

ORIGINAL ARTICLE Experimental Models of Allergic Disease

Long-term reduction in food allergy susceptibility in mice by combining breastfeeding-induced tolerance and TGF- β -enriched formula after weaning

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Summary

Background Oral tolerance induction in early life is a promising approach for food allergy prevention. Its success requires the identification of factors necessary for its persistence.

Objectives We aimed to assess in mice duration of allergy prevention by breastfeeding-induced oral tolerance and whether oral TGF- β supplementation after weaning would prolong it.

Methods We quantified ovalbumin (OVA) and OVA-specific immunoglobulin levels by ELISA in milk from the EDEN birth cohort. As OVA-specific Ig was found in all samples, we assessed whether OVA-immunized mice exposed to OVA during lactation could prevent allergic diarrhoea in their 6- and 13-week-old progeny. In some experiments, a TGF- β -enriched formula was given after weaning.

Results At 6 weeks, only 13% and 34% of mice breastfed by OVA-exposed mothers exhibited diarrhoea after six and seven OVA challenges vs. 44% and 72% in mice breastfed by naïve mothers ($P = 0.02$ and 0.01). Protection was associated with decreased levels of MMCP1 and OVA-specific IgE ($P < 0.0001$). At 13 weeks, although OVA-specific IgE remained low ($P = 0.001$), diarrhoea occurrence increased to 32% and 46% after six and seven OVA challenges in mice breastfed by OVA-exposed mothers. MMCP1 levels were not significantly inhibited. Supplementation with TGF- β after weaning induced a strong protection in 13-week-old mice breastfed by OVA-exposed mothers compared with mice breastfed by naïve mothers (0%, 13% and 32% of diarrhoea at the fifth, sixth and seventh challenges vs. 17, 42 and 78%; $P = 0.05$, 0.0043 and 0.0017). MMCP1 levels decreased by half compared with control mice ($P = 0.02$). Prolonged protection was only observed in mice rendered tolerant by breastfeeding and was associated with an improved gut barrier.

Conclusions In mice, prevention of food allergy by breastfeeding-induced tolerance is of limited duration. Nutritional intervention by TGF- β supplementation after weaning could prolong beneficial effects of breast milk on food allergy prevention.

Keywords animal models, food allergy, immunotherapy and tolerance induction, paediatrics, prevention

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Introduction

Nowadays, food allergy is becoming a major concern both at the public health and the individual level. Studies using oral challenge-proven food allergy, as the

most stringent criteria for food allergy diagnosis, indicate a point prevalence of 0.9% in Europe [1] and up to 8% in high prevalence countries such as United Kingdom and Australia [2]. After the respiratory allergy epidemic, which started in the sixties, there is a strong

impression that a second wave of food allergy epidemic has started since the nineties [2, 3]. Most of disease burden is seen in young children and cow milk and egg allergy usually was found as early as in the first year of life [2]. Recent data in a study performed in Australia showed that more than one-third of infants with atopic eczema were already sensitized to egg at only 4 months of age [4], suggesting earlier onset of food allergy than previously reported [3]. Furthermore, while food allergy to milk, egg, wheat and soya was known to resolve during childhood, recent studies suggest that both egg and milk allergies are now commonly persisting into late childhood and adolescence [2, 3]. Food allergies significantly affect the quality of life of sufferers because of painful and disabling symptoms which can affect the skin such as eczema, the gastrointestinal tract such as vomiting, abdominal pain, diarrhoea, and the respiratory tract such as asthma and in some cases, causing life-threatening anaphylactic reactions [5]. Accidental exposure is a constant fear for both children and parents and leads to social exclusion. Last but not least, food allergy, in particular egg allergy, is the strongest predictor of evolution into the atopic march and later sensitization to aeroallergens and development of asthma [6]. Although specific oral immunotherapy is now recognized as facilitating significant desensitization and sustained unresponsiveness in a subset of patients, currently, there is no cure to food allergy. Altogether, this indicates the critical need to elucidate the risks of and protective factors for disease to develop. Food allergy can be classified into non-IgE-mediated and IgE-mediated food allergies. The latter is due to inappropriate Th2 lymphocyte differentiation towards innocuous dietary antigen which leads to a symptomatic inflammatory response upon allergen exposure [7]. Recent data suggest that the skin may be a critical route for allergic sensitization to food antigen, while oral exposure may favour immune tolerance through the induction of Foxp3 expressing regulatory lymphocytes and/or IL-10- and IFN- γ -secreting Tr1 lymphocytes [7–10]. The conditions for oral immune tolerance induction to food need to be better understood. Oral exposure to food antigen in a specific time window has been the subject of recent interventional trials which showed that early introduction of solid food in the diet, such as egg (4–8 months of age [4]) and peanut (4–11 months) [11], may be beneficial. Introducing solid food while continuing to breastfeed has been recommended to enhance chances of tolerance induction and prevent food allergy due to the presence in breast milk of immunomodulatory factors such as TGF- β [12–16]. We further proposed that early oral exposure to potential allergens through breast milk could induce local and systemic tolerance [13, 14]. In a mouse model, we indeed found that mice exposed to

only ng/mL of egg ovalbumin antigen (OVA) through breast milk were protected from OVA-induced allergic airway disease [17, 18]. In contrast to the adult, we found that oral antigen exposure was not sufficient for tolerance induction in early life and that additional maternal milk factors such as TGF- β and maternal OVA-specific IgG were required for oral tolerance induction [17, 18]. These data showed that the reported heterogeneity of results on allergy prevention by breastfeeding [19] may be explained by variation in milk composition and in particular its content in allergen, TGF- β and allergen-specific immunoglobulin.

Many of the food allergies that a child may develop do only rarely show up at the first exposure but often months or years of exposure [20]. This suggests that tolerance to food antigen may wane with time and/or be broken by certain conditions and underscores the necessity to identify factors required for the maintenance of oral tolerance throughout life for better efficacy of allergy prevention. Time-related decrease in oral tolerance induced to OVA was also found in *princeps* papers published in adult mouse models [21], and dose of the antigen and the presence of microbiota were found to affect duration of tolerance [22]. Here, we explored in mice the hypothesis that controversy on prevention of food allergy by breastfeeding may result from limitation in duration of breastfeeding-induced tolerance. We further examined whether oral TGF- β supplementation after weaning would prolong breastfeeding-induced protection from food allergy.

Methods

Human breast milk samples

Mother–child pairs from the French EDEN ('Etude des Déterminants pre- et post-natal du développement et de la santé de l'Enfant') prospective birth cohort study were studied (<http://eden.vjf.inserm.fr>) [23]. From a total of 2002 children recruited in Nancy and Poitiers, six hundred and twenty-one ($n = 621$) had breast milk collected during the first week after delivery. For this study, 255 mothers who had milk samples collected between day 3 and day 7 post-partum (transitional breast milk) were randomly selected (Random Selection program by SAS). Maternal history of asthma or allergies was defined on the basis of a positive answer to a questionnaire administered by a nurse on physician-diagnosed asthma, allergic rhinitis, eczema or food allergies.

The study received approval from the ethics committee (CCPRB) of Kremlin Bicêtre University Hospital on 12 December 2002 and from CNIL (Commission Nationale Informatique et Liberté), the French data privacy

institution. Written consent was obtained from all mothers after they had been informed.

OVA and OVA-specific immunoglobulins detection in human breast milk

Transitional breast milk was collected using a sterile breast pump. The samples were aliquoted and stored at -80°C . Before analysis, breast milk samples were thawed and centrifuged for 20 min at 160 g, at 4°C . The top layer of fat and the pellet were discarded and the intermediate fluid fraction was used for measuring OVA concentration as previously described [18]; detection level was 50 pg/mL. Positive samples are defined as samples in which OVA level was above ELISA detection levels (50 pg/mL). Samples below the ELISA detection level were given an arbitrary value of zero, and OVA-specific IgG and IgA levels were also determined by ELISA. Briefly, 96-well plates were coated overnight at 4°C with 10 $\mu\text{g/mL}$ OVA protein (grade V, Sigma-Aldrich, St. Quentin Fallavier, France) in PBS buffer. The next day, plates were saturated with 5% dry skimmed milk (Régilait, France) in PBS Tween 0.05% for 1 hr at room temperature (RT). Samples were diluted twofold and 10-fold in blocking buffer (5% dry milk in PBS Tween 0.05%) for OVA-specific IgG analysis and fivefold and 20-fold for OVA-specific IgA analysis, added to plates and incubated for 2 h at RT with slow agitation. Bound OVA-specific IgG and IgA were revealed by incubation for 1 h at RT with anti-human IgG-peroxidase mAb (Sigma-Aldrich) or anti-human IgA-peroxidase mAb (Sigma-Aldrich) diluted in Buffer CGS2 (Cosmo Bio, Tokyo, Japan), respectively. TMB (3,3',5,5'-tétraméthylbenzidine, 50-65-00, KPL) was used as the chromogenic substrate, and the reaction was stopped by addition of 2N H_2SO_4 . Plates were extensively washed in PBS Tween 0.05% between each step. Absorbance was measured at 450 nm. Results were expressed as arbitrary units (AU) per mL, obtained by comparison with a serum for IgG or colostrum pool for IgA, both defined to contain 1000 AU/mL.

Mice

Synchronous pregnant BALB/c mice were purchased from Janvier Labs (France) and housed under SPF conditions at 'Centre Méditerranéen de Médecine Moléculaire (C3M)'. Experimental design and animal welfare were reviewed and approved by an IACUC (Institutional Animal Care and Use committee, Nestlé Research Center, Lausanne, Switzerland), and experiments were performed according to the CIEPAL animal ethics agreement NCE/2012-81.

Oral tolerance induction in mice through breast milk

Oral tolerance induction by OVA transfer through breast milk was assessed in mice breastfed by immunized mice as previously described [18] (Fig. 1a). Briefly, 10-week-old BALB/c mice were immunized by two intraperitoneal (IP) injections with 10 μg chicken OVA (grade V, Sigma-Aldrich) adsorbed on 2 mg aluminium hydroxide (alum) (Thermo Fisher Pierce, Illkirch, France) at intervals of one week and were mated 2 days after the last sensitization. To avoid maternal *in utero* effect, all pups were delivered by naïve non-sensitized mothers and fostered at birth by sensitized mothers. During the lactation period, sensitized mothers were given 2 mg OVA by intragastric gavage, three times a week. Control groups were breastfed by naïve mothers given water by intragastric gavage, three times a week.

TGF- β -enriched formula administration after weaning

A cow milk-derived basic whey protein fraction containing naturally occurring TGF- β 1 and TGF- β 2 at a concentration of 7 and 150 $\mu\text{g/g}$ powder, respectively (Vitalarmor® GF-100, Armor Protéines, France) [24], was added to a partially hydrolysed whey protein-based formula to obtain a final concentration of approximately of 65 pg/mL TGF- β 1 and 3 ng/mL TGF- β 2. From weaning, pups were on standard animal chow (A04 diet, Safe, France) and this 'TGF- β -enriched formula' was administered 5 days a week *ad libitum* to the pups from weaning until 12 weeks of age; it was then replaced by water. Control groups were given water or a non-supplemented formula *ad libitum* from weaning (Fig. 1c).

OVA-induced allergic diarrhoea

Sensitization and challenge procedures were conducted as previously published [25]. Briefly, female mice (progeny, 6 or 13 weeks of age, 7–11 mice/group) received two IP injections with 50 μg chicken OVA and 1 mg alum (Sigma-Aldrich) at 2-week intervals (Fig. 1b and c). Two weeks after the second sensitization, the mice were fasted for 3 h and challenged every 2–3 days with 50 mg OVA by intragastric gavage for a total of seven challenges. Mice were placed in individual cages and monitored for diarrhoea symptoms for one hour. The mice were scored according to faeces appearance as follows: score 0 = no change; score 1 = well formed; score 2 = soft, non-formed; score 3 = one episode of liquid diarrhoea; score 4 = at least two episodes of liquid diarrhoea. One hour after the last challenge, mice were sacrificed by lethal IP administration of Pentothal and blood and gut tissue harvested for further analyses.

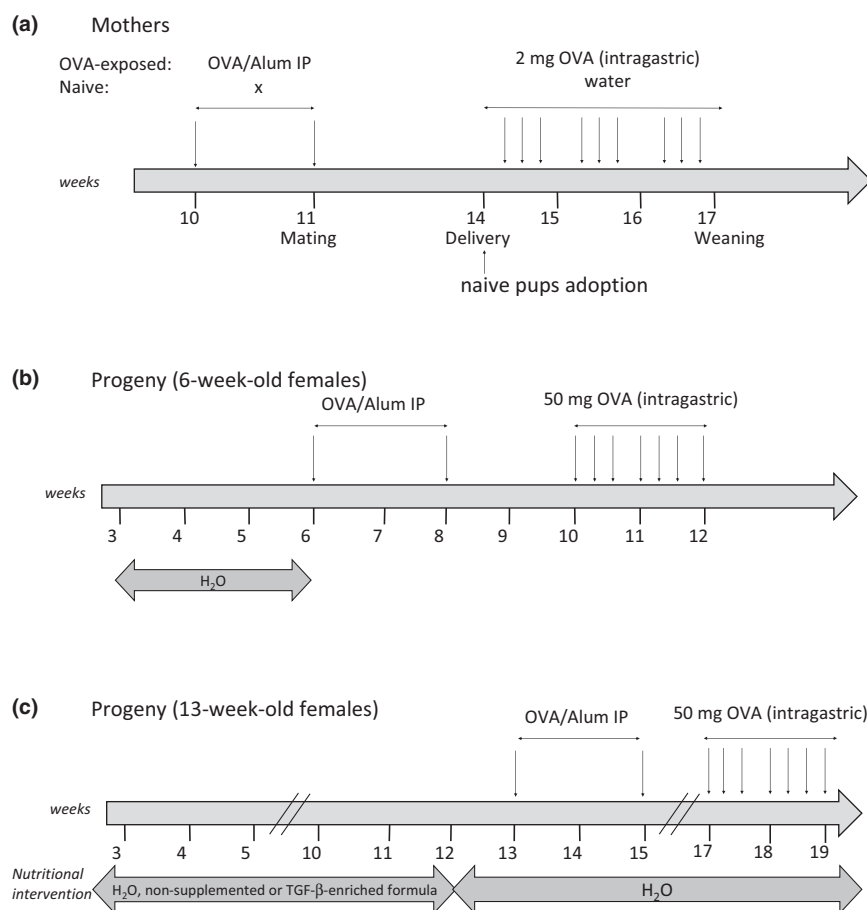


Fig. 1. Mouse model to assess impact of TGF- β -enriched formula on maintenance of food allergy prevention induced by breastfeeding. (a) Mother treatment. OVA-exposed and immunized mothers were obtained by two intraperitoneal immunizations with OVA in alum before mating and OVA intragastric administration three times a week, after delivery. Non-immunized naïve mothers only received water by intragastric administration after delivery. (b, c) Progeny treatment. All pups were born from naïve mothers and fostered at birth by naïve or OVA-immunized mothers. Food allergy was induced in 6-week-old (b) and 13-week-old (c) female mice by two intraperitoneal immunizations with OVA in alum followed by seven oral challenges with OVA (50 mg) by intragastric administration. Mice immunized at 13 weeks had free access to either TGF- β -enriched formula, non-supplemented formula or water from weaning until immunization in addition to the standard pellet diet.

Scoring was performed independently by two examiners. One hour after the last OVA challenge, mice were euthanized and serum was collected. MMCP1 levels in serum were quantified by ELISA (eBiosciences, Paris, France) to evaluate mast cell degranulation [25]. Allergic sensitization to OVA was evaluated by measuring the levels of OVA-specific IgE as previously described [18].

In vivo gut permeability

Twelve-week-old mice were fasted for 3 h and then given fluorescein isothiocyanate–dextran (MW 4.4 kDa) (Sigma-Aldrich) by intragastric administration at a dose of 0.4 mg/g body weight. After 4 h, 100 μ L blood was collected from the facial vein and the fluorescence of a twofold-diluted serum measured at 488 nm using a fluorimeter (Tecan Infinite, Männedorf, Switzerland). Concentration of dextran was calculated using a standard curve; detection limit was 0.1 μ g/mL.

FoxP3 regulatory T lymphocytes

Mesenteric lymph nodes were harvested in 12-week-old mice and isolated cells were analysed by flow cytometry after staining with anti-CD4 (eBiosciences, Paris, France) and anti-CD3 mAb (Becton Dickinson, Pont de Claix, France) for T-cell lymphocyte characterization. Cells were then fixed, permeabilized using the Fixation and Permeabilization Kit (eBiosciences) and stained with anti-FoxP3 mAb (eBiosciences). Cells were analysed by flow cytometry using a FACS Canto II and FACS Diva software (Becton Dickinson).

Statistics

Data were expressed as the median $\pm$ interquartile range. Mann–Whitney nonparametric *U*-test for continuous variables and Fisher's exact test for percentages were used to determine whether specified comparisons

were significantly different. Probability values of less than 5% were considered significant (P -value ≤ 0.05). Analysis was performed with GraphPad Prism 6 (GraphPad Software, San Diego CA, USA).

Results

Human breast milk from both allergic and non-allergic mothers contains OVA and OVA-specific immunoglobulins

The presence of egg antigen (OVA) and OVA-specific IgG and IgA was assessed in breast milk samples from the French mother–child cohort 'EDEN'[19]. Detectable amounts of OVA antigen were quantified in about 50% of milk samples, with a median value among positive samples of 129.1 pg/mL (Table 1). Similar levels were found in the breast milk of allergic and non-allergic mothers (Table 1). OVA-specific IgA and IgG were found in the near totality ($> 95\%$) of human milk samples irrespective of mother's allergic background status (Table 1). Levels of OVA-specific IgA were comparable between milk from allergic (114.6 AU/mL) and non-allergic (120.5 AU/mL) mothers, while OVA-specific IgG were found at higher levels in milk from allergic mothers compared with non-allergic mothers (127.3 vs. 82.6 AU/mL; $P = 0.03$) (Table 1).

Oral OVA exposure through breast milk of immunized mice prevents allergic diarrhoea in young offspring

As our human data indicate that almost all mothers are sensitized to egg, irrespective of their allergic status (Table 1), we next addressed whether it is possible to induce oral tolerance to OVA and prevent food allergy via the OVA transfer through breast milk of OVA-sensitized mice. We previously found that OVA exposure of

lactating OVA-immunized mothers resulted in the presence of both OVA and OVA-specific IgG and IgA milk [18]. We also found that early oral exposure to OVA through breastmilk in the presence of maternal Ig resulted in a Treg-dependent prevention of respiratory allergy [18]. Here, mice breastfed by naïve mothers or by mothers immunized to OVA and exposed to OVA during lactation were tested for their food allergy susceptibility when 6 weeks old (Fig. 1). We found that breastfeeding by OVA-immunized mothers exposed to OVA during lactation decreased allergic diarrhoea susceptibility in 6-week-old mice. Indeed, there was a threefold decrease (13 vs. 44, $P = 0.02$) and twofold (34.7 vs. 72, $P = 0.01$) decrease in the percentage of mice exhibiting diarrhoea after the 6th and 7th OVA challenges, respectively, in the group of mice breastfed by OVA-exposed mothers compared with the group of mice breastfed by naïve mothers (Fig. 2a). Decrease in diarrhoeic symptoms in mice breastfed by OVA-exposed mothers was accompanied by a decrease in the serum levels of mucosal mast cell protease 1 (MMCP1), a mast cell-derived mediator of allergic reaction (Fig. 2b, $P < 0.0001$), and by a decrease in serum levels of OVA-specific IgE (Fig. 2c, $P < 0.0001$). These data indicate that susceptibility to food allergy in young mice can be modulated by early oral exposure to OVA through breast milk of immunized mothers.

Prevention of allergic diarrhoea by breastfeeding is of limited duration

To assess whether prevention of allergic diarrhoea by OVA transfer through breast milk of sensitized mothers persisted throughout life, we analysed susceptibility to food allergy in 13-week-old mice. As shown in Fig. 2a, we found that the occurrence and severity of allergic diarrhoea were similar upon immunization of six- and

Table 1. OVA, OVA-specific IgA and OVA-specific IgG concentrations in human breast milk according to maternal history of asthma or allergies

Median (IQR), pg/mL	Total population	Allergic mothers	Non-allergic mothers	P^*
OVA	($n = 251$)	($n = 83$)	($n = 168$)	
Median (IQR)	0.0 (0.0–128.2)	50.0 (0.0–148.0)	0.0 (0.0–119.5)	
Mean (SD)	112 (244.1)	127.4 (287.9)	104.4 (219.8)	
Percentage of positive samples (n)	49.4% (124)	50.6% (42)	48.8% (82)	
Median (IQR) among positive samples	129.1 (80.2–250.7)	142.5 (91.8–252.0)	124.1 (74.9–249.0)	
OVA-specific IgA	($n = 253$)	($n = 84$)	($n = 169$)	
Median (IQR)	63.7 (42.3–131.2)	72.0 (43.6–141.9)	62.0 (41.3–129.8)	
Mean (SD)	118.6 (158.9)	114.6 (157.4)	120.5 (160.0)	
Percentage of positive samples (n)	96.4% (124)	97.6% (82)	95.8% (162)	
OVA-specific IgG	($n = 254$)	($n = 83$)	($n = 171$)	
Median (IQR)	53.8 (16.3–117.2)	67.0 (21.5–165.2)	49.1 (15.8–110.0)	0.04
Mean (SD)	97.2 (135.1)	127.3 (171.3)	82.6 (111.2)	0.03
Percentage of positive samples (n)	96.0% (244)	97.5% (81)	95.3% (163)	

* P -value of Mann–Whitney U -test comparing medians and P -value of t -test comparing means between allergic and non-allergic mothers.

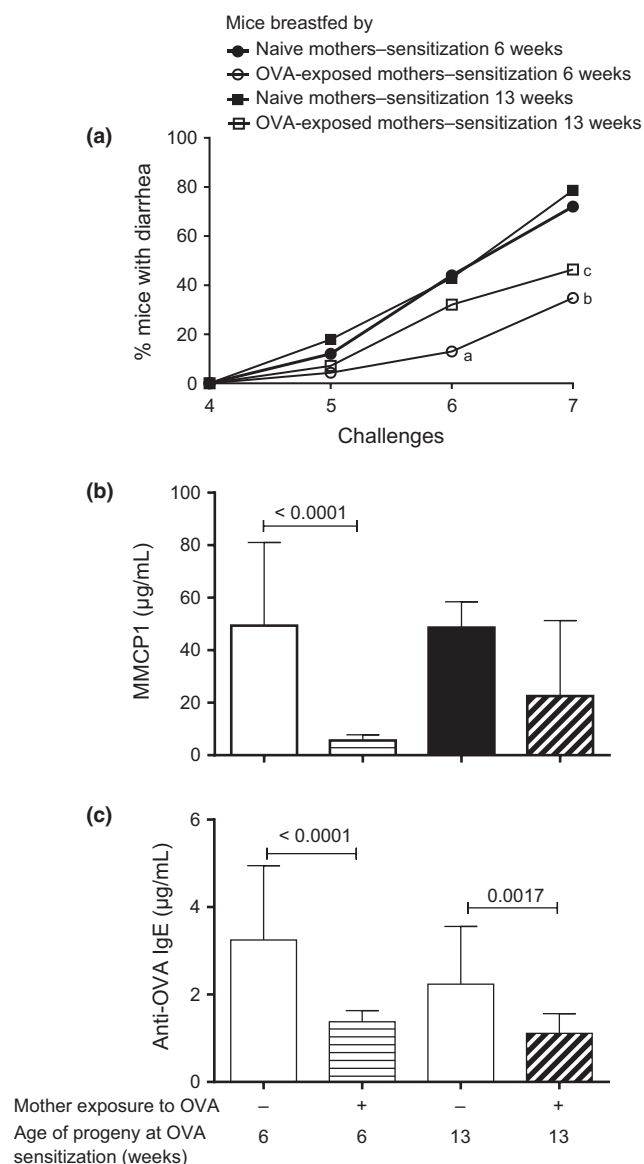


Fig. 2. Short-term prevention of allergic diarrhoea by oral OVA exposure through breast milk. (a) Percentage of diarrhoea (score ≥ 3) at each oral OVA challenge, (b) MMCP1 levels in serum and (c) OVA-specific IgE levels in mice which were breastfed by OVA-exposed and sensitized mothers or naïve mothers and which were submitted to a food allergy protocol at 6 or 13 weeks of age. Data show median $\pm$ interquartile range (b, c) and represent results obtained in three independent experiments; $n = 6$ –11 mice per group in each experiment. Fisher's exact test was used in Fig. 2a to determine whether % of diarrhoea were significantly different among groups: (a) $P = 0.02$ (b) $P = 0.01$ for mice breastfed by OVA-exposed vs. naïve mothers and sensitized at 6 weeks, and (c) $P = 0.02$ for mice breastfed by OVA-exposed vs. naïve mothers and sensitized at 13 weeks. Mann-Whitney nonparametric U -test was used in Fig 2b,c to compare groups; significant P values are indicated on the graphs.

13-week-old mice breastfed by naïve mothers. However, prevention of allergic diarrhoea by breastfeeding from OVA-exposed and sensitized mothers was

reduced in 13-week-old mice when compared to 6-week-old mice. At 13 weeks, we found no significant protection anymore after the 6th oral OVA challenge, and, after the 7th challenge, 46% of mice experienced diarrhoea instead of 34% at 6 weeks (Fig. 2a). Similar to observed impact on symptoms, OVA transfer through breast milk was not capable to prevent the increase in MMCP1 levels in 13-week-old mice submitted to a food allergy protocol (Fig. 2b). In contrast, OVA-specific IgE were still significantly inhibited (Fig. 2c). Taken together, these data indicate that the capacity of breastfeeding to prevent food allergy diarrhoeic symptoms by antigen transfer declines after weaning and that approaches are needed to promote longer term prevention.

Post-weaning feeding with TGF- β -enriched formula prolongs breastfeeding-induced prevention of allergic diarrhoea

Oral administration of TGF- β was shown to be critical for oral tolerance induction both in neonates [17, 26] and adults [27] (reviewed in [28]). Importantly, TGF- β was shown to be necessary for maintenance of regulatory T lymphocytes [29]. We therefore chose to investigate whether post-weaning oral supplementation with TGF- β using a TGF- β -enriched formula would prolong the beneficial effect of breastfeeding to prevent allergic diarrhoea. In a first series of experiments, we checked whether supplementation with a formula enriched with TGF- β for a prolonged period after weaning would be well tolerated. Mice which received TGF- β -enriched formula had no modification of stool consistency (data not shown). As shown in Fig. 3a, body weight increased similarly in mice receiving water or formula for 9 weeks after weaning. Due to the reported effects of TGF- β as a growth factor (reviewed in [28]), we also assessed the length of mouse colon and small intestine at the end of the 9 weeks of formula supplementation post-weaning. No significant impact of formula administration was observed on any of these parameters (Fig. 3b and c).

We next assessed the impact of post-weaning oral supplementation with TGF- β on prolonging the beneficial effect of breastfeeding to prevent food allergy. Mice were administered TGF- β -enriched formula for 9 weeks after weaning and at 13 weeks were tested for their susceptibility to allergic diarrhoea following OVA challenges (Fig. 1). In contrast to the limited protection at 13 weeks in mice breastfed by OVA-exposed mothers and receiving water after weaning, we observed a total abolition (0 vs. 17.9%, $P = 0.05$), a sixfold (7.1 vs. 42.9%, $P = 0.0043$), and a 2.5-fold (32.1 vs. 78.6%, $P = 0.0017$), decrease in diarrhoea occurrence at the fifth, sixth and seventh OVA challenges, respectively, in

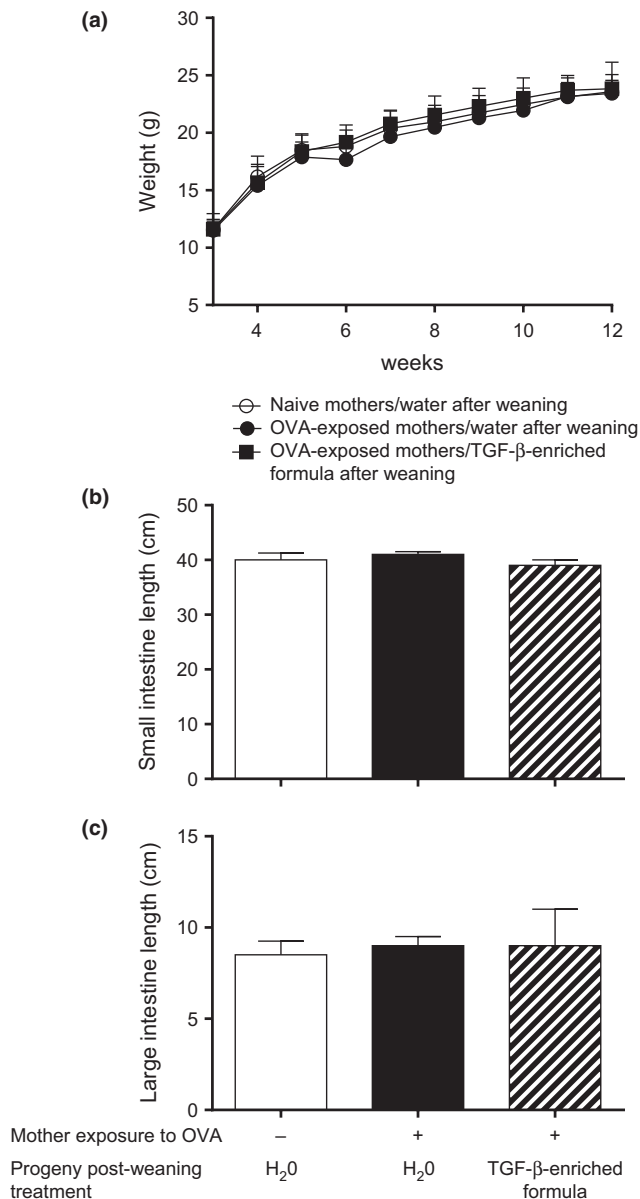


Fig. 3. Post-weaning feeding with TGF- β -enriched formula does not affect body weight gain nor gut growth. (a) Mice weight gain, (b) Small intestine and (c) Large intestine length in mice which were breastfed by OVA-exposed or naïve mothers and then received water or TGF- β -enriched formula after weaning for 9 weeks. Data represent mean $\pm$ SD (a) and median $\pm$ interquartile range (b and c). Results were obtained from one experiment with 10 (a) and 6 (b and c) mice per group.

mice breastfed by OVA-exposed mothers and supplemented with TGF- β -enriched formula after weaning compared with mice breastfed by naïve mothers (Fig. 4a). Administration of a formula containing TGF- β after weaning to mice breastfed by mothers exposed to OVA during lactation also decreased by half MMCP1 levels compared with levels found in control mice (Fig. 4b, $P = 0.02$). OVA-specific IgE levels were not

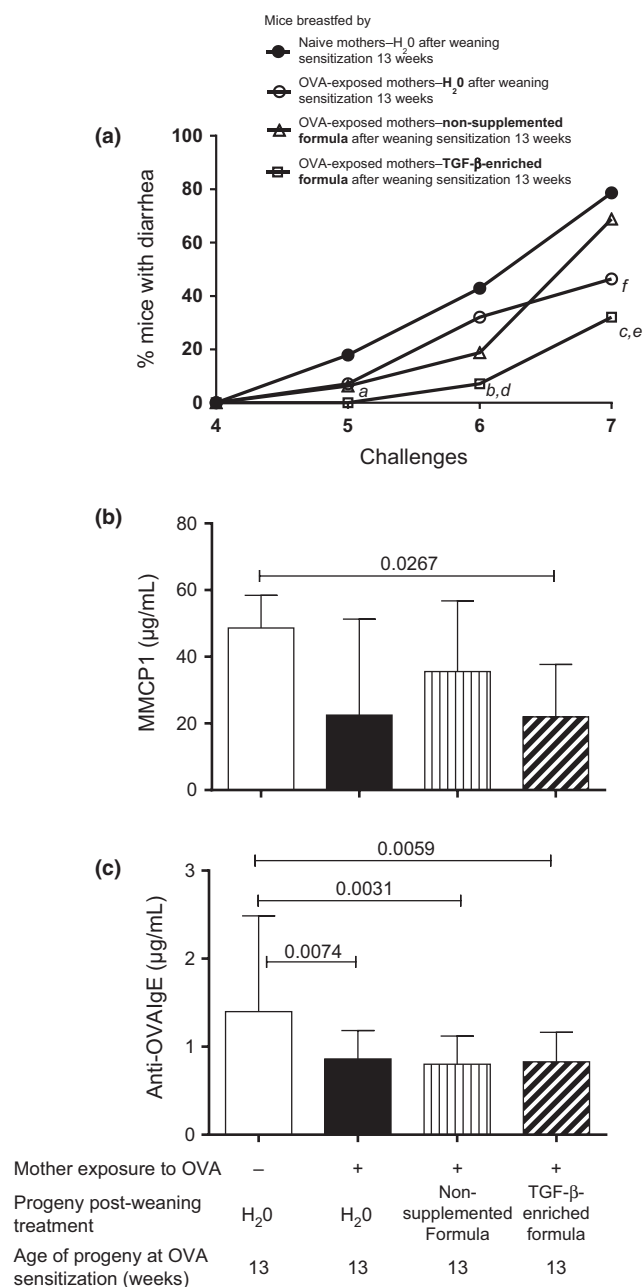
further affected by TGF- β -enriched formula administration after weaning compared with water administration after weaning in mice breastfed by OVA-exposed mothers (Fig. 4c). In contrast to results observed with a TGF- β -enriched formula after weaning, the administration of a formula not supplemented in TGF- β had no significant impact on allergic diarrhoea occurrence (Fig. 4a). The levels of MMCP1 and of OVA-specific IgE were comparable to the one found in mice breastfed by OVA-exposed mothers and receiving water after weaning (Fig. 4b and c).

Post-weaning feeding with TGF- β -enriched formula does not induce immune suppression

To determine whether oral TGF- β supplementation may induce immune suppression, we performed two separate series of experiments. In the first one, we investigated whether TGF- β -enriched formula administration after weaning would affect allergic diarrhoea susceptibility in mice which had not been tolerized by OVA transfer through breast milk. We found that diarrhoea occurrence (Fig. 5a) and MMCP1 levels (Fig. 5b) were not significantly affected in mice breastfed by naïve mothers and receiving oral TGF- β supplementation for 9 weeks after weaning, as compared to the group of mice with no supplementation. This is in contrast to what we had observed in mice breastfed by OVA-exposed mothers, where a significant difference was observed between mice who received TGF- β -enriched formula and those who did not (Fig. 4a–c). Furthermore, allergic sensitization to OVA was not affected by oral TGF- β supplementation after weaning in mice breastfed by naïve mothers (Fig. 5c). We therefore conclude that oral supplementation with TGF- β -enriched formula after weaning is maintaining breastfeeding-induced tolerance but has no immune suppressive effect on its own. We further assessed whether oral TGF- β supplementation after weaning would modify the endogenous pool of FoxP3-expressing regulatory CD4⁺ T lymphocytes (FoxP3⁺ Tregs). We found a similar representation of FoxP3⁺ Tregs in mesenteric lymph nodes of 13-week-old mice breastfed by OVA-exposed mothers with or without TGF- β -enriched formula supplementation after weaning as well as in mice breastfed by naïve mothers and without formula supplementation (Fig. 5d).

Gut barrier is improved in mice which received TGF- β -enriched formula after weaning

Increased gut permeability is known to favour allergic diarrhoea development (reviewed in [30]). We therefore evaluated whether oral TGF- β supplementation would strengthen gut barrier and thereby contribute



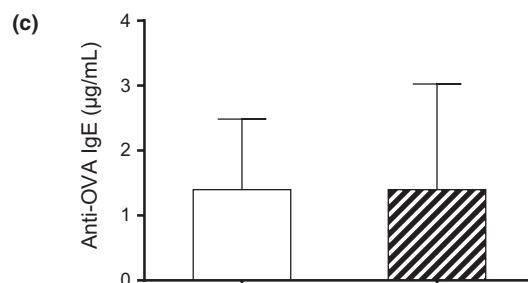
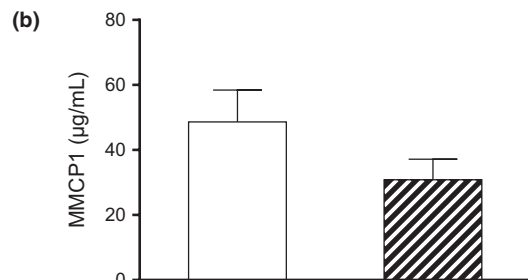
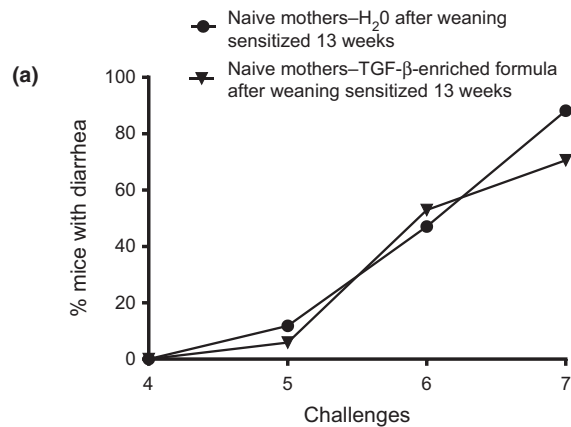
to the protection against food allergy. We analysed the *in vivo* gut permeability of 12-week-old mice which received TGF- β -enriched formula or not after weaning and before induction of the food allergy protocol. We found that gut permeability to 4.4 kDa dextran molecules was significantly decreased in mice who received OVA through breast milk and a TGF- β -enriched formula post-weaning (median dextran serum levels of 3.1 $\mu\text{g/mL}$) as compared to mice receiving OVA only through breast milk (4.5 $\mu\text{g/mL}$) or mice who did not receive OVA through breast milk or TGF- β -enriched formula after weaning (4 $\mu\text{g/mL}$) (Fig. 6).

Fig. 4. Post-weaning feeding with TGF- β -enriched formula prolongs prevention of allergic diarrhoea induced by oral exposure to OVA through breast milk. (a) Percentage of diarrhoea (score ≥ 3) at each oral OVA challenge, (b) MMCP1 levels in serum and (c) OVA-specific IgE levels in mice, which were breastfed by mothers exposed and sensitized to OVA or not and submitted to a food allergy protocol at 13 weeks of age with or without post-weaning feeding with TGF- β -enriched or non-enriched formula. Data show median $\pm$ interquartile range (b–d) and represent results obtained in two (non-enriched formula groups) and three independent experiments for all other groups; $n = 6$ –11 mice per group in each experiment. Fisher's exact test was used in Fig. 4a to determine whether % of diarrhoea were significantly different among groups: (a) $P = 0.05$ (b) $P = 0.0043$, (c) $P = 0.0011$ for mice breastfed by OVA-exposed mothers and receiving TGF- β -enriched formula after weaning compared with mice breastfed by naive mothers and receiving water after weaning; (d) $P = 0.04$ for mice breastfed by OVA-exposed and receiving TGF- β -enriched formula after weaning vs. water after weaning; (e) $P = 0.02$ for mice breastfed by OVA-exposed and receiving TGF- β -enriched formula after weaning vs. non-enriched formula after weaning; (f) $P = 0.02$ for mice breastfed by OVA-exposed mothers vs. naive mothers and receiving water after weaning. Mann-Whitney nonparametric U -test was used in Fig. 4b,c to compare groups; significant P values are indicated on the graphs.

Discussion

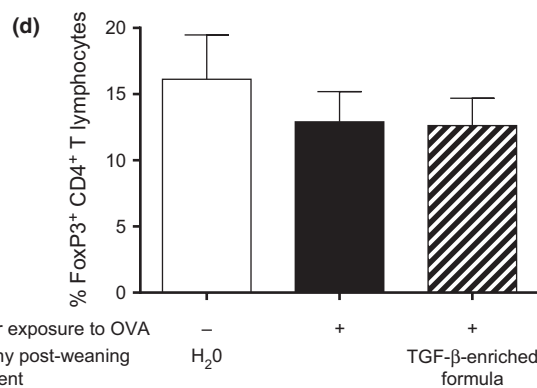
While there is increasing evidence both in mice and in humans that early oral exposure to food allergen can induce immune tolerance, less is known about how to maintain tolerance and ensure long-term prevention of food allergy. The necessity of continuous exposure of the immune system to allergen for tolerance maintenance is a matter of debate [31], and a recent interventional clinical trial showed that it may not be required [32]. Although it is possible to prevent allergic diarrhoea to egg by breastfeeding-induced oral tolerance in mouse models (this study and [33], we found here that protection was decreasing with time. This was observed in a mouse model of OVA transfer through breast milk, which reproduces human milk composition in terms of very low OVA content (Table 1 and [34]) and presence of OVA-specific Ig (Table 1). We previously showed this model to be associated with immune tolerance induction which relied on induction of OVA-specific FoxP3 Tregs [18]. We also found that maternal milk antigen-specific antibodies helped in the maintenance of a partial tolerance in a model of allergic airway inflammation [18]. We further demonstrated here that feeding mice with a TGF- β -enriched formula after weaning was sufficient to allow a prolongation of a potent breastfeeding-induced protection. Importantly, this approach was found to be safe as it did not affect mice body weight gain, intestine growth nor induce immune suppression in mice.

The capacity of breastfeeding to protect against food allergies is a matter of intense debate although the



Mother exposure to OVA
Progeny post-weaning treatment
Age of progeny at OVA sensitization (weeks)

Mother exposure to OVA	Progeny post-weaning treatment	Age of progeny at OVA sensitization (weeks)
-	H ₂ O	13
-	TGF- β -enriched formula	13



Mother exposure to OVA
Progeny post-weaning treatment

Mother exposure to OVA	Progeny post-weaning treatment
-	H ₂ O
+	TGF- β -enriched formula

recommendation is still to breastfeed for 6 months [10, 19, 35]. One of the major hypotheses, which has been assessed in epidemiological studies, is whether food allergen consumption during lactation would

Fig. 5. Absence of immune suppression induction by administration of TGF- β -enriched formula after weaning. (a) Percentage of diarrhoea (score ≥ 3) at each oral OVA challenge, (b) MMCP1 levels in serum and (d) OVA-specific IgE levels in mice which were breastfed by naïve mothers and submitted to a food allergy protocol at 13 weeks of age with or without post-weaning supplementation with TGF- β -enriched formula. Data show median $\pm$ interquartile range (b, c) and represent results obtained in two independent experiments, (d) % of cells expressing FoxP3 among CD4⁺ lymphocytes from mesenteric lymph nodes of 13-week-old mice breastfed by OVA-exposed or unexposed mothers with or without post-weaning supplementation with TGF- β -enriched formula. Data represent results obtained in one experiment with seven mice per group. Fisher's exact test was used in Fig. 5a and Mann-Whitney *U*-test in Fig. 5b-d.

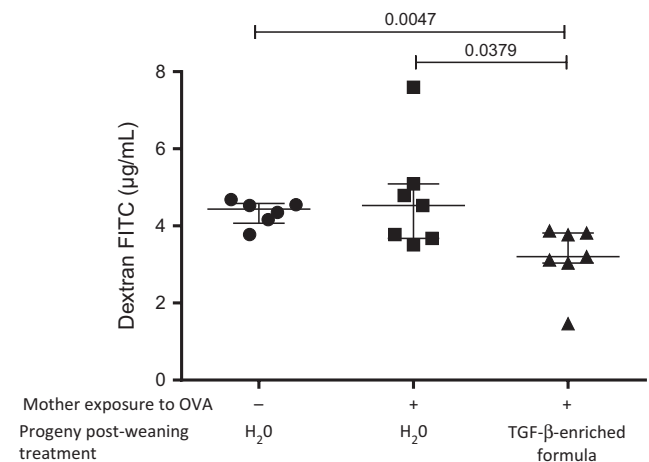


Fig. 6. Improved gut barrier in mice fed TGF- β -enriched formula after weaning. Permeability to FITC-dextran was assessed in 12-week-old mice breastfed by OVA-exposed or unexposed mothers with or without post-weaning feeding with TGF- β -enriched formula. Dextran was given orally by intragastric administration and fluorescence in sera was measured 4 h after the administration. Data represent results obtained in one experiment representative of two with 6-7 mice per group; horizontal bar represent median value and IQR.

affect allergy risk in infants. Meta-analysis and systematic reviews of intervention studies assessing the impact of maternal allergen avoidance during lactation have demonstrated the absence of significant effect of this strategy and led to the current recommendation by the American Academy of Allergy and Immunology [35] and the European Academy of Allergy and Clinical Immunology [10] of no allergen avoidance during lactation. Recently published and ongoing studies such as Quest trial developed by Debbie Palmer in Australia are now assessing the opposite approach consisting in deliberate allergen exposure during lactation and measure of its impact on food allergy. Recent data showed that multiple allergenic foods' introduction in breastfed children may be beneficial [16]. Important parameters which will be most probably influencing prevention of egg

allergy by egg consumption during lactation will be the concentration of egg antigen in breast milk which has been shown to vary a lot from one mother to another after egg ingestion [34, 36]. Our study further shows the great heterogeneity of egg antigen levels in maternal milk. Importantly, our work highlights in a mouse model that protection induced by antigen transfer through breast milk may not be long-lived and this could represent an additional important factor affecting results of impact of breastfeeding on food allergy. A recent meta-analysis from Dogaru et al. supports this hypothesis as it showed that breastfeeding was strongly protective for asthma in the first 2 years of life and diminished over time [37].

In this study, we tested the impact of an oral administration of a TGF- β -enriched formula on maintenance of food allergy prevention induced by breastfeeding. The formula was designed to contain similar amounts of TGF- β to those in human milk [38]. We found that prolonged administration of a TGF- β -enriched formula after weaning reduced allergic diarrhoea susceptibility after oral OVA challenges only when OVA had been transmitted through breast milk. These data are in agreement with previous observations made by Ando et al. who observed that orally administered TGF- β in adult mice did not modify allergic immune responses in mice in the absence of co-ingestion of food antigen [27]. Furthermore, allergic sensitization and the endogenous pool of FoxP3 Tregs in draining gut MLN were not affected by administration of the TGF- β -enriched formula. These data demonstrate that mice given TGF- β -enriched formula after weaning for a prolonged period of time do not undergo immune suppression and, in contrast, suggest that post-weaning oral administration of TGF- β is helping to extend breastfeeding-induced tolerance.

TGF- β is a critical cytokine for immune homeostasis. Lack of TGF- β and/or signalling in T cells results in severe generalized inflammation mostly due to an impact on the generation, maintenance and function of FoxP3 Tregs [39]. Recent research on the mechanisms of maintenance and function of FoxP3 Treg pools indicates that hypomethylation in FoxP3 DNA is necessary for stable Tregs function and that TGF- β may be involved in such epigenetic changes in induced Tregs [40]. More specifically, TGF- β in the gut mucosa is critical for oral tolerance induction. In conjunction with retinoic acid secreted by gut mucosal CD103+ dendritic cells, it plays a major role in the differentiation of FoxP3 Tregs from naïve T cells [41, 42]. In adult, endogenous secretion of TGF- β in the gut is sufficient for oral tolerance induction. This process can be further enhanced by oral administration of TGF- β , thus demonstrating persistence of oral TGF- β

bioactivity in spite of the presence of gut proteases [27]. However, both mice and human data indicate that oral tolerance and allergy prevention in early life may benefit from exogenous oral sources of TGF- β . Studies in knock-out offspring mice show that maternal milk provides TGF- β to the neonate at levels which are sufficient to prevent severe inflammatory disease [43]. Most human cohort analyses show an inverse correlation between levels of milk content in TGF- β and risk of allergy in infancy and early childhood [38]. Furthermore, studies in rodents indicate that lack of exogenous oral TGF- β administration during the lactation period is responsible for gut allergic inflammation [44] and allergic sensitization to cow milk in susceptible strains [26]. The need for exogenous TGF- β in early life may be related to insufficient endogenous TGF- β synthesis in infants. This is supported by data showing low expression of TGF- β in the small intestine of rats before weaning [45] and by analyses of TGF- β in infant stools which show that levels of TGF- β between birth and 6 months of age are 5 times lower than those at 1 year [46]. From all these data, we propose that in early life insufficient endogenous synthesis of TGF- β combined with insufficient exogenous sources may preclude long-term maintenance of tolerance and prevention of food allergy. Our data suggest that, when prolonged breastfeeding is stopped, a formula enriched with TGF- β may be a way to maintain naturally induced oral tolerance and ensure long-term prevention of food allergy. In addition to its effects on allergen-specific immune regulation, post-weaning administration of TGF- β -enriched formula may be also beneficial for prevention of allergy by its protective effects on gut barrier as supported by our *in vivo* data and previous publications on TGF- β impact on gut epithelial integrity [47, 48]. Indeed, intestinal barrier dysfunction is thought to contribute to the severity of food allergen-induced clinical symptoms [30].

In conclusion, this work supports the supplementation in TGF- β at weaning to ensure long-term prevention of food allergy by prolonging breastfeeding-induced protection; this should be now tested in clinical trials.

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Conflict of interest

The work performed by VV, AR, PM and MT has been funded by Nestec Ltd. SH, LD, AM and SN are employed by Nestec Ltd.

Author contributions

AR, PM, MT and VV performed and analysed experiments; IAM set up the Eden birth cohort, and NB and IAM analysed data of Eden birth cohort. SH and LD contributed to the analysis of the experiments. SN and VV designed the study with the contribution of AM. AR and VV wrote the paper. VV supervised the whole project. All authors read, commented on the manuscript and approved the final version.

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