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Immune response to tetanus booster in infants aged 15 months born prematurely with very low birth weight

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ABSTRACT

Objectives: To compare humoral and cellular immune responses to tetanus booster vaccination in infants born prematurely with those born at full term and identify factors associated with the humoral response.
Methods: A prospective study was carried out on children born prematurely and with a birth weight <1500 g and with infants born at full term. At 15 months (pre-vaccination) and 18 months (post-vaccination), anti-tetanus antibodies were measured by ELISA; the intracellular interferon-gamma percentages of CD4+ T and CD8+ T cells after in vitro stimulation with tetanus toxoid were determined by flow cytometry. Chi-squared or Fisher's exact test was used to compare categorical variables. Student's *t*-test or Mann–Whitney test was used to compare numerical variables. Regression analysis was performed to determine factors associated with humoral immunity. Statistical significance was considered if $p < 0.05$.
Results: Sixty-four premature and 54 full-term infants were studied. The proportion of children immune against tetanus at 15 and 18 months was similar in both groups. The geometric mean of the antibodies was lower among the premature children at 15 months ($p = 0.025$) and was similar in both groups at 18 months ($p = 0.852$). The percentages of CD4+ and CD8+ T cells expressing intracellular IFN-γ were similar in both groups at 15 and 18 months. Gestational age <32 weeks was associated with a reduction of −0.116 IU/mL in the level of antibodies at 15 months. Breastfeeding >6 months was associated with a 3.5-fold greater chance of optimal protective (≥0.1 IU/mL) antibody level against tetanus at 15 months and an increase of 0.956 IU/mL in the level of antibodies at 18 months.
Conclusions: Humoral and cellular response following a tetanus booster was similar in both groups. Premature infants exhibited lower levels of anti-tetanus antibodies at 15 months of age, with the lowest levels in those born at a gestational age of less than 32 weeks. Breastfeeding was associated with greater levels of antibody against tetanus.

1. Background

Studies suggest that even patients vaccinated against tetanus and with antibody levels considered protective may acquire tetanus, depending on the immune status of the host and amount of tetanus neurotoxin produced by Clostridium tetani [1,2]. Moreover, children born prior to a gestational age of 37 weeks may exhibit a lower immune response to vaccination and/or have an accelerated decay of serum antibodies [3–5].

Factors that contribute to the survival of premature infants, such as the use of prenatal steroids in women at high risk of giving premature birth [6] and the use of postnatal corticosteroids for the treatment of bronchopulmonary dysplasia [7], may also affect the immune response to vaccination in children born prematurely [5,8]. According to Slack et al. [5], the production of anti-tetanus antibodies in premature infants with a gestational age of less than 32 weeks is negatively associated with the number of doses of prenatal corticosteroids. Robinson et al. [8] found that antibody levels following vaccination for tetanus, diphtheria and whooping cough were lower in children with bronchopulmonary dysplasia treated with dexamethasone. Moreover, breastfeeding, less prevalent among premature infants, and nutritional status, which may be compromised in this population, are also involved in the immune response to vaccination [9,10].

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It is not known whether the compromised immune response to vaccination in premature infants is only related to vaccines administered in the first six months of life. However, Kirmani et al. [3] reported lower antibody levels following vaccination for diphtheria, tetanus toxoid, poliovirus, *Haemophilus influenzae* type b and hepatitis B in seven-year-old children born at a gestational age of less than 29 weeks and with a birth weight of less than 1000 g in comparison to children of the same age born at full term.

The aims of the present study were to compare the humoral and cellular immune response to a tetanus booster vaccine at 15 months of age in infants born prematurely with those born at full term and to identify factors associated with humoral immune response. Specifically with regard to immune response, the concentration of anti-tetanus antibodies and percentages of CD4+ T and CD8+ T cells expressing intracellular interferon-gamma after in vitro stimulation with tetanus toxoid were compared before and after the tetanus booster vaccination.

2. Methods

The present prospective study was carried out between September 2007 and January 2010 and received approval from the Ethics Committee of the institution. All parents/guardians of the participants signed a statement of informed consent.

The inclusion criteria were children aged 15 months, having received three doses of tetanus vaccine (at 2, 4 and 6 months of age) and not having yet received the tetanus booster vaccine. Participants were divided into two groups. The premature group included children born with a gestational age of less than 37 weeks and birth weight of less than 1500 g (very low birth weight preterm infants). These infants were assisted at the neonatal intensive care unit of the Federal University of São Paulo, SP, Brazil, where preterm infants with birth weight less than 1500 g were followed up at the multidisciplinary premature outpatient clinic of the institution. After delivery from the neonatal unit, these infants were routinely followed up by pediatricians, neurologists, physiotherapists, ophthalmologists, nutritionists, psychologists and dentists until 20 years of age.

The control group included children born at full term, adequate for gestational age, with no neonatal complications, discharged from the maternity unit at two to four days of life and in follow up at a pediatric outpatient clinic.

The exclusion criteria were: congenital malformation, children of HIV-infected mothers, primary immunodeficiency, children who received plasma or immunoglobulin transfusions five months before or three weeks after the booster dose or received the tetanus booster vaccination prior to being invited to participate in the study.

Infants included in the study were vaccinated according to the Brazilian immunization recommendations. Briefly, the routine vaccine schedule in Brazil is: BCG at birth; Hepatitis B at birth, 1, 2 and 6 months of age (the 1-month dose, only for children born with less than 2 kg); tetanus and diphtheria toxoids and pertussis (DTP) at 2, 4, 6 months and 4–6 years; *H. influenzae* type b (Hib) at 2, 4 and 6 months; oral poliovirus at 2, 4, 6 months and 4–6 years; rotavirus at 2 and 4 months; 10-valent pneumococcal conjugate vaccine at 3, 5, 7, 15 months; meningococcal C conjugate vaccine (Men C) at 3, 5, and 12 months; yellow fever vaccine at 9 months; measles–mumps–rubella vaccine at 12 months and 4–6 years of age.

Maternal demographic and clinical characteristics as well as children's data related to the period of hospitalization in the neonatal unit and clinical complications in the first year of life were collected. Gestational age was determined either by the best obstetric estimate or using the New Ballard method [11]. The adjustment of birth weight to gestational age was performed using the curve proposed by Alexander et al. [12]. Clinical severity score in the first 12 h of life was determined using the Score for Neonatal Acute Physiology, Perinatal Extension, Version II (SNAPPE II) [13]. Nutritional status at the time of vaccination was determined based on the recommendations of the World Health Organization [14].

Four mililiters of blood was collected for the determination of humoral and cellular immunity against tetanus toxoid at 15 months of age (prior to the booster vaccine dose against tetanus, diphtheria and whooping cough) and at 18 months of age (post-vaccination).

Double-antigen enzyme-linked immunosorbent assay (ELISA) was used to determine humoral immunity, as described by Kristiansen et al. [15]. The results were expressed in international units per milliliter (IU/mL) by comparisons of the curves of the plasma samples tested and the international reference standard. Concentrations of anti-tetanus antibodies equal to or greater than 0.1 IU/mL were considered optimal protective levels against tetanus, concentrations between 0.01 and 0.1 IU/mL were considered minimal protective levels and concentrations lower than 0.01 IU/mL, susceptibility to the disease [1,16].

Cellular immune response against tetanus toxoid was determined by the percentages of CD4+ T and CD8+ T cells expressing intracellular interferon-gamma after in vitro stimulation with tetanus toxoid by flow cytometry. A culture was done using full blood diluted to 1:10 in RPMI 1640 culture medium (Gibco, NY, USA) supplemented with L-glutamine, penicillin and streptomycin. The diluted blood was then distributed in polystyrene tubes in volumes of 1 mL. Following the addition of the antigen, the tubes were sealed and incubated at 37 °C for 72 h in an atmosphere with 5% CO_2. For all tests, one tube of blood stimulated with phytohemagglutinin was used as the positive control and another of non-stimulated blood was used as the negative control. Tetanus toxoid was obtained from Butantã Institute (São Paulo, Brazil). In the last 4 h of incubation, brefeldin A was added at a concentration of 10 μg/mL to all tubes (Sigma, St. Louis, USA). CD3-APC and CD8-PerCP conjugated monoclonal antibodies (BD Biosciences) were used for cell-surface staining. Cells were fixed, washed, resuspended with permeabilization buffer, and incubated for 10 min in the dark at room temperature. IFNγ-FITC-conjugated monoclonal antibody was then added. Finally, the cells were washed and kept at +4 °C in the dark until data acquisition. Sample acquisition was performed with FACSCalibur Cytometer (BD Biosciences) using Cell Quest software (BD Biosciences). The analysis was performed using FlowJo software (Tree Star, Ashland, USA). Fifty thousand events were acquired in the lymphocyte gate based on the forward scatter and side scatter dot plot. CD3+ T cells were selected based on the side scatter profile and CD3-APC fluorescence. CD8+ T lymphocytes were defined as CD3+/CD8+; due to down regulation of CD4 molecules during activation, CD4+ T lymphocytes were defined as CD3+/CD8−. Intracellular IFN-γ production was evaluated in CD3+/CD8+ and CD3+/CD8− cells. The final value of positive cells to each stimulus was obtained by subtracting the percentage of positive cells of the culture without stimulus (negative control) from the culture in the presence of stimulus.

2.1. Statistical analysis

The numerical variables were compared using either the Student's *t*-test (normal distribution) or the Mann–Whitney test (non-normal distribution). The categorical variables were compared using either the chi-squared (χ^2) test or Fisher's exact test. Multiple linear regression analysis was performed to determine factors associated with tetanus antibody levels measured at 18 months. Variables associated with optimal protective antibody level (≥0.1 IU/mL) against tetanus at 15 months of age were studied by multiple logistic regression analysis. The statistical analysis

was carried out using the Statistical Package for Social Sciences for Windows, version 17.0 (SPSS, Chicago, IL, USA), with the level of significance set to 5% ($p < 0.05$).

3. Results

Among the 89 premature infants in follow up at the premature outpatient clinic of the institution during the study period, 20 fulfilled the exclusion criteria. Sixty-nine premature infants and 60 full-term infants fulfilled the inclusion criteria. Among these, 5 (3.9%) premature infants and 6 (10.0%) full-term infants were excluded because the parents abandoned the study prior to the blood collection for the immunity analyses. Thus, data on 118 patients (64 in the premature group and 54 in the control group) were analyzed (Fig. 1).

Premature infants had mean gestational age of 29.9 ± 2.2 weeks (variation: 25.6–34.4 weeks), birth weight of 1185 ± 216 g (variation: 714–1480 g), 23 (35.9%) were small for gestational age, and 48 (75.0%) had antenatal corticosteroids exposure. During the neonatal period, 36 (56.3%), 17 (26.6%), 29 (45.3%), 36 (56.3%), and 16 (25.0%) had respiratory distress syndrome, patent ductus arteriosus, clinical sepsis, intraventricular hemorrhage, retinopathy of prematurity, respectively. Also, during the neonatal period, 40 (62.5%) neonates were submitted to mechanical ventilation on median for 6 days (variation: 1–57 days), 25 (39.1%) were on need of oxygen therapy at 28 day of life, 6 (9.4%) received corticosteroids during hospitalization in the neonatal unit, 31 (48.4%) received at least one red blood cells transfusion, 2 (3.1%) received plasma and 4 (6.3%) received at least one platelet transfusion.

Table 1 summarizes the differences between the premature and full-term infants.

At the beginning of the study, the premature infants had lower weight (8119 ± 1122 g vs. 9743 ± 1100 g; $p < 0.001$), stature (69.9 ± 3.4 cm vs. 75.0 ± 2.8 cm, $p < 0.001$) and body mass index (BMI) (16.5 ± 1.5 vs. 17.3 ± 1.3; $p = 0.005$), in comparison to the full-term infants. Four premature infants (6.3%) had a BMI below the −2 *z*-score and 22 (34.3%) premature infants had a stature/age *z*-score < −2, whereas all full-term infants were within the normal range for these indices.

Regarding clinical evolution following discharge from the neonatal unit, 18 (28.1%) premature infants developed pneumonia, 41 (64.1%) exhibited wheezing and 24 (37.5%) required prednisolone, 5.7 ± 4.5 months before booster dose at 15 months, at a dose of 1 mg/kg/day for five days. Moreover, 24 (37.5%) required hospitalization, with a median value of 1 (range: 1–12) hospitalization per premature infant hospitalized. Only one child in the control group developed pneumonia and required hospitalization.

Mother's milk was administered to 37 (57.8%) premature infants and 48 (88.9%) full-term infants ($p < 0.001$). Breastfeeding continued for more than six months among 9 (14.1%) premature infants and 32 (59.3%) full-term infants ($p < 0.001$) and for more than one year among 0 (0%) premature infants and 15 (27.8%) full-term infants ($p < 0.001$). Mean duration of breastfeeding was shorter among the premature infants (3.2 ± 3.7 months vs. 9.1 ± 6.3 months; $p < 0.001$).

Premature infants were older at the time of the first dose (mean age, 2.4 ± 0.8 months vs. 2.1 ± 0.2 months; $p = 0.002$), second dose (4.6 ± 0.9 months vs. 4.2 ± 0.3 months; $p = 0.001$) and third dose (6.9 ± 1.2 months vs. 6.2 ± 0.4 months; $p < 0.001$) of the tetanus vaccine in comparison to the full-term infants.

The tetanus booster dose was administered at a mean age of 15.2 ± 0.3 months.

The percentage of infants with optimal protective humoral immunity was similar in both groups prior to and following vaccination (Table 2). Among infants with minimal humoral immunity for tetanus at 15 months, a greater percentage of them had been breastfed for less than six months (37% vs. 17%; $p = 0.026$). Geometric mean of the anti-tetanus antibody levels was lower in the premature infants at 15 months (0.147 ± 0.2 vs. 0.205 ± 0.3; $p = 0.025$) and similar in both groups at 18 months (1.997 ± 2.2 vs. 1.867 ± 2.5; $p = 0.852$).

Regarding cellular immunity, the percentages of CD4+ T and CD8+ T cells expressing intracellular interferon-gamma were similar in both groups at pre-booster and 3 months post-booster (Table 3).

Multiple linear regression and multiple logistic regression analyses were performed to determine an association between demographic/clinical factors and humoral immune response to anti-tetanus vaccination. The following independent variables were incorporated into all regression models: use of at least one cycle of antenatal corticosteroids; gestational age <32 weeks; small for gestational age; clinical severity score assessed by SNAPPE II; need for erythrocyte transfusions; BMI; and breastfeeding for more than six months. After controlling for these variables, the final linear regression model showed that having been born at a gestational age of less than 32 weeks was associated with a reduction of −0.116 IU/mL (95% CI: −0.219 to −0.014; $p = 0.027$) in the level of antibodies and breastfeeding for more than six months was associated with an increase of 0.956 IU/mL (95% CI: 0.080–1.832; $p = 0.033$) in the level of antibodies after booster dose. Likewise, after controlling for the same variables, the logistic regression revealed that breastfeeding for more than six months was associated with a 3.455-fold (95% CI: 1.271–9.395; $p = 0.015$) greater chance of having optimal protective antibody level (≥0.1 IU/mL) against tetanus at 15 months when compared to breastfeeding for less than six months.

4. Discussion

In the present study, the proportion of children with minimal protective (≥0.01–≤0.09 IU/mL) antibody levels and potentially susceptible to tetanus was similar between groups at 15 months of age. However, mean anti-tetanus antibody levels were lower among the premature infants at 15 months of age in comparison to the full-term infants. This finding is important, as delayed vaccination is more common among infants born prematurely, when compared to the general population, which may lead some of these children to become more susceptible to tetanus [17].

Mean age at administration of the first three doses of the tetanus vaccine was significantly greater among the premature infants than the full-term infants, but probably not enough to confer a better immune response for premature infants [18]. Moreover, the fact that premature infants have lower levels of maternal antibodies than full-term infants may be an additional factor involved in the better humoral immune response to vaccination [19,20]. In the same way, Baxter et al. referred that 100% and 98.3% of 121 premature infants born less than 32 weeks of gestation developed, respectively, a minimum and optimum antibody levels after a 3 dose primary series of tetanus vaccine [21]. However, despite these factors, the premature infants analyzed in the present study had lower levels of antibodies. This finding suggests the influence of premature birth and/or possible factors associated with a lower immune response to vaccination or a faster decay in antibody levels resulting from the primary vaccination in premature infants in comparison to those born at full term [22].

It is possible that the immune response to the tetanus vaccine in the first six months of life was lower among the infants born prematurely, especially those born with a gestational age of less than 32 weeks, in comparison to those born at full term, as described by other authors [5]. These results are in agreement with data

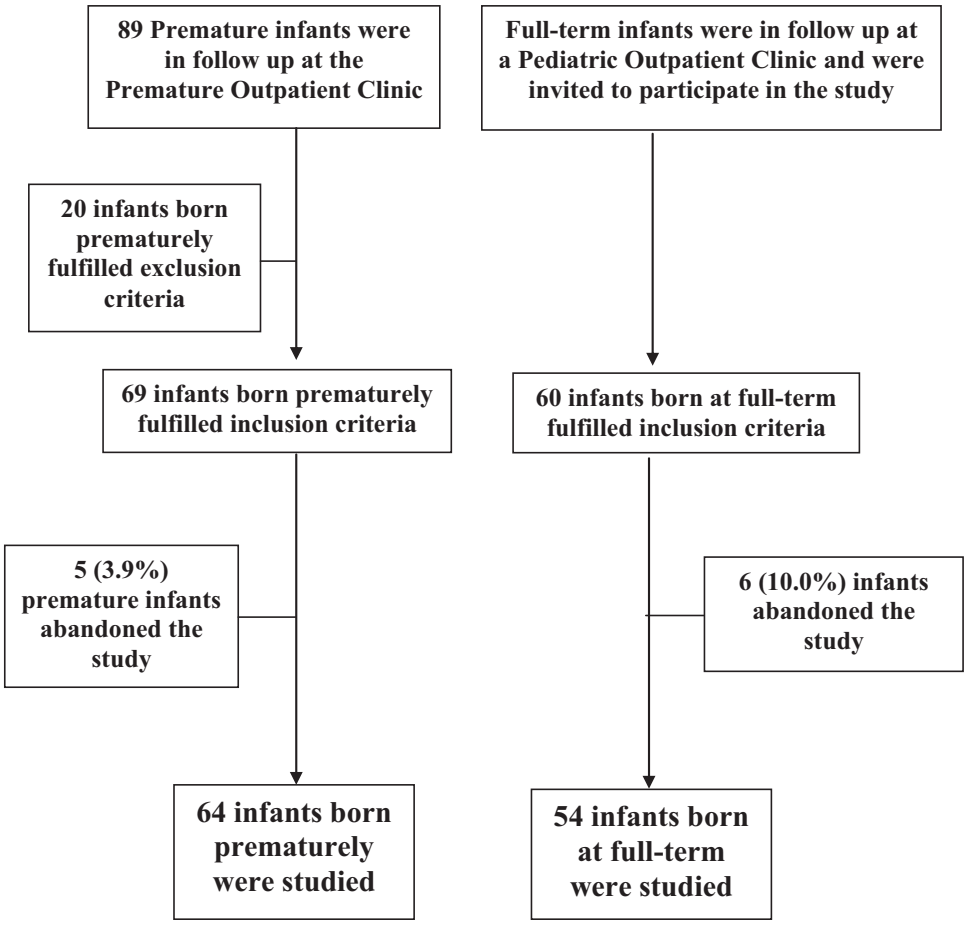

Fig. 1. Flow diagram showing the flow of the studied infants born premature and at full-term.

Table 1
Demographic and clinical characteristics of the studied infants, during the perinatal period, expressed by mean ± sd or number and percentage.

	Premature ($n = 64$)	Full-term ($n = 54$)	p
Mother age (years)	28 ± 8	26 ± 6	0.138*
Number of gestations	2.4 ± 1.5	1.7 ± 1.0	0.001**
Number of antenatal appointments	5.5 ± 3.6	7.9 ± 1.9	<0.001**
Hypertensive syndromes	24 (37.5%)	0 (0%)	<0.001***
Diabetes mellitus	5 (7.8%)	0 (0%)	0.062***
Chorioamnionitis	6 (9.4%)	0 (0%)	0.031***
Cesarean section	48 (75.0%)	31 (57.4%)	0.043****
Male	31 (48.4%)	28 (51.9%)	0.712****
1st minute Apgar	7.1 ± 1.7	8.3 ± 0.6	<0.001*
5th minute Apgar	8.6 ± 0.7	9.2 ± 0.4	<0.001*
Gestational age (weeks)	29.9 ± 2.2	39.0 ± 1.0	<0.001*
Birth weight (g)	1185 ± 216	3231 ± 269	<0.001*
Days of hospital stay	61 ± 33	3 ± 1	<0.001**

* p: t test.
** p: Mann–Whitney test.
*** p: exact Fisher test.
**** p: χ^2.

Table 2
Humoral immunity against tetanus: geometric mean (variation) of antibody levels before (at 15 months) and after (at 18 months) vaccination against tetanus, expressed in IU/mL and number and percentages of children, according to their immunity status.

	n	Premature	n	Full-term	p
Pre-vaccination (IU/mL)	63	0.147 (0.015–0.969)	54	0.205 (0.012–1.080)	0.025*
Post-vaccination (IU/mL)	62	1.997 (0.280–9.959)	53	1.867 (0.164–12.506)	0.852*
>0.1 IU/mL at 15mo	63	42 (66.7%)	54	41 (75.9%)	0.271**
0.01–0.1 IU/mL at 15mo	63	21 (33.3%)	54	13 (24.1%)	0.271**
>0.1 IU/mL at 18mo	62	62 (100%)	53	53 (100%)	

mo: months; * p: Mann–Whitney test; ** p: χ^2. Missing data occurred due to difficult venous access in three preterm infants. Parents of one term infant abandoned the study after the first blood collection at 15 months.

Table 3
Percentages of CD4+ T and CD8+ T cells expressing intracellular interferon-gamma after in vitro stimulation with tetanus toxoid before (at 15 months) and after vaccination (at 18 months).

	n	Premature	*n*	Full term	*p*[*]
CD4+ T cells-15mo	53	2.10 ± 8.89	54	1.01 ± 3.18	0.514
CD8+ T cells-15mo	53	2.66 ± 9.66	54	2.06 ± 6.02	0.254
CD4+ T cells-18mo	54	1.55 ± 6.02	52	0.75 ± 3.36	0.686
CD8+ T cells-18mo	54	1.97 ± 4.78	52	1.18 ± 3.58	0.564

IFN-γ: interferon-gamma; mo: months. Missing data occurred due to difficulty venous access that led to blood clotting or a small blood volume which allowed only the assessment of humoral immune responses. Parents of one term infant abandoned the study after the first blood collection at 15 months.

[*] *p*: Mann–Whitney.

described in the literature stating that immune response in premature infants may be lower in the first six months of life due to the lower number of circulating T and B lymphocytes and T helper cells as well as the lower CD4/CD8 ratio in comparison to children having been born at full term [23].

In the present study, the premature group was recruited among children born prematurely with a birth weight of less than 1500 g and the control group was composed of children born at full term, adequate for gestational age, with no clinical complications in the neonatal period and discharged from hospital by four days of life. Moreover, the control group had children within the ideal weight range and a good breastfeeding index until six months of age, whereas 77% of the premature group had been born at a gestational age of less than 32 weeks, had a high rate of hospitalization following discharge from the neonatal unit and a low breastfeeding index. Nonetheless, the humoral response to the tetanus booster was similar between groups and cell immunity was similar between groups at 15 and 18 months of age. These findings show that the two groups had a similar good memory response after a booster dose at 15 months after having received a 3 dose primary series vaccine against tetanus.

Vermeulen et al. compared 48 premature infants who were born at less than 31 weeks of gestational age after vaccination at 2, 3, and 4 months of chronological age with an acellular or a whole-cell tetravalent diphtheria–tetanus–pertussis–polio vaccine. While assessing cellular immune response, the authors found that most of the preterm infants developed a gamma interferon (IFN-γ) response to the *Bordetella pertussis* antigens. This finding suggests that most preterm infants are able to mount a specific cellular immune response [24].

In the present study, the time of immune evaluation, three months after the booster dose, could be stated as a limitation. It is possible that the antibody titers and numbers of circulating tetanus-specific T cells may have decayed from peak levels three months after vaccination. Antibody levels following a booster dose usually peak after 15 and 30 days. The antigen-specific IFN-producing cells most probably are found among circulating Peripheral blood mononuclear cells 1–2 weeks after vaccination very transiently, thereafter, they rapidly reach the lymph nodes and then decay with time [24–27].

With the increase in the survival rate of premature infants at progressively younger gestational ages and the growing use of therapeutic resources, premature infants currently exhibit different characteristics from those of past decades [28,29] and factors other than prematurity itself may be involved in the immune response. Thus, apart from the direct comparison of antibody levels between groups, linear and logistic regression analyses were performed to control for variables that may affect the response to vaccination. It should be pointed out that the same independent variables were incorporated into all multiple linear and logistic regression models, which contributes to the consistency of the findings.

Breastfeeding for more than six months was associated with a 3.5 fold increase in the chance of having optimal protective antibody levels against tetanus at 15 months of age, and a 0.96 IU/mL (95% CI: 0.08–1.83) increase of antibody levels 3 months after the booster dose. However, given the significantly lower rates of breastfeeding in premature infants, the effect observed of breastfeeding could be a confounding of other factors (e.g. gestational age, affinity maturation, etc.) that could influence the antibody response levels in these infants. However, this effect has also been described by Greenberg et al. [30], who found high levels of antibodies among children who received a conjugated vaccine against *H. influenzae* type b and tetanus toxoid and had been breastfed until at least six months of age. Jeppesen et al. [31] found a correlation between breastfeeding and the population of T CD8+ cells. It is suggested that breastfeeding contributes to the structural and functional development of the thymus and the control of the apoptosis of immature thymocytes, which subsequently transform into CD4+ T and CD8+ T cells [32].

The use of antenatal corticosteroids, nutritional status and erythrocyte transfusions were not associated with the humoral response to the tetanus vaccine at 15 and 18 months, which is in agreement with findings described in previous studies [5,8–10,33].

In conclusion, the levels of anti-tetanus antibodies were lower among the premature infants at 15 months (pre-booster) when compared to children born at full term, although the percentage of children with optimal protective antibody level against tetanus was similar in both groups. At 18 months, the premature and full-term infants had similar humoral and cellular immune responses to the tetanus booster vaccine. Moreover, breastfeeding increased the odds of optimal protective antibody level against tetanus at 15 months of age and raised levels of antibodies concentration following the tetanus booster vaccine.

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