving the mechanism by which RSV induces pathogenesis is essential for developing new effective vaccines (Farrag and Almajhdi, 2016; Arruvito et al., 2015; Jorquera et al., 2016; Higgins et al., 2016).

4.6. Hardy Weinberg equilibrium

Finally, regarding the HWE, we must remember that the HWE assumes an ideal population, without the interference of evolutionary factors. However, in genes as the involved in immunity, inflammation, and infection controlling, the HWE imbalance may appear secondarily associated with the selection mechanisms that favored a particular allele that can bring a more effective response. The disequilibrium does not invalidate the association study since the groups are part of the same population.

4.7. Study limitations

There are a number of limitations in our study, including: (i) superficial characterization of the population of healthy controls; (ii) because miscegenation in our population is extensive and present in both groups evaluated, the ethnicity assessment was performed in a self-reported manner and we did not study the ancestry, in the controls or in the patients; (iii) sample size reduction due to the non-identification of SNP genotypes for some patients and the difficulty in obtaining the clinical and laboratory markers of all the patients included in the study; (iv) number of evaluations carried out at the same time may lead to confounding; (v) limitations on the size of the sample included in the study and the power of the sample obtained for all the analyzes performed (need for correction by multiple tests with high denominator); (vi) use of candidate genes as a study model, rather than genome-wide association studies, due to technical limitations and high cost involved in the laboratorial analysis.

5. Conclusions

Our findings provide some evidence that genetic variation in selected immune genes may influence the outcomes of severe bronchiolitis but replication in other datasets is needed. The determination of polymorphisms in immune response genes could be used in future work to help predict high-risk infants who might benefit from preventive measures. Knowledge of SNPs associated with severe bronchiolitis will also contribute to an understanding of disease pathogenesis and the innate immune response to its infection. This will be useful in guiding efforts to develop more effective treatment for this potentially fatal infection.

Ethics approval and consent to participate

This study was approved by the National Research Ethics Commission (number: 00869612.7.0000.5404). Legal representatives of patients, and participants of the control group, received explanations about the research and gave written informed consent.

Potential conflicts of interest

The authors declare that they have no conflicts of interest.

Financial disclosure

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Authors' contributions

AEA conceived and designed this study, selected patients, performed clinical evaluations of patients included in the study, acquired data, analyzed statistical data, drafted, revised, approved and submitted the final manuscript.

FALM conceived and designed this study, performed polymorphism screening, analyzed statistical data, drafted, revised and approved the final manuscript.

CSB conceived and designed this study, performed polymorphism screening, revised and approved the final manuscript.

CWA and JCSB conceived and designed this study, performed viral

identification, revised and approved the final manuscript.

ECEB and ATT conceived and designed this study, revised and approved the final manuscript.

MTNR, MBM, CCBA, TO, PGS, EC, MLFM, MCR and JVP selected patients, acquired data, revised and approved the final manuscript.

JDR conceived and designed this study, analyzed statistical data, drafted, revised and approved the final manuscript.

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Appendix A. Supplementary data

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