INTRODUCTION

Despite the universal use of thimerosal-containing vaccines (TCV) in immunization of children, our knowledge of the toxicokinetics (TK) and toxicodynamics (TD) of their Hg metabolite (ethylmercury—EtHg) is derived mostly from methylmercury (MeHg) studies. The mechanistic significance of thimerosal neurotoxicity through EtHg is unquestionable, but its toxicological significance is the result of direct demonstration in animal and theoretical models. Our ability to understand the safety of TCV-EtHg is unsatisfactory; although there is no proven direct association of neurodevelopmental disorders in children immunized with TCV-EtHg, its plausibility has been shown by neurotoxic disasters caused by organic Hg compounds (1). However, there are no studies pointing to neurologic disorders being an unambiguous result of TCV-EtHg; rather, the associative studies have so far emerged amid intense scientific debate.

Rodier (2) and many others have discussed the vulnerability of the young brain to neurotoxic substances over the early neonatal period. Compared to other organs, the infant brain takes an unusually long period to form. Besides the blood—brain barrier (BBB), which is not fully developed until the middle of the first year of life, neuron proliferation and migration also continue in the postnatal period. Additionally, myelin production, along with development of receptors and transmitter systems, is part of the great postnatal activity of the central nervous system (CNS). Organic Hg compounds are among the neurotoxic substances with effects that depend on the neuron structure at time of exposure (2). Coincidently, during the first 6 months, Brazilian infants are impacted by a heavy vaccination schedule: six i.m. injections of TCV (Hepatitis-B at 0, 30 and 180 days; DTP at 60, 120 and 180 days) containing 0.01% of thimerosal as preservative (25 μgHg/dose).

Nursing infants are exposed to Hg from formulas and breast milk (3); however, the extent of exposure during breastfeeding depends on the maternal Hg that diffuses into milk. The amount of Hg in the blood of the nursing mother enters breast milk at a rate (inorganic Hg more than MeHg) that depends on its protein-binding capacity. Oskarsson et al. (4) observed that, compared to organic Hg (MeHg), there was a more efficient transfer of inorganic Hg from blood to breast milk. They estimated that infant exposure from breast milk ranges up to 0.3 μgHg/kg/day; approximately 50% was inorganic Hg. While the breast milk Hg exposure is proportional to milk intake, it is amortized in several sucking events during the day; furthermore, the newborn is not exposed until lactation is established and during this time breastfeeding is acting on the CNS priming. On the other hand, TCV are given immediately after parturition by parenteral route. In our infants, five vaccines are given in the first 6 months, two of them given at the same time (180 days) and providing a double dose of TCV (50 μgHg/dose). Indeed, breastfed vaccinated infants have shown a substantial increase in hair-Hg concentrations during the first 6 months (5).

Abstract

Aim: Brazilian newborns immunized with hepatitis-B (thimerosal containing vaccine, TCV) receive the first dose within 24 h if delivered in public hospitals, but at a later time if born in private hospitals. We compared neurodevelopment (ND) in infants born in a state hospital (immunized within 24 h) and in privately run hospitals (immunized 2–4 days postnatally).

Methods: We used the Gesell Developmental Schedules in 82 healthy exclusively breastfed infants at 6 months to assess motor skills, language development, comprehension capacity and social skills.

Results: Compared to the group immunized 2–4 days after hospital discharge, the group immunized within 24 h showed no significant difference in ND delays. Despite the variation in gestational age (range 36–42 weeks) and TCV-ethylmercury (EtHg) dose (5.7–11.3 μgHg/kg b.w.) at birth, time of exposure to TCV showed no significant association with ND. Gesell Developmental Score was not significantly correlated with total parenteral EtHg/unit of body mass neither with the relative increase in hair-Hg (as an additional challenge to prenatal Hg exposure).

Conclusion: In breastfed infants, differences in early exposure to TCV-EtHg cannot portend clinical neurodevelopmental delays at 6 months. We speculate that breastfeeding remains a significant strategy to improve central nervous system protection of infants facing early exposure challenges.
Guidelines to EtHg use as vaccine preservatives are derived from MeHg toxicity studies. These guidelines from various countries vary from 0.1 to 0.4 μg/kg of MeHg for adults (1). Harry et al. (6) found that in immature mice, when EtHg was injected, either as the chloride or as thimerosal, less of the actual delivered dose reached the brain as compared to an injection of MeHg. They concluded that MeHg does not appear to be a good model for EtHg-containing compounds. Therefore, serum transport of MeHg and EtHg can modulate toxic differences (in the neonate) by regulating the relative levels of free forms transported and available to cross the BBB. Additionally, there are differences in initial weight loss that are more pronounced in breastfed infants. Macdonald et al. (7) identified neonatal median time of maximum weight loss around 2.7 days. Clearly, this can further increase differences in TCV-EtHg exposures.

The inability of the neonatal liver to secrete bile, coupled with an immature renal system, mean that Hg excretion takes longer than in adults. This is particularly relevant for the breastfed infant; because maternal milk usually comes in at between 2–4 days after birth, for breastfed infants this is the time of the lowest expected bowel output (8). Together with lower albumin binding capacity, the immaturity of the transport-protein system in neonates enhances the risk of mercury toxicity. Perinatal immaturity of the CNS and of other physiological functions (detoxifying enzymes, renal and gastrointestinal organs) that interferes with Hg binding and excretion is well established. Additionally, both birth weight (9) and gestational age (10) are positively associated with childhood psychometric intelligence. The TCV-EtHg impact on CNS remains unstudied; gestational age and birth weight are modulators of TK and TD of TCV. The parenteral delivery of TCV-EtHg, albeit in very small quantities, reaches the neonate circulation very quickly and gains unimpeded access to the immature BBB and the blood cerebrospinal fluid barrier (BCSF). Therefore, the early postnatal days are important windows of opportunity for CNS insults.

Neurodevelopment delay can occur in infants exposed to prenatal maternal neurotoxic substances; its etiology is multifactorial and includes Hg. Given the immaturity of newborns, after an acute parenteral exposure of thimerosal, there is more chance of infants accumulating Hg more readily and excreting it more tardily. When there are differences in time of first exposure there might be additional risk factors; there is, therefore, more likelihood of heightening the risks of untoward neurological effects. Special population groups, such as term neonates, have a variety of constitutional modifying risk factors: (a) parenteral exposure at very early time; (b) sequence of high magnitude EtHg exposures (first days, 1, 2, 4 and 6 months); (c) differences in maturity at birth (gestational ages 36–42 weeks); (d) differences in body mass at birth and (e) additive toxicity of TCV-EtHg to prenatal Hg exposure gradients (especially in fish-eating Amazonian populations).

Because of the complexity of studying neurodevelopment and ethical issues involving study design with TCV we took advantage of an existing research project and analysed recorded data of early and relatively later TCV-EtHg exposure of breastfed infants. Therefore, our primary aim was to compare the effect of time differences in the first exposure to TCV on ND at 6 months. Our secondary aim was to identify modifying factors associated with prenatal Hg exposure.

**MATERIALS AND METHODS**

The data used in this study were not collected with the purpose of evaluating the impact of immunization on ND. In a recent publication (11) we described the protocol to assess growth and development of children exposed to prenatal background Hg; when the research project started we were not aware of the TCV issue in paediatric vaccines. The parent publication (11) dealt with prenatal Hg exposure; after that, when studying the changes in hair Hg concentrations (5, 11) we realized that because Hospital de Base is a public health facility, only the babies born at this Hospital (66%) received the hepatitis-B vaccine within the first day postpartum. Babies born at the Hospital Panamericano and the Hospital Regina Pacis received the hepatitis-B vaccine immediately after the mother’s discharge (2–4 days postpartum). This appeared as an opportunity to study differences in timing of immunization and ND outcomes; additionally, Hospital de Base as a state-run facility receives mostly poor mothers.

The previous publications detailed the characteristics of the population of Porto Velho (West Amazonia); the research protocol was approved by the Ethics Committee of Studies for Humans of the Universidade Federal de Rondonia (11). Briefly, pregnant mothers between the ages of 15 and 43 years were enrolled as volunteers; mothers were in good health, reporting no illness or complaints and were willing to breastfeed. They were introduced to plain-language information about the study, and a written consent form was presented and signed by the volunteering mother. Mothers were selected by a nurse during their routine visits to the Prenatal Clinics (Hospital de Base, Hospital Panamericano and Hospital Regina Pacis). Hospital de Base is a state-run facility while Hospital Panamericano and Hospital Regina Pacis are not. Excluding factors were occupational exposure to toxic chemicals and hereditary neurological illnesses.

After birth, the newborns were clinically examined with special attention to vitality, perinatal reflexes, maturity and congenital malformations; weight, length, head circumference and Apgar scores were recorded. While in the maternity wards, we collected samples of hair from mothers and respective infants (fetal hair); the hair sampling was repeated at 6 months. Hair strands were cut from the occipital region and placed in plastic bags, with the root end stapled on a paper sheet. These samples were analysed by routine laboratory procedures described in previous publications (5,11).

The mothers were closely monitored by nurses to guarantee full support for breastfeeding and pre and postnatal care; immunization scheme followed the Brazilian vaccination program. The first vaccine (Hepatitis B) was taken either before hospital discharge (in Hospital de Base) or a few days latter, and at 30, 60, 120 and 160 days. After hospital discharge the mothers were taken under our supervision to a state-run clinic where vaccines are distributed free.
At 6 months of age, only 82 of the 100 original mother-infant pairs reported for the programmed clinical and neurobehavioural examination when infants were weighed and measured for length, and hair samples were collected again (5,11).

The neurodevelopment tests were conducted by trained professionals using the Gesell Developmental Schedules (12,13). These tests included all reactions (voluntary, spontaneous or learned) and reflexes. We also evaluated postural reactions, hand pressure, locomotion and coordination, constructive ability (which is influenced by motor development), visible and audible communication, individual reactions regarding people and stimuli (depending mainly on the temperament of the child and the surroundings). The results were expressed as developmental scores for the Adaptive Ability, Language and Motor deficiencies (gross motor ability), and Social/Emotional ability.

Estimated exposure to EtHg from vaccines (injected thimerosal):
Integrated weight gains at 30, 60, 120 and 180 days were estimated from differences in infants' weight at birth and at 6 months. Differences in infants' weight at birth and at 6 months were used to estimate daily weight gain and integrated gain at 30, 60, 120 and 180 days. As stated by manufacturers, vaccines contained 0.01% thimerosal; the Hg concentration of the doses delivered through vaccines was 25 \( \mu \text{gHg}/0.5 \text{ mL} \) for hepatitis-B (Korea Green Cross Corporation, Kheung-Eup Youin-Goon Kivityngi-Do, Korea; Euvax B injectable, LG Life Sciences, Jeonbuk-Do, Korea) and diphtheria, tetanus and pertussis-DTP (Triple Antigen, Serum Institute of India Ltd., India; Vacina Tríplice, Instituto Butanta, São Paulo, Brazil). The exposure to EtHg derived from vaccines was based on the current national immunization program of the Ministry of Health of Brazil.

Statistical analysis
Statistical analysis for testing differences between groups was done using the Statistica (StatSoft, Inc., v.6.0) statistical package. One-way analysis of variance was used with Tukey's procedure. The Shapiro-Wilk test of normality was applied and data transformed when required. For the association between variables Pearson's correlation was calculated and tested for significance; we accepted a value of <0.05 as statistically significant.

RESULTS
Parameters and outcome differences due to timing of the first dose of TCV (hepatitis-B) are shown in Table S1; no significant differences were observed in neurodevelopmental scores at 6 months. The doses of Hg derived from injected thimerosal varied greatly as a function of birth weight. Although they may seem comparable between groups, actually because the estimation was based on birth weight, the infants that took the vaccine at a later time (mean of 3 days) were probably underestimated due to obligatory neonatal weight loss. As a function of body mass, the mean TCV-EtHg perinatal dose was equivalent to the two vaccine (third dose of hepatitis-B and third dose of DTP) doses (50 \( \mu \text{gHg} \)) at 6 months when infants had double their body weight. Despite differences in socio-economic status between women delivering at the public hospital and at private run facilities, no significant differences were observed in infant anthropometry (and relative TCV dose) at birth or at 6 months.

Because of variation in body mass gain during the first 6 months there was an asymmetry of TCV-EtHg exposure at time of immunization; this is illustrated by percentage distributions in Figure S1 (a, b and c). During this period (six shots, 150 \( \mu \text{gHg} \)) there was considerable variation in individual thimerosal exposure as a function of body mass. The exposure to TCV-EtHg on the first and last vaccination day (birth and 180 days) exceeds the recommended tolerable daily intake of MeHg for adults. The amount of Hg per unit of integrated body-mass gained over 4 months (Fig. S1c) shows how high the immunized infant can be exposed to EtHg. Despite a wide variation in birth weight, the correlation of first dose of EtHg with Gesell scores was not statistically significant (Fig. S2a).

Continuous variables were analysed as single linear correlations; Pearson correlations were not statistically significant and are illustrated in the scatter plots of Figures S2 and S3. The hair-Hg representing antenatal (fetal hair-Hg at 0 day) and postnatal (hair-Hg of 180 days) Hg exposure events are plotted as percent change during the study period (Fig. S3). The percentage increase in hair-Hg concentrations was not significantly correlated with Gesell scores (Fig. S2a); it should also be noted that that the change in hair-Hg was not dependent of changes in body mass (Fig. S2b). Indeed, Gesell scores at 6 months (Fig. S2c) were independent of body mass increase.

DISCUSSION
The main finding of this work is that differences in time of the first exposure to TCV (hepatitis-B) did not predispose infants to clinical neurodevelopment delays at 6 months. Furthermore, percent change in hair-Hg (antenatal to 6 months hair-Hg, after the additional load of 150 \( \mu \text{gHg} \)) was not significantly correlated with neurodevelopment delays of these breastfed infants. The effect-modifiers of NDD risk considered in this study [(a) time of exposure of first EtHg challenge, (b) sensitivity due to immaturity (birth weight/gestational length) and (c) prenatal Hg exposure] could have been overruled by breastfeeding.

The maturation of the infant brain is a process that extends over a long period; during this time, brain fine structure and functioning are susceptible to neurotoxic substances. Mercury is one of such substance and the earlier the exposure the higher the risk of adverse effects. We realize that the relatively small amount of thimerosal in vaccines is unlikely to cause overt clinical neurological alterations (at least in non-susceptible populations). However, given the time differences (0 day versus 3 days), maturation gradients related to gestational age (36–42 weeks) and attendant birth weight, these results shed some light on the uncertainties raised
by theoretical models. Furthermore, because TCV-EtHg is quickly excreted through stools (14) possible differences due to bile production, bowel movements that could coincide with differences in time of immunization did not seem to affect neurological landmarks.

Thimerosal has been used in vaccines since the 1930s without dispute but since then breastfeeding rates have fallen considerably. While the adverse effects of TCV-immunization on children have not been satisfactorily demonstrated, the absence and/or limited duration of breastfeeding has been amply demonstrated to negatively affect neurodevelopment in short- and long-term studies. It is recognized that the consequences of early exposure to mercury can be subtle and may be evident after a prolonged latency (15–17). Therefore, a causal relationship between EtHg (from vaccines) exposure and an adverse neurological effect is difficult to establish in the relatively short time of 6 months, especially when infants had the benefit of breastfeeding. Nevertheless, the lack of a linear correlation between neurodevelopment delays with parameters related to gestational age/birth weight (Fig. S2) indicates that either infant sensitivity to small doses of injected thimerosal lies beyond these clinical tests or may manifest itself at a later time.

The Gesell developmental quotient seems adequate in gauging neurodevelopmental changes; it was able to detect differences in mean gross motor development at 1 year of age between term-infants fed formula with alpha-linolenic acid (18). The 6-month old breastfed infants in this study were not susceptible to identifiable effects caused by TCV-EtHg. It is plausible that estimates of the actual neurotoxic risk from TCV-EtHg exposure could be overruled by the CNS protective functions of breastfeeding. It is worth noting that the small number of observations did not provide the necessary material for multivariate analysis that could adjust important confounding factors. Therefore, suboptimal neural development due to TCV, if present in the more susceptible individuals, could only be detected in much larger epidemiological studies. Indeed, subtle NDD which amortized against the broad spectrum of the Brazilian population could reach significant numbers. Indeed, the large number of infants born annually in Brazil (5.7 million) has national DTP coverage of 90% in 72% of the Brazilian districts (19).

We can predict outcomes of positive effects of breastfeeding on neuromotor development, but can only indicate the possibility that NDD may occur as a result of TCV-EtHg. Furthermore, breastfeeding has been shown to have an immunomodulating effect (20) and to significantly increase antibody levels of DTP to a greater degree than in those that are formula fed (21). Although it is possible that untoward events in neurodevelopment may not be detected at 6 months, it is also possible that breastfeeding could act as a modifying-risk factor. At this young age, few cognitive skills are available to the infant; the basic neuronal constitution that modulates motor and perception involves time-critical processes. Therefore, adaptive functions or acquisition of skills that might be compromised by neurotoxic insults may take much longer than 6 months, to manifest themselves. Indeed we had a small number of individuals with a considerable overdose of thimerosal-EtHg (Fig. S1a). In such individuals, subtle neurodevelopment may lag behind but impairments could be detected only in large epidemiological studies.

Although postnatal exposure to toxic metals can occur as a result of milk feedings (3), exposure to EtHg occurs only through immunization with TCV. However, contrary to formula feeding, breastfeeding carries an established advantage of CNS-priming substances and maternal stimuli that aid infant neurodevelopment; together these neuroprotective effects of breastfeeding have a lifelong benefit on CNS integrity and general health status. Studies of perinatal events related to neurodevelopment and breastfeeding are rare. Radzymski (22) has noted that intact and functioning CNS is crucial for breastfeeding behaviour. His study compared breastfeeding behaviours and neurobehaviours at birth and at 24 h of age in neonates of mothers who received epidural analgesia during labor and controls; the higher the infant scored in relation to neurobehavioural functioning, the higher the infant scored on breastfeeding behaviours.

The NDD effects of thimerosal depend on the interplay between TK and TD of EtHg. In infants, these features have not been sufficiently studied (particularly in neonates with a wide range of birth weight and maturation—gestational age); in the neonate, the fast surge of serum thimerosal could be a factor regulating the relative levels of free forms of EtHg available to cross the BBB. On the other hand, the TD of thimerosal have been based on animal (mostly small rodent) models due to ethical difficulties in conducting such studies in humans. The lack of even observational studies, like the present, has made our understanding of the TK and TD of thimerosal depends on expert opinion (23). Therefore, it is reassuring to have the opportunity to observe no clinical neurodevelopment delay as a result of early immunization timing. However, it is important to emphasize that breastfeeding could have played a role in overruling subtle effects that such early impact of EtHg might have caused. Therefore, it is recommendable that breastfeeding should be considered as a frontline procedure to minimize uncertainties related to neurotoxic effects of early TCV-EtHg exposure.

CONCLUSION

This paper draws on information derived from a singular situation in which breastfed infants, opportunistically divided into early (<1 day) and late (mean of 3 days) thimerosal-EtHg exposure groups, did not show untowards effects on developmental landmarks at 6 months.

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References

Supplementary material
The following supplementary material is available for this article:

Table S1 Mean (range) of anthropometry, pre and perinatal Hg exposure, and Gesell Scores of infants

Figure S1 Percent distribution of Thimerosal-EtHg/ unit of body weight at birth (a—first dose of hepatite-B, 25 µgHg) and at 180 days (b—third dose of hepatite-B and third dose of DTP, 50 µgHg); (c) percent distribution of Thimerosal-EtHg (second dose of hepatite-B, first and second dose of DTP, 75 µgHg)/weight gain between first and last vaccines.

Figure S2 Plot illustrating correlation between Gesell scores and (a) dose of injected TCV-EtHg as function of body mass (r = −0.1724; p = 0.1214), (b) between relative change in hair-Hg at 6 months (r = 0.0753; p = 0.5037) and (c) relative weight gain at 6 months (r = −0.1185; p = 0.2892).

Figure S3 Plot illustrating correlation between change in hair-Hg at 6 months and respective change in body mass (r = −0.0287; p = 0.7988).

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