



Bimodal determination of immunoglobulin E by fluorometry and ICP-MS by using platinum nanoclusters as a label in an immunoassay

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Abstract

The authors describe the use of platinum nanoclusters (PtNCs) as bimodal labels in a competitive immunoassay for immunoglobulin E (IgE). Both fluorometry and inductively coupled plasma – mass spectrometry (ICP-MS) are used. Optimization of the PtNCs synthesis using lipoic acid as ligand was carried out. The time for synthesis and the effect of NaOH added to the PtNCs precursor mixture was optimized with the aim to obtain PtNCs with strong red fluorescence and low size dispersity. Maximal fluorescence was obtained at excitation/emission wavelengths of 455/620 nm. The average diameter (1.5 nm) and crystal structure (face-centered cubic structure) of the PtNCs were determined by HR-TEM. It was calculated that each PtNC contains 116 Pt atoms at average. Labelling of the antibody (Ab) against IgE with PtNCs was optimized in terms of recognition capabilities and fluorescence intensity. A molar ratio (Ab:PtNCs) of 1:11 is found to be best. A competitive immunoassay for IgE was developed and detection was carried out by using both ICP-MS (by measuring ¹⁹⁵Pt) and fluorometry. The limit of detection (LOD) of the fluoroimmunoassay is 0.6 ng mL⁻¹ of IgE. The LOD of the ICP-MS method is as low as 0.08 ng mL⁻¹. The method was evaluated by analyzing four (spiked) serum samples by ICP-MS. No sample pretreatment excepting dilution is needed. Results compared favorably with those obtained by a commercial ELISA kit.

Keywords Fluoroimmunoassay · Lipoic acid · One-pot synthesis · Carbodiimide crosslinking · Immunoprobe · Elemental mass spectrometry

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Introduction

Elemental labels have shown a high potential for sensitive detection of biomolecules by inductively coupled plasma – mass spectrometry (ICP-MS) [1–3]. Within this context, single chelates [4], commercial polymers containing several lanthanide atoms covalently attached [5], metal nanoparticles (NPs) most commonly made of gold [6, 7] with diameters in the order of 20–50 nm, and quantum dots (QDs) [8, 9] have been successfully investigated as elemental labels. The use of labels with many atoms of a given metal per tag will allow high signal amplification, and thus, good sensitivity for detection of the corresponding biomolecule. Pure metal nanostructures, such as AuNPs, contain a core with atoms of just one metal. Thus they possess a higher number of detectable atoms per label unit volume [10] as compared to QDs (such as CdSe QDs) or to polymers containing lanthanides. Consequently, it is expected that pure metal nanostructures will provide greater signal amplification when comparing similar label sizes.

On the other hand, water soluble fluorescent metal nanoclusters (NCs) with sizes below 3 nm have shown a burgeoning interest for biomedical and biosensing applications [11, 12]. The small volume of these NCs makes of interest their use for labelling of biomolecules such as antibodies (Ab), since they will have a lower risk of hindering the Ab recognition capabilities. Gold is, by far, the most common metal employed for the synthesis of fluorescent NCs [13]. Silver NCs [14, 15] and copper NCs [16] are also frequently investigated. Nowadays, the synthesis of fluorescent NCs of other metals is pursued, such as iridium [17] and molybdenum [18].

Special efforts are being addressed towards the synthesis of platinum NCs (PtNCs). PtNCs show lower toxicity than QDs [19] and templates such as proteins, DNA, polymers and dendrimers have been investigated for the synthesis of water soluble PtNCs [20–24]. However, major drawbacks of big scaffolds are the difficulties both to remove the remaining free templates from the NCs in solution and to isolate the NCs from the big scaffold. In addition, when using polymers and dendrimers as templates it has been observed that reaction blanks often emit a noticeable fluorescence. In contrast, the synthesis of PtNCs using small thiolated ligands as stabilizing agents is an attractive alternative to easily isolate and purify fluorescent PtNCs [25, 26]. Previous work in our laboratory has shown that the synthesis of fluorescent PtNCs using lipoic acid is feasible [26]; however, emission was low and studies about polydispersity were not undertaken.

Sensitive dual detection can be carried out with immunoprobe labelled with fluorescent NCs for biomolecules determination by fluorescence and elemental MS. Each of the detection methods will offer advantages and limitations. Therefore it is convenient to compare the analytical performance of both. Some works have been published about the bimodal detection concept using fluorescence and ICP-MS [8, 27–29]. Bustos et al. [8] reported a comparison of both detection modes with CdSe/ZnS QDs through the measurement of Cd in the case of ICP-MS. Other examples include the use of a nanoprobe composed of europium chelated by 1,10-bis(50-chlorosulfo-thiophene-20-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone [28], the synthesis of hybrid labels containing both a DTPA chelate (coordinated with either ^{165}Ho or ^{111}In) and a Cy5 fluorescent dye [27], or by combining a fluorescent dye (Cy3) with upconversion NPs [29]. Those probes are composed either of just one metal atom or of atoms of different heteroatoms (e.g. CdSe), reducing the amplification capabilities by ICP-MS detection. Therefore, the described probes are not optimum when comparing expected signal amplification by ICP-MS and label size. Within this context, fluorescent NCs with small size and many atoms of a given metal are worth to be studied as labels for bimodal detection by fluorescence and ICP-MS.

In this work, the one pot synthesis of PtNCs with lipoic acid and sodium borohydride is optimized in terms of high

fluorescence, low size dispersity and PtNCs stability. As a proof of concept, the synthesized PtNCs were bioconjugated, via carbodiimide, to anti-human IgE Ab (anti-h IgE) and evaluated for the determination of IgE in human serum by fluorescence and ICP-MS. We have selected this protein because IgE levels typically present in human serum are very low. Therefore, an analytical methodology with low limits of detection is required for its correct determination. To corroborate the IgE content obtained with the methodology using PtNCs labels, a commercial ELISA kit was employed for the same samples.

Experimental

Reagents and materials

The synthesis of PtNCs was carried out using H_2PtCl_6 (8% wt; Sigma-Aldrich, USA; <https://www.sigmaaldrich.com>), lipoic acid (>98% powder; Across Organics, Belgium; <http://www.acros.com>) and NaBH_4 (98% powder, Sigma Aldrich). Labelling of the selected Ab with the PtNCs was carried out with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (98% powder; Across Organics) and N-hydroxysuccinimide (NHS) (> 98% powder; Sigma-Aldrich). Amicon ultra centrifugal filter units (3 and 100 kDa pore size, Merck Millipore, Darmstadt, Germany; <http://www.merckmillipore.com>) were employed for purification.

ELISA microtitration plates (96 well; Thermo Fisher Scientific, Germany; <https://www.thermofisher.com>) were used to evaluate the recognition capabilities of the Ab labelled with PtNCs. A polyclonal anti-goat IgG labelled with horseradish peroxidase (whole molecule, produced in rabbit; Sigma-Aldrich) and a TMB substrate kit (Thermo Fisher Scientific) were employed in such studies.

The immunoassay for human-IgE determination was accomplished using an immunoprobe consisting of polyclonal anti-h IgE Ab (ϵ -chain specific, produced in goat; Sigma Aldrich) labelled in our laboratory with the PtNCs. Native human IgE protein (Abcam, UK; <https://www.abcam.com>) was used as analytic standard. Bovine serum albumin (BSA) (99% powder, Merck, Germany) and phosphate buffered saline (PBS) (Sigma-Aldrich) solution with 0.05% of surfactant Tween 20 (Sigma-Aldrich) were employed in the immunoassay procedure. These immunoassays were carried out in two different mountings depending on the detection mode. Poly-L-lysine surface coated microscope slides (Electron Microscopy Sciences, Hatfield, PA, USA; <http://www.electronmicroscopysciences.com>) with an adhesive press-to-seal silicon isolator (Grace bio-labs, Oregon, USA; <https://gracebio.com>) were used for confocal microscopy in fluorescent detection, whereas the immunoassays were

performed in ELISA microtitration plates (96 well; Thermo Fisher Scientific) when ICP-MS detection was performed.

Commercial human serum (from human male AB plasma, USA origin, sterile-filtered, Sigma-Aldrich) was used to investigate the applicability of the immunoassay with PtNCs. In addition, three clinical serum samples without any signs of relevant pathologies were supplied by the Instituto Oftalmológico Fernández-Vega (Spain). The study adheres to the tenets of the Declaration of Helsinki and full ethical approval was obtained for the Clinical Research Ethics Committee at the Asturias Central University Hospital (Spain). To validate the methodology, the IgE (human) ELISA Kit (Abnova, Taiwan; <http://www.abnova.com>) was used.

Other chemicals were aqua regia, prepared by mixing concentrated nitric acid (65%, Merck, Germany) and hydrochloric acid (37%, Fisher Scientific, USA) in a volume ratio of 1:3, used for PtNCs digestion prior to ICP-MS analysis, NaOH (97%, Merck) and H₂SO₄ (Fisher Chemical, Thermo Fisher Scientific). Boric acid (99.5%, Sigma Aldrich) and KCl (99%, Sigma Aldrich) were employed to prepare the borate buffered saline (BBS). Deionized ultrapure water, resistivity 18.2 MΩ cm⁻¹ (Purelab Flex 3&4, UK; <https://www.elgalabwater.com>), was used in all the experiments.

Instrumentation

Fluorescence measurements in solution were performed with a spectrofluorimeter (LS-50-B, Perkin Elmer, USA; <https://www.perkinelmer.com>). Also, a spectrophotometer (Cary 60 UV-VIS, Agilent Technologies, USA; <https://www.agilent.com>) was employed for absorption measurements of the synthesis precursors and PtNCs suspensions. A Suprasil quartz cuvette model 101-Qs from Hellma® (Sigma-Aldrich) was required for such purpose. A high-resolution transmission electron microscope (HR-TEM) (JEOL JEM-2100, Tokyo, Japan; <https://www.jeol.co.jp>) with an energy dispersive x-ray (EDX) spectrometer for microanalysis allowed morphological characterization of the PtNCs. Measurement of the Z-potential of PtNCs suspensions at different pHs was carried out with a Zetasizer Nano ZS (Malvern analytical Ltd., UK; <https://www.malvernpanalytical.com>).

A laser confocal microscope (DM IRE2; Leica, Germany, Germany; <https://www.leica-microsystems.com>) with a 63x oil immersion objective was employed for measurement of the immunoassays by fluorescence. Measurements with ICP-MS (Model 7500; Agilent Technologies) were taken for different purposes: to determine the platinum concentration in the PtNCs synthesis process, to confirm the platinum mass balance during the bioconjugation procedure, and to quantify the IgE protein in the competitive immunoassay measuring the platinum signal in each well of the ELISA plate.

An absorbance microplate reader (ELx800; Bio-Tek, USA; <https://www.biotek.com>), an ultrasonic bath (J.P. Selecta,

Spain; <http://grupo-selecta.com>), a centrifuge (Gyrozen and Co., Rep of Korea; <https://www.gyrozen.com/index.php>), a magnetic stirring plate (Fisher Scientific), a vortex mixer (Labbox Labware, Spain; <https://esp.labbox.com/>) and a stove (Memmert, Spain, <https://www.memmert.com>) were other equipments used.

Fluorescence data processing was carried out using Image J software (National Institute of Health, Bethesda, USA; <https://www.nih.gov>) and immunoassay curves representation was done with the online data analysis tool MyAssays Ltd.

Synthesis of PtNCs and labelling of anti-h IgE

50 μmol (10.5 mg) of ligand (lipoic acid) was dissolved in 10 mL of deionized water in which 30 μL of freshly 2 M NaOH was previously added. The mixture was then homogenized with an ultrasonic bath until the ligand was completely dissolved. Then, 200 μL of 50 mM H₂PtCl₆ (1:5 M final ratio of Pt:ligand) was added under stirring and 5 min later 400 μL of a 25 mM NaBH₄ solution was added dropwise (employing a syringe). After 15 h at room temperature under stirring, the excess of reagents were removed by ultrafiltration (3 kDa pore size Amicon): a first cycle at 1100 g for 10 min and then three washing steps with deionized H₂O at 1600 g for 10 min were carried out for such purpose. Once the volume was reduced, a small volume of yellow colored solution containing the PtNCs remained on the filter. Then, initial volume was restored. The purified solution was stored in the fridge.

100 μL of anti-h IgE Ab (100 μg mL⁻¹) was added in an Eppendorf tube followed by an excess of the PtNCs purified solution (198 μL, 1:20 M ratio of Ab:PtNCs). As soon as the PtNCs solution was poured, vortex stirring was started. Then, 10 μL of a solution containing EDC and NHS were added with a molar ratio Ab:EDC:NHS of 1:1500:1500. After 2 h at room temperature with constant stirring, purification was performed by ultrafiltration (100 kDa pore size Amicon) with a first cycle at 1600 g for 10 min and three washing steps with deionized H₂O at 1600 g for 10 min. The purified solution was stored in the fridge.

Optimization of the immunoprobe composition

The ratio between the PtNCs label and the anti-h IgE in the immunoprobe was optimized through a spectrophotometric immunoassay, using a secondary Ab labelled with horseradish peroxidase. For such purpose, IgE was immobilized (100 μL/well of 5 μg mL⁻¹ IgE) in an ELISA plate and incubated for 6 h at 37 °C. Then, the excess protein solution was removed and 200 μL/well of 1% BSA solution in 10 mM PBS (pH 7.4) was added to block the empty places in the well. The plate was incubated for 2 h at room temperature. Next, 100 μL/well of the anti-h IgE (1 μg mL⁻¹) was added and kept for 2 h at 37 °C. In this step, the Ab labelled with different molar ratios

of PtNCs was employed for optimization purposes. After washing with 0.05% PBS-Tween 20, 100 μL /well of the anti-goat IgG peroxidase-labelled Ab (diluted 1:20000 in a buffer containing 1% PBS-BSA and 0.05% Tween 20) was added and incubated as above. Finally, the plate was washed and treated with 100 μL /well of the TMB kit. The enzymatic reaction was stopped with 2 M sulfuric acid (100 μL /well) and detected in an absorbance plate reader ($\lambda = 450 \text{ nm}$).

Immunoassay with fluorescent detection using PtNCs as label

100 μL /well of IgE protein (10 $\mu\text{g mL}^{-1}$) containing EDC (1500:1 M ratio of EDC:IgE) were added to wells on a poly-L-lysine coated microscope slide (EDC allowed to improve immobilization on the slides) and incubated during 2 h at 37 $^{\circ}\text{C}$. Next, the IgE still in solution was removed and 200 μL /well of 1% BSA in 10 mM PBS (pH 7.4) was added to each well. The microscope slide was then incubated for 2 h at room temperature. Then, all wells were washed three times with 10 mM PBS (pH 7.4) and 0.05% Tween 20 (200 μL /well).

Concurrently, incubation of the immunoprobe containing PtNCs as label (100 μL of 10 $\mu\text{g mL}^{-1}$, expressed as Ab concentration) with 100 μL of the IgE standards or serum samples in an Eppendorf tube was carried out. To carry out the competitive reaction, this mixture was added to the previously treated wells (as described in the above paragraph) and incubated for 2 h at 37 $^{\circ}\text{C}$. After the washing steps (three times) with PBS-Tween 20, the fluorescence was measured.

Immunoassay with ICP-MS detection using PtNCs as label

Despite the immunoassay steps were the same as those described in the above section, the mounting was changed because of the final step of the required protocol. An ELISA microwell plate was used for this purpose using ICP-MS detection. Once the last washing step was performed, 50 μL /well of a solution of 2 M H_2SO_4 was added in order to extract the PtNCs into the solution. After 10 min, the acid solutions were extracted from the wells to carry out the PtNCs digestion. 200 μL of aqua regia (HNO_3 :3HCl)/tube were added to oxidize and dissolve platinum and then the tubes were introduced 20 min in an ultrasonic bath. Finally, a 1:200 dilution (5 mL final volume) of the samples was required to minimize the percentage of acid introduced into the ICP-MS (<3% v/v). ICP-MS measurements to determine the platinum concentration of PtNCs solutions were carried out by external calibration with pure platinum standards (concentrations from 0.05 ng g^{-1} to 20 ng g^{-1}) using iridium as internal standard (isotopes ^{195}Pt and ^{193}Ir were measured, respectively).

Human serum analysis

The applicability of the immunoassays with PtNCs was evaluated for the analysis of a commercial human serum and three clinical human serum samples (denoted as Serum 1, Serum 2 and Serum 3) using the same immunoassay procedure as for the IgE standards. Dilution of the sera was required to fit the concentrations of the samples into the linear range of the calibration plots. A 1:1000 dilution with PBS (pH 7.4) was carried out in the case of the commercial serum and the “Serum 1” clinical sample, whereas a 1:500 dilution was used for the rest of the sera.

The methodology with PtNCs as label was validated using a commercial ELISA kit.

Results and discussion

Synthesis of fluorescent PtNCs, their bioconjugation to a specific Ab and their subsequent application as label for detection of IgE is described. PtNCs have been chosen because they are fluorescent and also high amplification can be expected by ICP-MS detection (each label is composed of a cluster of platinum atoms). Therefore, they can be used for dual detection. NaBH_4 (as reducing agent) and lipoic acid (as small thiolated stabilizing agent) were used for the synthesis of PtNCs.

Synthesis of PtNCs using lipoic acid as ligand

PtNCs synthesized following the procedure described in the *Experimental section* showed fluorescent emission with a maximum at 620 nm (see Fig. 1). The formation of the PtNCs was confirmed through absorbance measurements: as can be seen in Fig. S1 (*Electronic Supplementary Material*),

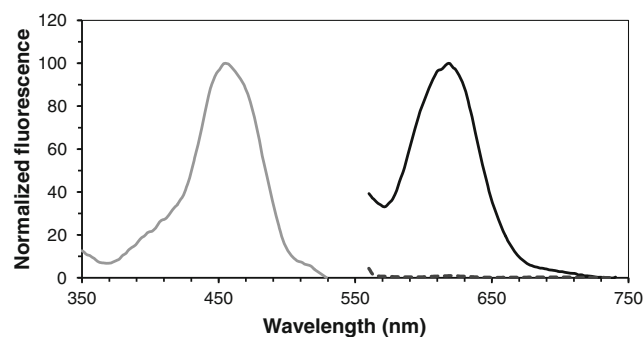


Fig. 1 PtNCs fluorescence characterization. Excitation (gray line) and emission (black line) spectra of PtNCs in ultrapure water ($\lambda_{\text{exc}} = 455 \text{ nm}$, $\lambda_{\text{em}} = 620 \text{ nm}$). The dotted black line corresponds to the emission spectrum of the synthesis blank. The plot depicts normalized intensities, given 100 value to the maximum fluorescence signal observed at 620 nm

the PtNCs suspension does not have the characteristic absorbance band of superficial plasmon resonance typical of bigger NPs. The blank of the synthesis was prepared under exactly the same steps than the PtNCs synthesis, excepting that the platinum salt was not added. As it is collected in Fig. 1, the emission signal for the reaction blank was negligible.

Lower NaBH_4 ratios decrease the reaction kinetics, giving rise to metal NCs of higher quality [14]. Therefore, in order to obtain PtNCs with higher fluorescence and less dispersity than those previously reported [26], the selected NaBH_4 molar ratio was lower than in the previous work (Pt:lipoic acid: NaBH_4 of 1:5:1 versus the 1:5:25 previously used). In addition, two experimental variables were evaluated in the optimization of the PtNCs synthesis procedure: the synthesis duration and the effect of 2 M NaOH volume added to the PtNCs precursor mixture. Regarding the synthesis time, maximum signals were achieved after 13 h of synthesis at room temperature (Fig. S2 in *Electronic Supplementary Material*). This time period is longer than the 6 h previously recommended by Fernández et al. [26] and it can be justified in terms of the slower PtNCs synthesis kinetics due to the lower NaBH_4 molar ratio employed.

Regarding the effect of 2 M NaOH volume, Fig. 2 shows a comparison of fluorescence spectra for the purified PtNCs suspension obtained under synthesis with three different volumes of 2 M NaOH. Fluorescence signals were higher when using 30 μL as compared to 20 μL of 2 M NaOH. Furthermore, the concentration of platinum was measured by ICP-MS both in the filtered solutions collected during the purification process (i.e. the unreacted free platinum) and in the reconstituted suspensions (containing the PtNCs retained in the ultracentrifugation filter). The PtNCs synthesis yield was 60% when using 20 μL of 2 M NaOH, while the PtNCs

synthesis yield was found to be 84% for the addition of 30 μL of 2 M NaOH to the reaction mixture. Here it should be noted that, as it is depicted in Fig. 2, the increase of fluorescence is higher than the 24% synthesis yield difference, thus indicating that PtNCs of higher quality are synthesized with 30 μL of 2 M NaOH.

HR-TEM allowed to measure diameters of the synthesized PtNCs (Fig. 3a shows, as example, the PtNCs synthesized with 30 μL of 2 M NaOH). Average diameters were determined from individual PtNCs randomly observed in the HR-TEM photographs. A smaller size and lower dispersity (1.49 ± 0.025 nm for $n = 1800$, 99% confidence interval, with the standard normal distribution) was found for the synthesis using 30 μL of 2 M NaOH as compared to 20 μL of 2 M NaOH (1.73 ± 0.044 nm for $n = 1800$, 99% confidence interval, with the standard normal distribution). In addition, EDX measurements confirmed that platinum and sulphur were major elements of the synthesized PtNCs (the presence of sulphur is attributed to the lipoic acid used in the synthesis).

Concerning the NaOH volume employed in the synthesis, Fig. 2 also collects the fluorescence of the purified PtNCs synthesized with 50 μL of 2 M NaOH. Fluorescence signals were lower than in the case of 30 μL of 2 M NaOH and the PtNCs synthesis yield measured by ICP-MS was 73%. Considering that both fluorescence and synthesis yield were lower than for the synthesis with 30 μL of 2 M NaOH, no further studies under these experimental conditions were carried out.

From the above experiments, the addition of 30 μL of 2 M NaOH to the synthesis precursor mixture was selected. The PtNCs have a crystalline structure, face-centered cubic structure of platinum atoms, as observed by the selected area electron diffraction pattern (Fig. 3b). The knowledge of the crystal structure allowed to calculate the volume occupied by a unit cell ($6.04 \cdot 10^{-2}$ nm³/cell), by considering the mass of a unit cell ($1.30 \cdot 10^{-21}$ g/cell) and the platinum density (21.45 g cm⁻³). Additionally, dividing the average PtNC volume (diameter measured by HR-TEM and assuming spherical shape) by the unit cell volume it was calculated 29 crystal lattices per NC. As a face-centered cubic structure contains four platinum net atoms per unit cell, it can be determined that each PtNC is composed by 116 atoms of platinum on average. This figure is used to transform the platinum molar concentration determined by ICP-MS in the PtNCs purified suspension into PtNCs molar concentration. A value of $6.75 \cdot 10^{-6}$ mol PtNCs L⁻¹ was experimentally obtained. In addition, the number of platinum atoms per NC is of interest to calculate the amplification expected in experiments with antibodies labelled with PtNCs and ICP-MS detection.

The effect of the pH was evaluated with the synthesized PtNCs. As can be observed in Fig. S3a (*Electronic Supplementary Material*), the fluorescence signal at 620 nm of the PtNCs suspension increased when lowering the pH

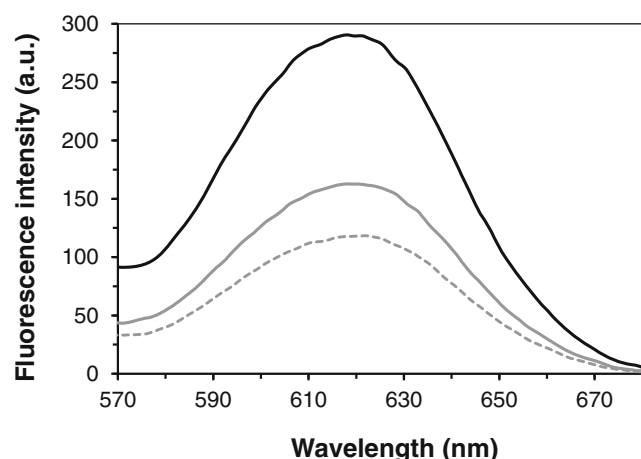


Fig. 2 Effect of 2 M NaOH volume added to the synthesis mixture of precursors on PtNCs fluorescence. Continuous black color: 30 μL NaOH, continuous gray color: 50 μL NaOH, dotted gray color: 20 μL NaOH. For comparison purposes the fluorescence was measured at the same pH for the three syntheses

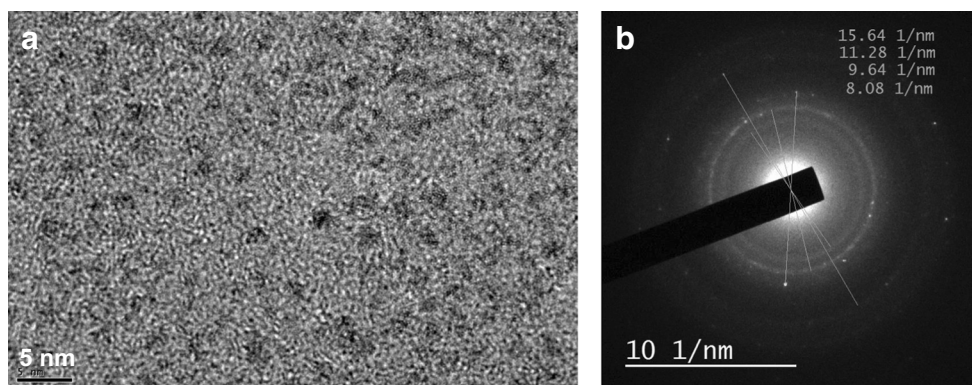


Fig. 3 Structural characterization of PtNCs. (a) HR-TEM image of the PtNCs synthesized under the optimum experimental conditions, and (b) Selected area electron diffraction pattern of a PtNC determined by HR-TEM where the four inner electron diffraction rings from the NC are

distinguished. The rings diameter in nm^{-1} units are collected at the top right of the image. The radius of the rings diffraction (d_{spacing}) confirms the crystal structure of the PtNCs to be compared with the Pt metal d_{spacing} collected in bibliography

from 12.4 up to 3.5. In addition, Z-potential measurements (Fig. S3b in the *Electronic Supplementary Material*) confirmed that PtNCs were stable at pHs higher than 3.5. Finally, stability experiments of PtNCs under different storage conditions and in different media were evaluated. Fig. S4a (*Electronic Supplementary Material*) shows high stability of the fluorescence signal (up to 3 months, which was the maximum period investigated) in ultrapure water and BBS medium. In the case of PBS, sensitivity starts to decrease after 1 day of storage. Fig. S4b (*Electronic Supplementary Material*) collects the time-drive of the PtNCs in ultrapure water during 2 h in the sample compartment of the fluorimeter ($\lambda_{\text{exc}} = 455 \text{ nm}$). No significant changes were observed in the fluorescence emission.

Selection of anti-h IgE:PtNC molar ratio in the bioconjugate

Different Ab:PtNC molar ratios (1:1, 1:10, 1:15, 1:20, 1:30 and 1:50) were assayed in the bioconjugation solution to label the anti-h IgE. To check if the Ab kept its functionality after being labelled with PtNCs, a study was made for the different ratios through a spectrophotometric immunoassay. For such purpose, IgE was immobilized in an ELISA plate and a secondary Ab labelled with horseradish peroxidase was employed for detection. Figure 4a depicts the absorbance at the different Ab:PtNCs molar ratios investigated. The recognition capabilities remained constant for 1:1, 1:10, 1:15 and 1:20 Ab:PtNC molar ratios (these values correspond to ratios added in the solution to label the Ab). However, the 1:30 ratio showed slight lower absorbance and the recognition capabilities were drastically lost for the Ab:PtNC molar ratio of 1:50. Therefore, in order to avoid the blocking of the recognition capabilities of the anti-h IgE due to the labelling process, Ab:PtNC molar ratios up to 1:20 were considered in the solutions to label the Ab.

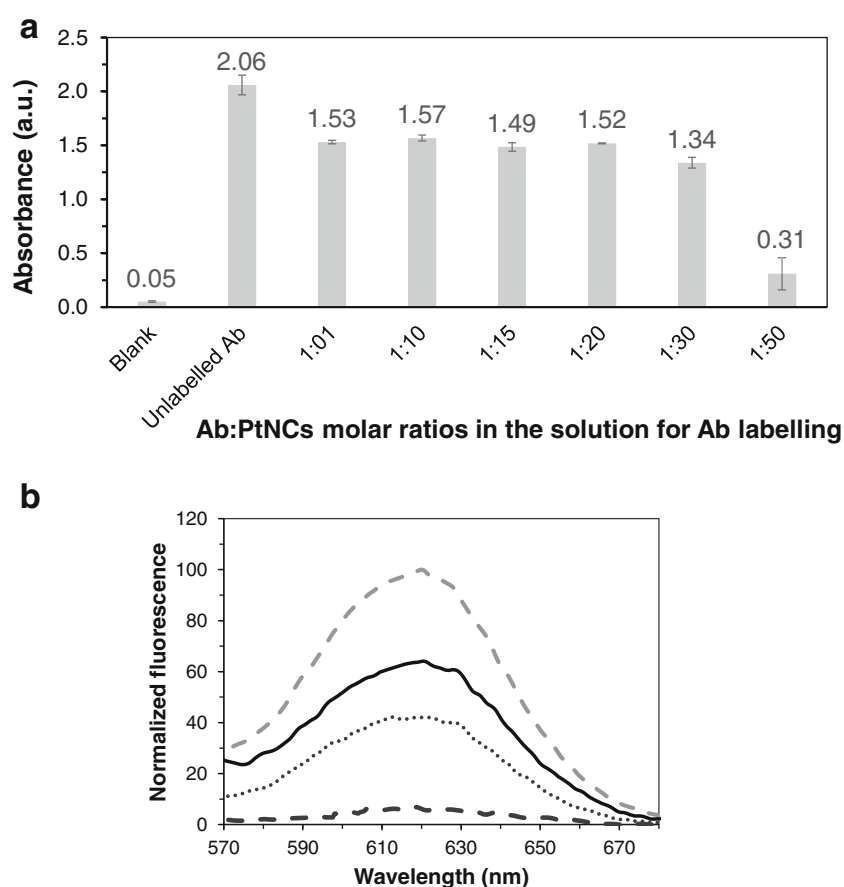
Once the labelling of the Ab with PtNCs was achieved, the real number of PtNCs per Ab was calculated by measuring the platinum concentration (by ICP-MS) in labelled-Ab solutions containing a known amount of Ab. As it is collected in Table 1, labelling yields decreased with higher PtNCs ratios. For the 1:1 initial molar ratio in the labelling solution, not all Ab was labelled (77.6% labelling yield, thus corresponding to a real 1:0.77 Ab:PtNC molar ratio), while for the 1:20 initial ratio the labelling yield decreased up to 55.7%, corresponding to a real 1:11.13 Ab:PtNC molar ratio. Figure 4b collects a comparison of the emission spectra of the bioconjugate containing 1:0.77 (1:1), 1:8.69 (1:15) and 1:11.13 (1:20) Ab:PtNC molar ratio and a PtNCs solution with the same dilution factor. It should be also highlighted that a linear regression was observed when plotting the different real bioconjugate molar ratios (up to 1:11.13 Ab:PtNC) versus measured signals, both for fluorescence at 620 nm and for platinum concentration determined by ICP-MS (data not shown).

Finally, platinum retained in the ELISA well plates (same experiment with data presented in Fig. 4a) was measured by ICP-MS. It is worth to highlight that a linear relationship was obtained by plotting the detected platinum mass (in ng) versus the real Ab:PtNCs molar ratio (Fig. S5 in *Electronic Supplementary Material*).

Immunoassays for IgE determination with fluorescence and ICP-MS detection

The determination of IgE using the immunoprobe containing PtNCs as label (with a Ab:PtNCs real molar ratio of 1:11.13) was carried out with a competitive immunoassay. Figure 5a shows the inhibition curve by fluorescence detection following the optimized protocol detailed in the *Experimental section* (i.e. immunoassay with fluorescent detection using PtNCs as label) for IgE standards. The calibration plot was fitted with a four-parameter equation with MyAssays Ltd. and different inhibitory concentration (IC) values were extracted

Fig. 4 PtNCs bioconjugation optimization. (a) Absorbance measured in ELISA well plates for different Ab:PtNC molar ratios employed in the reaction solution for Ab labelling. Uncertainties represent the standard deviations of the mean of three independent measurements, and (b) Comparison of emission spectra for PtNCs (dotted gray line) and Ab labelled with PtNCs using different Ab:PtNC molar ratios, 1:20 (black line), 1:15 (tinny dotted line) and 1:1 (dotted black line), in the bioconjugation process (same pH and same PtNCs dilution in the spectra)



from the graph. The limit of detection (LOD), corresponding to IC_{10} , was 0.6 ng mL^{-1} of IgE, while the linear range (IC_{20} – IC_{80}) extended from 1.6 ng mL^{-1} up to 60 ng mL^{-1} of IgE. It is interesting to note that this LOD is slightly worse than the LOD previously reported using AuNCs as fluorescent labels (0.2 ng mL^{-1} of IgE) [30].

The inhibition curve by ICP-MS detection is collected in Fig. 5b. The calibration plot was fitted, as above, using a four-parameter equation. The LOD (IC_{10}) in this case is 0.08 ng mL^{-1} of IgE, which is lower than by fluorescent measurements with the same PtNCs immunoprobe. Such LOD is lower than the LOD of 0.2 ng mL^{-1} for IgE with fluorescent detection using an immunoprobe labelled with AuNCs [30]. Also, it compares favorably with most recently published methods for IgE determination in human serum (see Table 2) [30–38]. With ICP-MS detection of platinum, the linear range (IC_{20} – IC_{80}) extended from 0.10 ng mL^{-1} up to 2.6 ng mL^{-1} of IgE. In addition, it is interesting to note that for

this linear interval we have observed that the plot of IgE molarity (x axis) used for calibration versus the detected platinum molarity (y axis) follows a linear trend, with a slope of 1264 units. This slope is in agreement with the signal amplification expected (the immunoprobe contains an average of 11.13 PtNCs per Ab and each NC is composed of 116 atoms of Pt on average).

Finally, considering the excellent LOD with ICP-MS, the immunoprobe labelled with PtNCs was investigated for the determination of IgE in human serum samples by ICP-MS. To evaluate the effect of the serum matrix, concentrations of $1 \cdot 10^{-4}$, $1.5 \cdot 10^{-4}$, $1.5 \cdot 10^{-3}$ and $2 \cdot 10^{-3} \text{ } \mu\text{g mL}^{-1}$ of IgE were added to a commercial serum diluted 1:1000 and results were introduced (grey marks) into the inhibition curve (Fig. 5b). As can be observed, the spiked samples fit into the calibration plots, demonstrating that there are no matrix effects and it is possible to quantify IgE in diluted serum samples through calibration with PBS. Next, determination of IgE in four

Table 1 Labelling yield of the Ab in the bioconjugation process determined by ICP-MS

Ab:PtNC molar ratio in the solution for Ab labelling	1:1	1:10	1:15	1:20
Labelling yield, % (ICP-MS measurements)	77.60%	62.41%	57.92%	55.67%
Measured (“real”) Ab:PtNC molar ratio in the labelled Ab	1:0.77	1:6.24	1:8.69	1:11.13

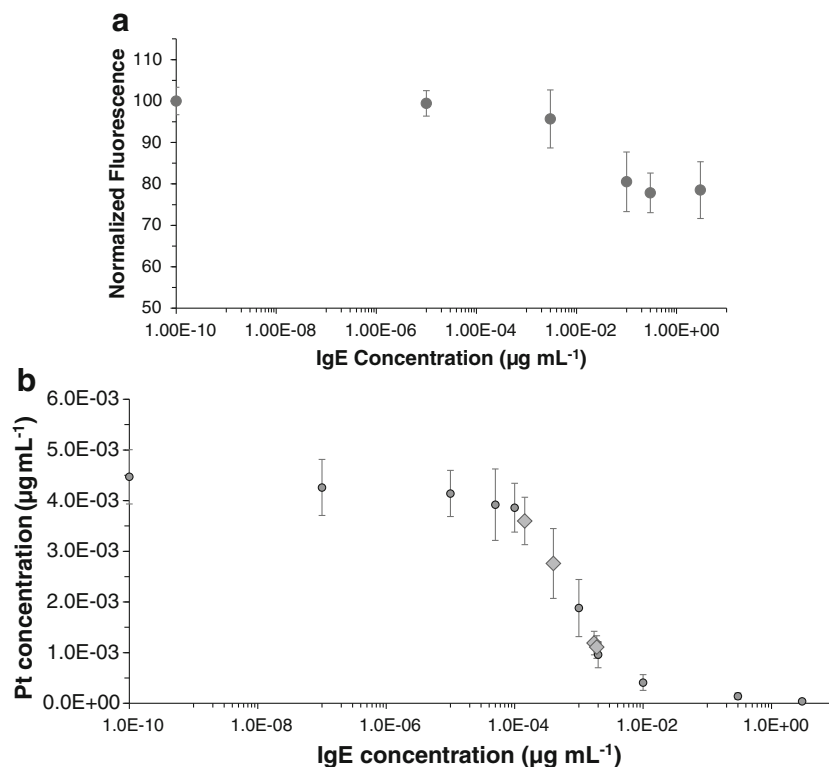


Fig. 5 Calibration curves for IgE determination by competitive format, using the IgE immunoprobe containing the molar ratio 1:11.13 Ab:PtNCs. (a) Calibration plot by fluorescence detection with laser confocal microscopy ($\lambda_{\text{ex}} = 470$ nm, range $\lambda_{\text{em}} = 610\text{--}650$ nm). The y-axis corresponds to the normalized integrated density signal fluorescence from the PtNCs in each well (integrated by ImageJ program). Uncertainties represent the standard deviations of the mean of three independent measurements. (b) Calibration plot by ICP-MS (^{195}Pt ion signal intensity was

measured). The y-axis corresponds to the concentration of Pt, calculated by an external calibration curve with internal standard (^{193}Ir). The black marks belong to the IgE protein standards in PBS solution for building the calibration curve and the circle marks correspond to the enriched sera (with different concentrations of IgE standard). Uncertainties represent the standard deviations of the mean of three independent measurements"

Table 2 Overview of recently reported nanomaterial and/or based optical sensitive methods for determination of Ig E

	Materials used	Method	LOD (ng mL^{-1})	Ref.
Commercial kits	–	Classical ELISA absorbance (ABNOVA).	12	–
	–	Microarray chip ImmunoCAP ISAC (Thermo Fisher)	0.24	–
Published methods	Superparamagnetic Fe_3O_4 NPs	Magnetophoretic immunoassay	0.1	[31]
	Tosyl-activated superparamagnetic beads	Immunoaffinity capillary electrophoresis. Fluorescence detection	2.4	[32]
	Chemiluminescence reagent acts as the signaling molecule and polystyrene beads as the amplification platform	Aptamer-barcode enhanced chemiluminescence immunosensor	0.87	[33]
	Poly(dimethylsiloxane) microfluidic electrokinetic concentration chip	Aptamer affinity probe capillary electrophoresis. Fluorescence detection	7	[34]
	Bioconjugated AuNPs	Lateral flow microarrays	2.4	[35]
	CdS nanospheres and AuNPs	Electrochemiluminescence aptasensor	0.02	[36]
	Aptamer modified with AgNPs	Visual microarray	20	[37]
	Polystyrene and super-paramagnetic particle complexes	Microparticle-based competitive immunoassay and visible light absorption	24	[38]
	AuNCs	Competitive fluorimmunoassay	0.20	[30]
	PtNCs	Competitive immunoassay (fluorescence and ICP-MS)	0.60 (fluorescence) & 0.08 (ICP-MS)	This work

human serum samples was carried out with the immunoprobe labelled with PtNCs and results are collected in Table S1 (*Electronic Supplementary Material*). The applicability was proved through the successful comparison of the IgE concentrations with this methodology and those with a commercial kit (results are also collected in Table S1).

Conclusions

Fluorescent PtNCs were synthesized and studied in terms of yield, size dispersity, platinum atoms per NC and PtNCs concentration. These PtNCs were used to label an anti-h IgE antibody as a proof of concept. Results showed that the immunoprobe selected as optimum contains an average of 11.13 PtNCs per Ab, which corresponds to about 1300 Pt atoms per Ab. Further studies to improve the size dispersity will be appropriate in order to use them in applications such as single-cell proteins detection.

The bimodal detection capabilities (fluorescence and ICP-MS) of PtNCs were investigated and compared through a competitive immunoassay for sensitive detection of IgE. The huge amplification with ICP-MS detection gave rise to a better LOD as compared to the fluoroimmunoassay. In any case, it should be kept in mind that fluorescence measurements are cheaper than those performed by ICP-MS. Therefore, the fluoroimmunoassay can be selected when sensitivity requirements allow it. In contrast, the low LOD by ICP-MS points out to the use of this detection system for those analyses requiring high sensitivity or for small sample volumes. In addition, it should be noted that in our experiments we have detected ^{195}Pt which has a 33.775% isotopic abundance. Therefore, the LOD by ICP-MS should be improved with isotopically-enriched PtNCs.

Finally, it is worth to highlight that the combination of the PtNCs immunoprobe with ICP-MS detection opens the door to multiplexed protein analysis by using isotopically-enriched NCs. In the particular case of platinum, it is possible to obtain six different immunoprobes (^{190}Pt , ^{192}Pt , ^{194}Pt , ^{195}Pt , ^{196}Pt , and ^{198}Pt) with the same synthesis protocol, thus increasing both the multiplexing capabilities and the sensitivity of the analysis. In addition, future research related to bioimaging studies by laser ablation coupled to ICP-MS using PtNCs labels should be expected.

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Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

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Electronic Supporting Material on the Microchimica

Acta publication entitled:

**Bimodal determination of immunoglobulin E by
fluorometry and ICP-MS by using platinum
nanoclusters as a label in an immunoassay**

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Results and discussion

Synthesis of PtNCs using lipoic acid as ligand

The measurement of the absorbance of the reagents used for the synthesis and the PtNCs solution (after 15 hours under stirring) shows the disappearance of the absorbance peaks corresponding to the isolated reactants when the PtNCs are formed (Fig S1).

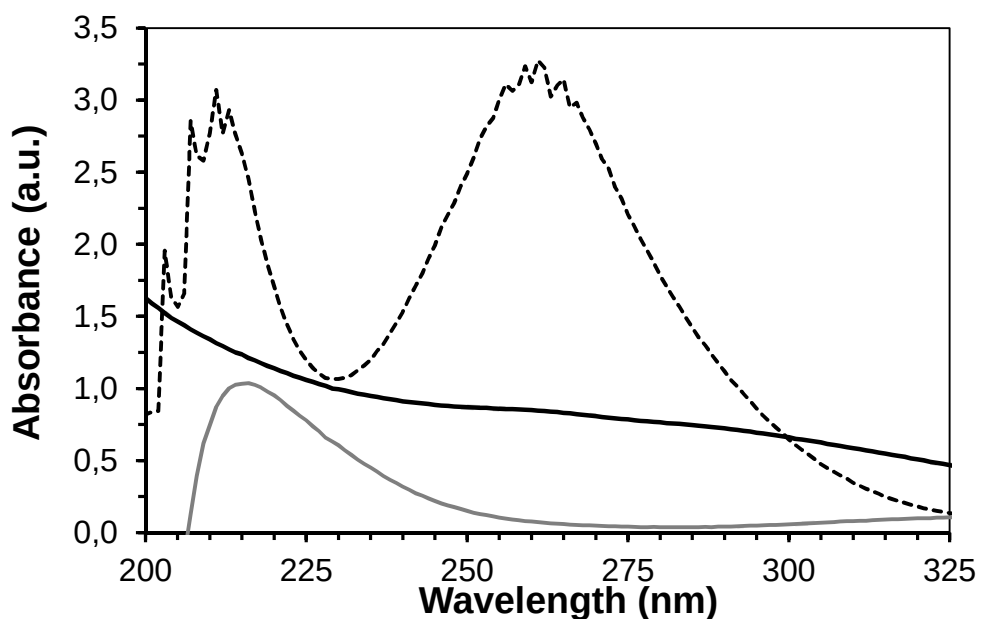


Fig. S1. Absorbance spectra of the reagents used in the synthesis and the synthesized PtNCs (continuous black line: PtNCs, dotted black line: H_2PtCl_6 , continuous gray line: lipoic acid).

In order to evaluate the time necessary to complete the synthesis, aliquots from the same synthesis were measured by fluorescence (at the maximum emission peak at 620 nm). Results showed maximum fluorescence after 13 hours under stirring (Fig. S2).

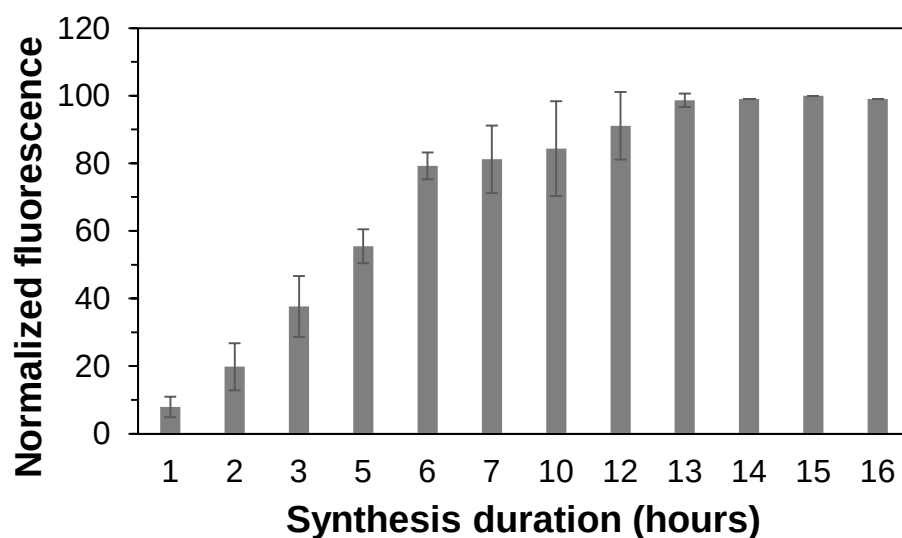


Fig. S2. Effect of synthesis time on the fluorescence signal for a Pt:lipoic acid:NaBH₄ molar ratio of 1:5:1 (normalized intensities; given 100 value to the maximum fluorescence signal obtained for 13 h and above). Uncertainties represent the standard deviations of the mean of three different synthesis measurements.

Moreover, the effect of pH on the fluorescence and stability of NCs was evaluated. For such purpose, aliquots from the same synthesis were adjusted at different pHs and measured by fluorescence (Fig. S3a) and Z-potential (Fig. S3b). As can be seen in Fig. S3b, Z-potential confirmed that the PtNCs are stable for pHs higher than 3.

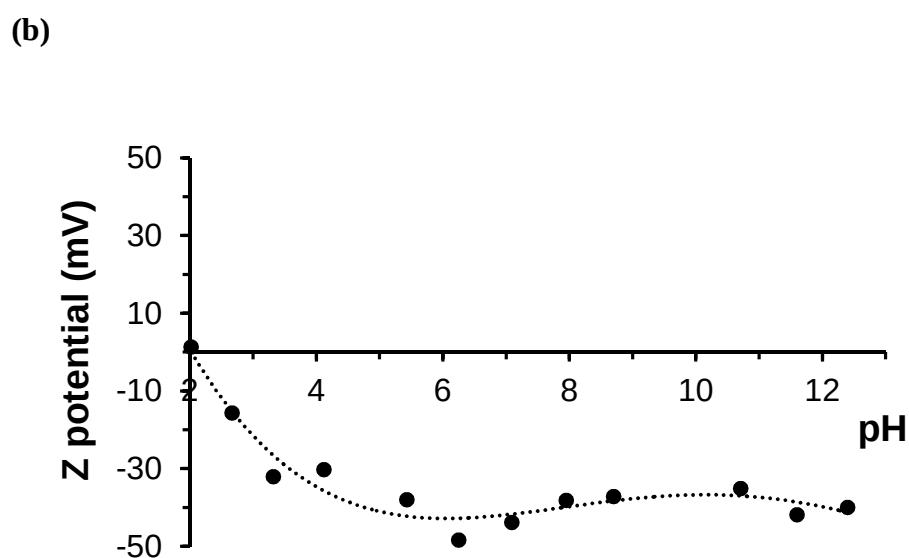
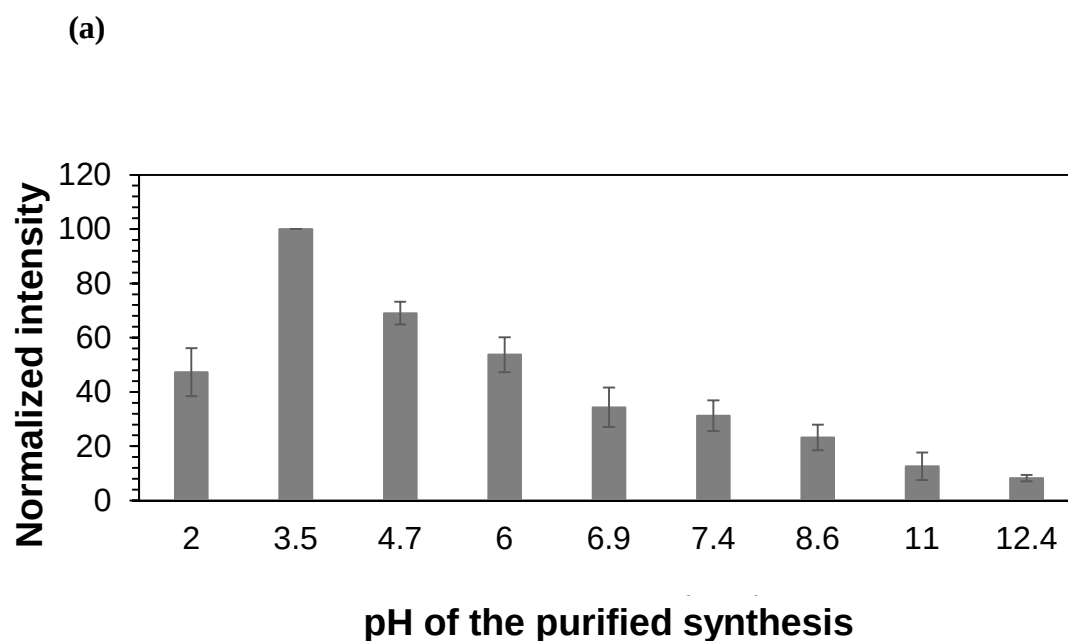
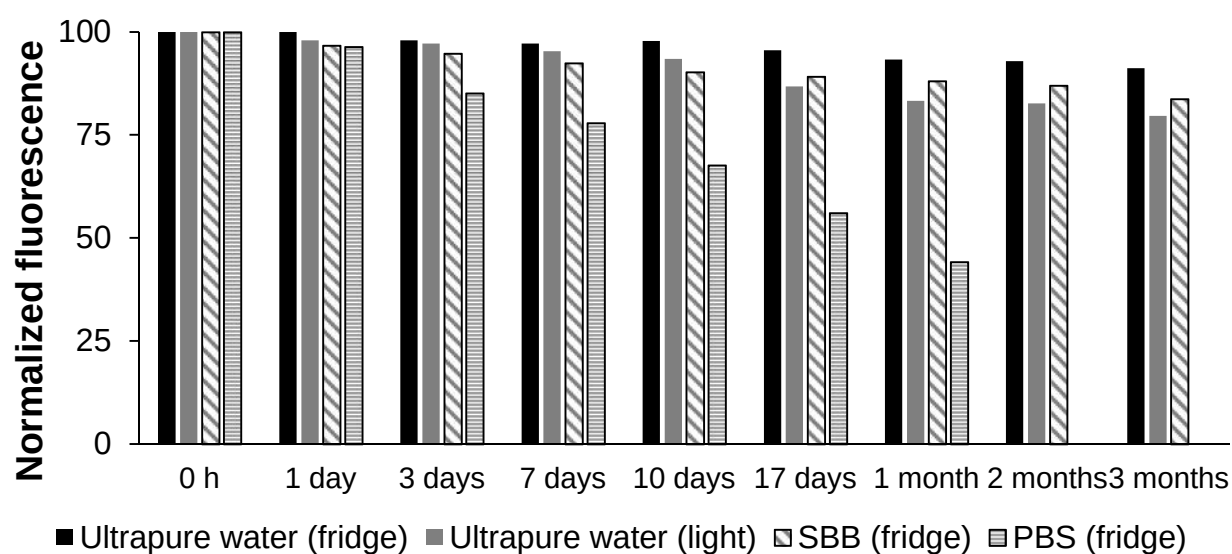


Fig. S3. Effect of pH in the suspension of purified PtNCs.. (a) Fluorescence signal at 620 nm. Uncertainties represent the standard deviations of the mean of three different synthesis measurements (b) Z-potential.

In order to evaluate the stability of synthesized PtNCs in ultrapure water and two different buffers (BBS and PBS), emission of PtNCs was measured at different storage conditions (into the fridge and ambient light). As can be observed in Fig. S4a,

fluorescence emission kept constant when PtNCs were in water and no differences were found between storage in cold/dark and ambient conditions. Additionally, while fluorescence emission of the purified PtNCs in BBS buffer was constant, PtNCs in PBS were less stable. Furthermore, the stability in the fluorimeter, exciting at 455 nm, was evaluated and the fluorescence stayed during at least 2 hours, as can be observed in Fig. S4b.

(a)



(b)

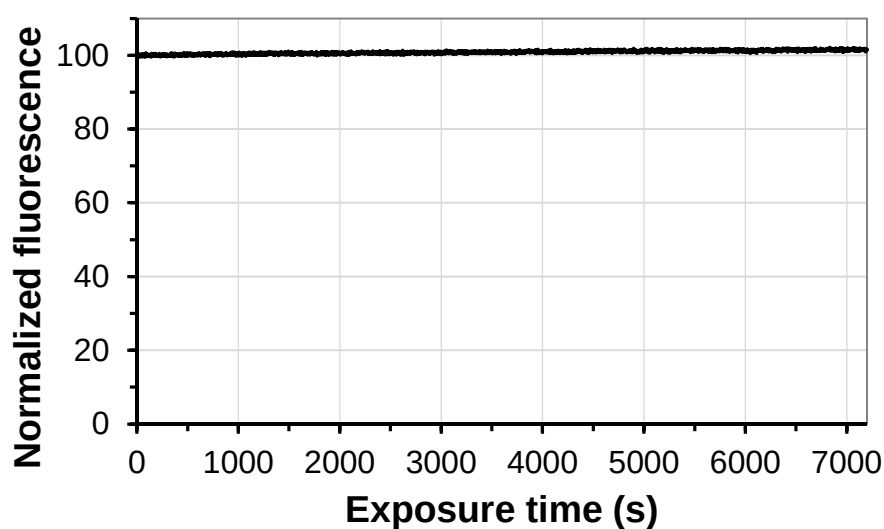


Fig. S4. Stability of the fluorescence of synthesized PtNCs. (a) Normalized intensity of PtNCs in different media and storage conditions (100 value was given to the maximum

fluorescence signal in each corresponding medium at the initial moment of measurement). b) Time-drive in the fluorimeter with $\lambda_{\text{exc}}=455$ nm and the PtNCs in ultrapure water.

Selection of anti-h IgE:PtNC molar ratio in the bioconjugate

In order to corroborate the relation between the quantities of platinum measured per well (after the noncompetitive immunoassay was carried out) and the real molar ratios of PtNCs labelled, the plot collected in Fig. S5 was drawn. As can be seen, the increasing amount of platinum found in each well is directly proportional to the increasing molar ratio.

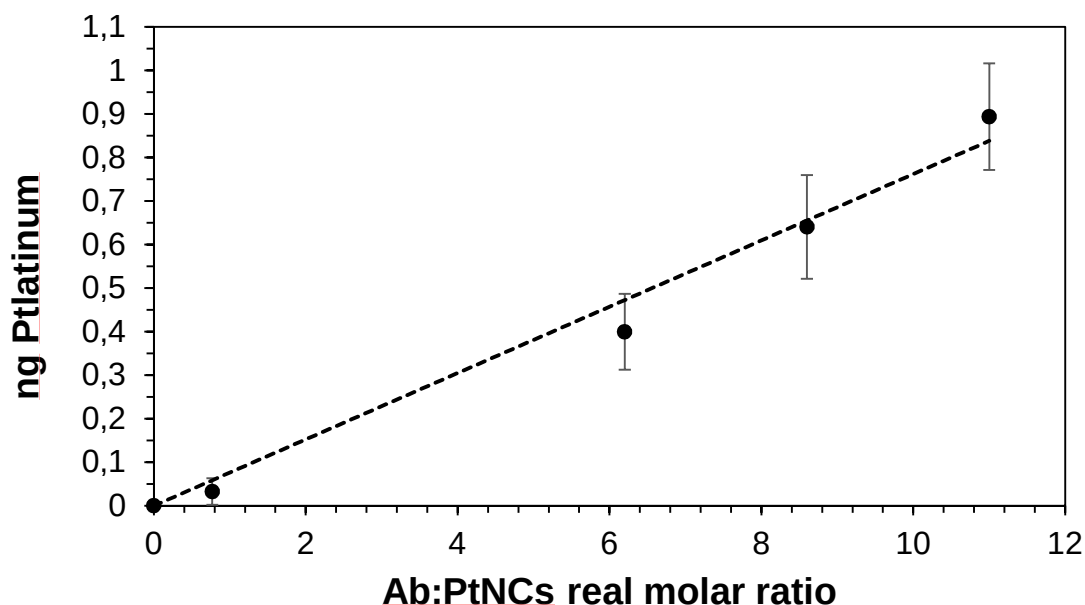


Fig. S5. Plot collecting the relationship between the real Ab:PtNCs ratio and the platinum mass detected by ICP-MS in the ELISA wells of the experiments collected in Fig. 4a. Uncertainties represent the standard deviations of the mean of three independent measurements.

Immunoassays for IgE determination with fluorescence and ICP-MS detection

The elemental detection of platinum by ICP-MS as mark to quantify the IgE in serum was selected because of its higher sensitivity. Comparable results for the quantification of IgE in the serum samples with the validation kit were obtained, as it is collected in Table S1.

Table S1. Determination of IgE in four serum samples by the PtNCs immunoassay using ICP-MS detection and comparison with concentrations found with a commercial ELISA kit. In both cases uncertainties represent the standard deviations of the mean of three independent measurements.

Sample	[IgE] by PtNCs immunoassay and ICP-MS detection ($\mu\text{g mL}^{-1}$)	[IgE] by commercial ELISA kit ($\mu\text{g mL}^{-1}$)
Commercial serum	0.285 ± 0.030	0.280 ± 0.025
Serum 1	0.214 ± 0.012	0.226 ± 0.020
Serum 2	0.0914 ± 0.013	0.0893 ± 0.012
Serum 3	0.0520 ± 0.0091	0.0536 ± 0.0099

Primary Prevention of Cow's Milk Sensitization and Food Allergy by Avoiding Supplementation With Cow's Milk Formula at Birth

A Randomized Clinical Trial

Mitsuyoshi Urashima, MD, MPH, PhD; Hidetoshi Mezawa, MD; Mai Okuyama, MD; Takashi Urashima, MD; Daishi Hirano, MD; Noriko Gocho, MD; Hiroshi Tachimoto, MD

IMPORTANCE Cow's milk formula (CMF) is used to supplement breastfeeding (BF) at birth without clear clinical evidence to support the practice.

OBJECTIVE To determine whether avoiding supplementation with CMF at birth can decrease risks of sensitization to cow's milk protein and/or clinical food allergy, including cow's milk allergy (CMA), overall and in subgroups stratified by 25-hydroxyvitamin D (25[OH]D) levels.

DESIGN, SETTING, AND PARTICIPANTS The Atopy Induced by Breastfeeding or Cow's Milk Formula (ABC) trial, a randomized, nonblinded clinical trial, began enrollment October 1, 2013, and completed follow-up May 31, 2018, at a single university hospital in Japan. Participants included 330 newborns at risk for atopy; of these, 312 were included in the analysis. Data were analyzed from September 1 through October 31, 2018.

INTERVENTIONS Immediately after birth, newborns were randomized (1:1 ratio) to BF with or without amino acid-based elemental formula (EF) for at least the first 3 days of life (BF/EF group) or BF supplemented with CMF (≥ 5 mL/d) from the first day of life to 5 months of age (BF plus CMF group).

MAIN OUTCOMES AND MEASURES The primary outcome was sensitization to cow's milk (IgE level, ≥ 0.35 allergen units [U_A]/mL) at the infant's second birthday. Secondary outcomes were immediate and anaphylactic types of food allergy, including CMA, diagnosed by oral food challenge test or triggered by food ingestion, with food-specific IgE levels of at least $0.35 U_A$ /mL. Subgroup analysis was prespecified by tertiles of serum 25(OH)D levels at 5 months of age.

RESULTS Of the 312 infants included in the analysis (160 female [51.3%] and 152 male [48.7%]), 151 of 156 (96.8%) in the BF/EF and BF plus CMF groups were followed up until their second birthday. The primary outcome occurred in 24 infants (16.8%) in the BF/EF group, which was significantly fewer than the 46 infants (32.2%) in the BF plus CMF group (relative risk [RR], 0.52; 95% CI, 0.34-0.81). The middle tertile of the 25(OH)D subgroup, but not the low and high tertiles, had a significant interaction with the intervention (RR, 0.19; 95% CI, 0.07-0.50; $P = .02$). The prevalence of food allergy at the second birthday was significantly lower in the BF/EF than in the BF plus CMF groups for immediate (4 [2.6%] vs 20 [13.2%]; RR, 0.20; 95% CI, 0.07-0.57) and anaphylactic (1 [0.7%] vs 13 [8.6%]; RR, 0.08; 95% CI, 0.01-0.58) types.

CONCLUSIONS AND RELEVANCE The evidence suggests that sensitization to cow's milk and food allergy, including CMA and anaphylaxis, are primarily preventable by avoiding CMF supplementation for at least the first 3 days of life.

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Food allergy mediated by IgE is becoming a global concern because its prevalence and severity are worsening.¹⁻³ To overcome this issue, prolonged exclusive breastfeeding (BF) with or without supplementation with hypoallergenic formulas for infants at risk has been recommended.⁴ However, these preventive strategies have recently been called into question.⁵ For example, a cohort study⁶ demonstrated that the frequency of IgE-mediated cow's milk allergy (CMA) was lower in infants who began receiving regular cow's milk formula (CMF) within the first 14 days of life and therefore recommended CMF supplementation at birth. However, the effects of supplementing BF with CMF at birth or within the first 3 days of life on the risk of CMA^{7,8} and atopy⁹⁻¹¹ are still controversial.

Many Japanese maternity wards encourage BF but allow mothers or nurses to supplement BF with CMF—for example, approximately 6 to 10 hours after birth or even earlier—based on maternal preferences but not clinical evidence. However, more than 20 to 30 years ago, sugar water was given instead of CMF supplement at birth. Thus, we hypothesized that early exposure to CMF at birth is, at least in part, associated with the recent increase in children with food allergy. Therefore, a randomized clinical trial (RCT), Atopy Induced by Breastfeeding or Cow's Milk Formula (ABC) trial, was conducted to assess whether the risk of CMF sensitization and food allergy is decreased by avoiding or supplementing with CMF at birth.

Excessive and/or deficient levels of vitamin D during the neonatal and infantile periods were suggested to raise the risk of food allergy.¹²⁻¹⁵ Although vitamin D supplementation for lactating mothers or infants is not common in Japan, a previous RCT¹⁶ demonstrated that food allergy was increased in the offspring of mothers supplemented with vitamin D compared to placebo. Moreover, no consensus exists about cutoff points for low, middle, and high levels of 25-hydroxyvitamin D (25[OH]D). Thus, subgroups stratified by tertiles of serum 25(OH)D levels, a biomarker of vitamin D status, were pre-specified for exploratory analyses.

Methods

Trial Design

At the departments of obstetrics and pediatrics of Jikei University Hospital, Tokyo, Japan, newborns were randomly assigned immediately after birth to avoiding supplementation with CMF for at least the first 3 days of life (ie, BF with or without an amino acid-based elemental formula [EF; BF/EF group]) or adhering to BF supplemented with CMF (≥ 5 mL/d) from the first day of life (BF plus CMF group) and then followed up to their second birthdays. The trial protocol was approved by the ethics committee of the Jikei University School of Medicine, and parents of infants provided written informed consent. This trial used an external data and safety monitoring committee. The full protocol for this trial is available in [Supplement 1](#). This study followed the Consolidated Standards of Reporting Trials (CONSORT) reporting guideline for RCTs.

Key Points

Question At birth, are the risks of sensitization to cow's milk formula and food allergy decreased by avoiding or by supplementing with cow's milk formula?

Findings In this randomized clinical trial involving 312 newborns, risks of sensitization to cow's milk and immediate-type food allergy, including cow's milk allergy and anaphylaxis, were decreased by avoiding supplementation with cow's milk formula for at least the first 3 days of life.

Meaning Results suggest that sensitization to cow's milk and clinical food allergies may be preventable by avoiding cow's milk formula supplementation at birth, which is easily and immediately applicable to clinical practice worldwide without the cost and time of therapy.

Participants

The inclusion criterion was an infant at risk for atopy, with at least 1 of the father, mother, or siblings having current and/or past atopic diseases (eg, asthma). The exclusion criteria consisted of parents who intended exclusive BF or exclusive CMF before birth or infants who were born at less than 36 weeks' gestational age, had a birth weight less than 2000 g, or had serious congenital anomalies (eg, cleft palate). Pregnant women and their husbands were informed about the trial at childbirth classes during the third trimester of pregnancy.

Randomization and Blinding

The computer-generated and centrally administered randomization used permuted blocks of 4. Participants were randomized in a 1:1 ratio without stratification. Owing to the nature of the interventions, the participants were not blinded. However, levels of cow's milk-specific IgE (CM-IgE) as the primary outcome and other food-specific IgEs were measured by outsourcing to a clinical laboratory testing company (SRL, Inc; Tokyo, Japan), and parts of food allergy as the secondary outcomes were diagnosed by an oral food challenge (OFC) test administered by pediatricians who were blinded to the allocation group. The secondary outcomes were finally determined by the end point committee, who were blinded to the intervention arms.

Intervention

At the maternity ward of Jikei University Hospital, participating mothers were informed of their allocation group. Mothers allocated to the BF/EF group were asked to feed their offspring by BF and continue to the first blood test at 5 months of age, but they were allowed to add amino acid-based EF when they believed that amounts of BF were not enough. If the mother added more than 150 mL/d of EF to BF for 3 consecutive days, EF was switched to CMF after the fourth day. Thus, offspring allocated to BF/EF could avoid CMF for at least the first 3 days of life. On the other hand, mothers allocated to the BF plus CMF group were asked to supplement BF with at least 5 mL of CMF from the first day or within the first 24 hours after delivery and with at least 40 mL/d after 1 month and to continue to the first blood test at 5 months of age or before start-

ing solid food. Both CMF (Hohoemi) and EF (Meiji Elemental formula) were purchased from Meiji Holdings Company, Ltd.

Outcomes

The primary outcome was atopic sensitization to cow's milk protein (ie, serum levels of CM-IgE of ≥ 0.35 allergen units [U_A]/mL at 24 months of age). Serum levels of CM-IgE at 5 and 24 months of age as a continuous variable were used as reference values for the primary outcome. Other atopic sensitizations to egg white, wheat, and mites at levels of at least 0.35 U_A/mL at 24 months of age were also evaluated to be compared with cow's milk sensitization, but not for the primary outcome.

In this trial, the secondary outcomes were clinical food allergies, defined as immediate and anaphylactic types. Immediate types of food allergy presented with at least 1 organ symptom of the skin (eg, urticaria), respiratory tract (eg, wheezing), gastrointestinal tract (eg, vomiting), or circulatory system (eg, loss of consciousness) triggered by an OFC test conducted by pediatricians trained to administer it (eTable in Supplement 2) or by food ingestion in daily life that appeared within minutes to a few hours, in combination with serum levels of the suspected food-specific IgE level (≥ 0.35 U_A/mL). The anaphylactic type of food allergy presented when symptoms were derived from at least 2 organ systems (eg, urticaria and wheezing). These secondary outcomes were evaluated by cumulative incidence if an infant even once experienced an immediate allergic reaction with or without outgrowing it by the second birthday and by prevalence at the second birthday without outgrowing it. Atopic conditions other than food allergy were planned to report separately. Safety outcomes were admission or emergency department visit for any reason and growth retardation (height and weight) at the second birthday. Serum levels of 25(OH)D were measured at 5 months using radioimmunoassay (SRL, Inc), as described.¹⁷

Follow-up

Participating infants were examined at 1, 2, 3, 4, 5, 7 to 8, 9 to 10, 12, 14 to 15, 18, and 24 months of age at the outpatient clinic of Jikei University Hospital. All participants underwent evaluation of blood samples obtained at 5 and 24 months of age, by which total IgE and antigen-specific IgE levels, cow's milk, egg white, wheat, mites, Japanese cedar pollen, dog dandruff, and cat dandruff were measured with specific IgE additive allergens (ImmunoCAP; Thermo Fisher Diagnostics KK) at SRL, Inc. Unscheduled visits were made to Jikei University Hospital, and blood tests were performed when a participating infant showed the immediate type of food allergy (eg, with immediate reaction to walnuts, walnut-specific IgE was measured by blood sampling at the unscheduled visit).

For infants who had food antigen-specific IgE-positive findings (≥ 0.35 U_A/mL) at 5 months of age, the OFC test was performed at approximately 1 year of age at Jikei University Hospital. After blood examination at 5 months of age, if the infants had already taken the allergenic food without clinical allergic signs or, conversely, the infants had already showed a strong allergic or anaphylactic reaction to the allergenic food, the OFC test was not performed.

Sample Size

The primary outcome, CM-IgE level of at least 0.35 U_A/mL at 24 months of age, would be found in 10% in one group and 25% in the other group with a 2-sided type I error of 5%, a power of 90%, and an assumption of 3% loss to follow-up. We estimated that 300 participants with a ratio of 1:1 would be sufficient to detect this difference. Assuming 10 participants per month could participate in this trial, the accrual period was estimated to be 2.5 years to enroll 300 participants. With final participant follow-up 2 years after enrollment, the total duration of the planned trial was 4.5 years.

Statistical Analysis

Data were analyzed from September 1 through October 31, 2018. All participants who underwent randomization were included in this analysis. Outcomes were assessed according to the randomization group even in cases that did not adhere to the intervention. Effects of the intervention, such as avoiding supplementation with CMF at birth, on the risk of outcomes were estimated using risk ratios (RRs) and 95% CIs. Levels of CM-IgE as a continuous variable were compared using the Mann-Whitney test. Subgroups were prespecified according to 25(OH)D levels sampled at 5 months of age into low, middle, and high tertiles. To clarify whether the intervention significantly affected these subgroups, *P* values for interaction were analyzed using the Mantel-Haenszel test. These analyses were not corrected for multiple comparisons. However, because of the potential for type I error due to multiple comparisons, findings for subgroup analyses should be interpreted as explanatory.

All data were analyzed using Stata, version 14.0 (Stata Corp). Interim analyses were planned twice at reaching 100 and 200 participating infants who had their second birthday. Statistical significance at the interim analysis was set at 2-sided *P* < .001 according to Peto group stopping boundaries.¹⁸

Post Hoc Analysis

Infants in the BF/EF group were further divided into the following 3 subgroups: (1) continued regimen at 5 months of age; (2) switched from EF to CMF on day 15 and after; and (3) switched from EF to CMF on day 14 or before. Levels of CM-IgE were then compared among these 3 groups and infants in the BF plus CMF group (ie, total of 4 groups).

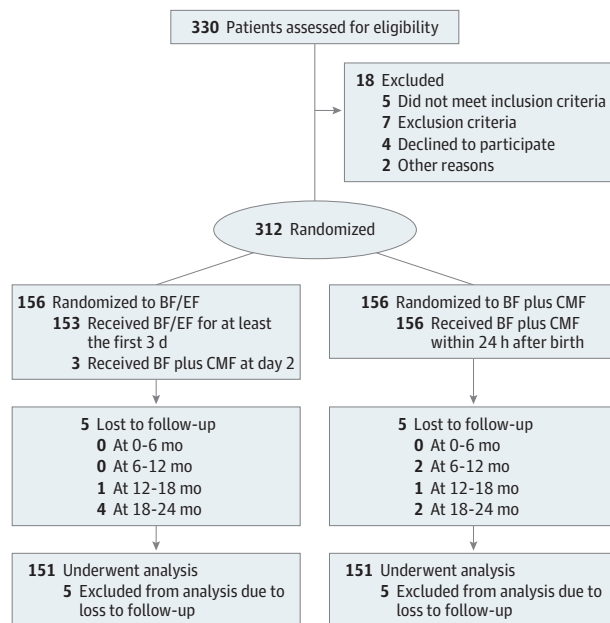
Results

Study Population

Enrollment began October 1, 2013, and follow-up was completed May 31, 2018. After excluding 18 families according to the inclusion and exclusion criteria, 312 of 330 infants (94.5%) (160 female [51.3%] and 152 male [48.7%]) were randomized to the BF/EF (*n* = 156) or the BF plus CMF (*n* = 156) groups at birth (Figure 1). At the second birthday, 151 infants (96.8%) in the BF/EF group and 151 (96.8%) in the BF plus CMF group could be followed up and were included in the analysis.

The participants' characteristics were similar between the 2 groups (Table 1). Because most participating mothers who

Figure 1. Patient Flow Through the Atopy Induced by Breastfeeding or Cow's Milk Formula (ABC) Trial



BF indicates breastfeeding; CMF, cow's milk formula; and EF, elemental formula.

attended the childbirth class were first-time parents, most of the participating infants did not have older sibling(s).

Effects of Avoiding CMF at Birth on Sensitization

To examine atopic sensitization, blood samples were available in 309 of 312 infants (99.0%) at 5 months and in 286 of 302 infants (94.7%) at 24 months of age. Participating infants were divided into the following prespecified subgroups according to tertiles of serum levels of 25(OH)D at 5 months of age: low (6-20 ng/mL [$n = 102$]), middle (21-36 ng/mL [$n = 101$]), and high (37-83 ng/mL [$n = 100$]) (to convert 25(OH)D to nanomoles per liter, multiply by 2.496).

Effects of avoiding CMF at birth on sensitization, reported as food antigen-specific IgE level of at least 0.35 U_A/mL at 24 months of age, are given in Table 2. Significantly fewer infants had the primary outcome, cow's milk sensitization, in the BF/EF group (24 of 143 [16.8%]) than in the BF plus CMF group (46 of 143 [32.2%]; RR, 0.52; 95% CI, 0.34-0.81). In the subgroup of middle 25(OH)D levels, cow's milk sensitization was significantly less frequent in the BF/EF group than in the BF plus CMF group (4 of 44 [9.1%] vs 22 of 45 [48.9%]; RR, 0.19; 95% CI, 0.07-0.50), but it was not significant in the low- or the high-level subgroup. The interaction between the intervention and middle tertile of 25(OH)D levels was significant ($P = .02$ for interaction).

In contrast, the number of infants with sensitization to egg white or other food antigens was not significantly different between the 2 comparative groups. However, in the subgroup of middle 25(OH)D levels, egg white sensitization was significantly lower in the BF/EF group than in the BF plus CMF group (RR, 0.51; 95% CI, 0.28-0.92), but it was not significant in the

Table 1. Participant Characteristics

Characteristic	Study Group	
	BF/EF ($n = 156$)	BF Plus CMF ($n = 156$)
Familial Background		
Age, mean (SD), y		
Paternal	37.0 (6.1)	37.7 (6.8)
Maternal	35.0 (4.5)	35.3 (4.3)
Current/previous atopic disease of mother, No. (%)		
Bronchial asthma	9 (5.8)/24 (15.4)	6 (3.8)/20 (12.8)
Atopic dermatitis	18 (11.5)/32 (20.5)	13 (8.3)/32 (20.5)
Food allergy	28 (17.9)/18 (11.5)	15 (9.6)/15 (9.6)
Allergic rhinitis	36 (23.1)/17 (10.9)	40 (25.6)/13 (8.3)
Pollen allergy	85 (54.5)/12 (7.7)	89 (57.1)/11 (7.1)
Current/previous atopic disease of father, No. (%)		
Bronchial asthma	4 (2.6)/22 (14.1)	11 (7.1)/24 (15.4)
Atopic dermatitis	7 (4.5)/14 (9.0)	13 (8.3)/25 (16.0)
Food allergy	20 (12.8)/4 (2.6)	17 (10.9)/9 (5.8)
Allergic rhinitis	37 (23.7)/11 (7.1)	41 (26.3)/17 (10.9)
Pollen allergy	80 (51.3)/11 (7.1)	79 (50.6)/3 (1.9)
Current/previous atopic disease of siblings, No. (%)^a		
Bronchial asthma	2 (66.7)/1 (33.6)	2 (22.2)/1 (11.1)
Atopic dermatitis	0/0	3 (33.3)/0
Food allergy	1 (33.3)/2 (66.7)	5 (55.6)/1 (11.1)
Allergic rhinitis	2 (66.7)/0	3 (33.3)/0
Pollen allergy	1 (33.3)/0	1 (11.1)/0
Gestational age, median (IQR), wk	39 (38-39)	39 (38-39)
Nonvaginal delivery, No. (%)		
Planned cesarean delivery	16 (10.3)	17 (10.9)
Emergency cesarean delivery	21 (13.5)	20 (12.8)
Female, No. (%)	82 (52.6)	78 (50.0)
Apgar score at 5 min, No. (%)^b		
8 points	4 (2.7)	10 (6.5)
9 points	131 (84.0)	128 (83.7)
10 points	15 (9.6)	15 (9.8)
Placental weight, mean (SD), g	585 (103)	563 (97)
pH of cord blood, mean (SD)	7.28 (0.06)	7.29 (0.08)
Anthropometry at birth, mean (SD)		
Body weight, g	2994 (313)	2994 (314)
Body height, cm	48.7 (1.7)	48.8 (1.8)
Chest circumference, cm	31.5 (1.3)	31.6 (1.4)
Head circumference, cm	34.0 (1.3)	34.1 (1.2)

Abbreviations: BF, breastfeeding; CMF, cow's milk formula; EF, elemental formula; IQR, interquartile range.

^a Number of siblings was 3 in the BF/EF group and 9 in the BF plus CMF group.

^b Includes 150 infants in the BF/EF group and 153 in the BF plus CMF group.

Table 2. Effects of Avoiding CMF at Birth on Antigen-Specific IgE Levels^a

Subgroups	Study Group, No. (%) of Infants			RR (95% CI) ^b	P Value for Interaction ^c
	Total	BF/EF	BF Plus CMF		
Cow's Milk-Specific IgE Level ≥0.35 U _A /mL					
Overall (n = 286) ^d	70 (24.5)	24 (16.8)	46 (32.2)	0.52 (0.34-0.81)	NA
Subgroups stratified by 25(OH)D levels ^e					
Lower tertile (n = 94)	17 (18.1)	8 (17.0)	9 (19.1)	0.89 (0.38-2.11)	.02
Middle tertile (n = 89)	26 (29.2)	4 (9.1)	22 (49.0)	0.19 (0.07-0.50)	
Higher tertile (n = 96)	26 (27.1)	12 (25.0)	14 (29.2)	0.86 (0.44-1.66)	
Egg White-Specific IgE ≥0.35 U _A /mL					
Overall (n = 286) ^d	107 (37.4)	53 (37.1)	54 (37.8)	0.98 (0.73-1.32)	NA
Subgroups stratified by 25(OH)D levels ^e					
Lower tertile (n = 94)	32 (34.0)	16 (34.0)	16 (34.0)	1.00 (0.57-1.78)	.02
Middle tertile (n = 89)	33 (37.1)	11 (25.0)	22 (49.0)	0.51 (0.28-0.92)	
Higher tertile (n = 96)	38 (39.6)	23 (47.9)	15 (31.3)	1.53 (0.92-2.56)	

Abbreviations: BF, breastfeeding; CMF, cow's milk formula; EF, elemental formula; NA, not applicable; RR, risk ratio; 25(OH)D, 25-hydroxyvitamin D; U_A, allergen units.

^a Other antigen-specific IgEs (wheat, mites, Japanese cedar pollen, dog dandruff, and cat dandruff) were not significantly different overall and in the subgroups.

^b Estimated as the risk of the effects of intervention (ie, avoiding supplementation with CMF at birth) on the risk of outcomes.

^c To clarify whether the intervention significantly affected these subgroups, P values for interaction were analyzed using the Mantel-Haenszel test.

^d Blood samples were available in 286 of 302 (94.7%) infants at 24 months of age (143 in each group).

^e Blood samples for measuring 25(OH)D levels were available in 303 infants at 5 months of age, but the number of infants who had data at 5 months and food antigen-specific IgE data at 24 months of age was reduced to 279.

low- or the high-level subgroup ($P = .02$ for interaction). Serum levels of CM-IgE as a continuous variable were lower in the BF/EF group (median, 0.05 U_A/mL [interquartile range {IQR}, 0.05-0.05 U_A/mL]) than in the BF plus CMF group (median, 0.05 U_A/mL [IQR, 0.05-0.10 U_A/mL]) at 5 months of age (Figure 2A). Similar results were found at 24 months of age in the BF/EF group (median, 0.05 U_A/mL [IQR, 0.05-0.16 U_A/mL]) compared with the BF plus CMF group (median, 0.05 U_A/mL [IQR, 0.05-0.48 U_A/mL]) (Figure 2B).

Effects of Avoiding CMF at Birth

The risks of secondary outcomes of each clinical food allergy are shown in Table 3. The immediate type of food allergy occurred in 22 of 151 infants (14.6%) in the BF/EF group, which was significantly less than 45 of 151 infants (29.8%) in the BF plus CMF group. Of a total of 67, 43 infants (64.2%) outgrew the allergy. At their second birthday, 4 infants (2.6%) in the BF/EF group had not yet outgrown the allergy, which was significantly less than the 20 infants (13.2%) in the BF plus CMF group (RR, 0.20; 95% CI, 0.07-0.57). Anaphylaxis developed in 1 infant (0.7%) in the BF/EF group, which was significantly fewer than the infants 13 (8.6%) in the BF plus CMF group (RR, 0.08; 95% CI, 0.01-0.58), all of whom did not outgrow the allergy by the second birthday. The OFC tests were performed in 20 infants in the BF/EF group and 24 in the BF plus CMF group, of whom 1 (5.0% of those tested and 0.7% of total) and 11 (45.8% of those tested and 7.3% of total), respectively, had positive results and were diagnosed as having a food allergy. At the second birthday, all infants with positive OFC findings in the BF/EF group could eat the allergenic food, whereas 9 (6.0%) with OFC-positive findings in the BF plus CMF group could not.

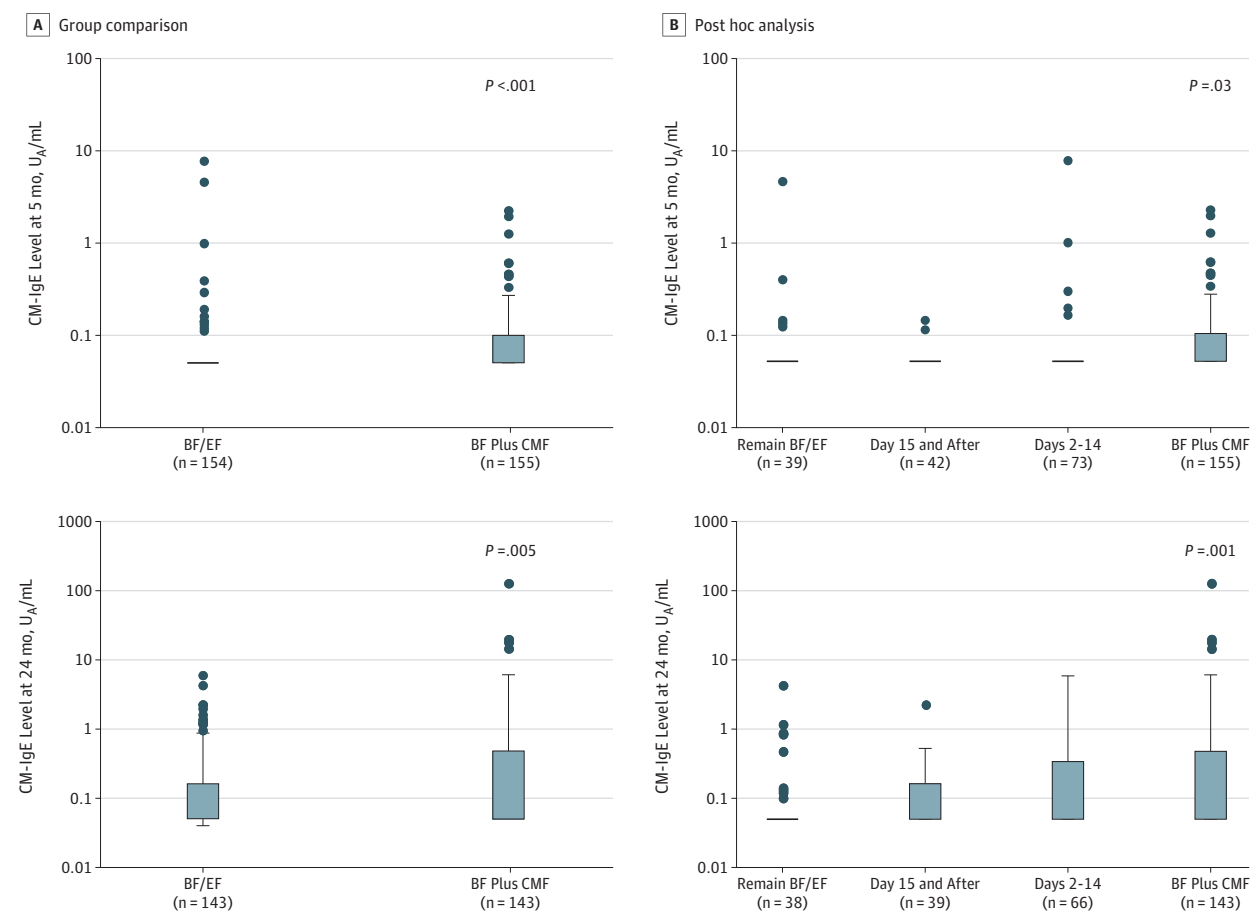
Incidence of CMA was significantly less in the BF/EF group (1 [0.7%]) than the BF plus CMF group (10 [6.6%]). Of the total

11 infants with CMA, 7 (63.6%) outgrew CMA by the second birthday, leaving no infants in the BF/EF group and 4 (2.6%) in the BF plus CMF group with CMA. Similarly, egg allergy occurred significantly less in the BF/EF (17 [11.3%]) than in the BF plus CMF (30 [19.9%]) group. Of the 47 infants with incidence of egg allergy, 38 (80.9%) had outgrown it by the second birthday, leaving 1 (0.7%) in the BF/EF group and 8 (5.3%) in the BF plus CMF group. Incidence of wheat allergy was lower in the BF/EF (1 [0.7%]) than in the BF plus CMF (7 [4.6%]) group, as was prevalence (0 and 5 [3.2%], respectively). The cumulative incidence of other food allergies (eg, walnuts) was 3 (2.0%) in the BF/EF group and 9 (6.0%) in the BF plus CMF group. In the subgroup of middle 25(OH)D levels, fewer immediate food allergies developed in the BF/EF vs BF plus CMF groups (0 vs 8 [17.0%]). Similar results were found for anaphylactic food allergies (0 vs 6 [12.8%]). In both instances, their interactions were not significant.

Post Hoc Analysis

In the BF/EF group, by maternal preferences, 73 infants were switched from BF/EF to BF plus CMF within 14 days and 42, after 14 days of life (total of 115 infants). A residual 39 infants continued to receive BF/EF until 5 months of age. In a post hoc analysis, levels of CM-IgE at 5 months of age did not show a clear trend (Figure 2B, upper panel), whereas those at 24 months of age showed a trend for earlier start of supplementation with CMF to be associated with higher levels of CM-IgE (BF/EF until 5 months of age, median of 0.05 U_A/mL [IQR, 0.05-0.05 U_A/mL]; switched to BF plus CMF after 14 days of life, 0.05 U_A/mL [IQR, 0.05-0.16 U_A/mL]; switched to BF plus CMF within 14 days of life, 0.05 U_A/mL [IQR, 0.05-0.34 U_A/mL]; BF plus CMF group, 0.05 U_A/mL [0.05-0.48 U_A/mL]) (Figure 2B, lower panel). Among these 115 infants, CMF sensitization was en-

Figure 2. Cow's Milk-Specific IgE (CM-IgE) Levels Among Study Groups



A, CM-IgE levels are compared between infants in the groups receiving breastfeeding with or without amino acid-based elemental formula for at least the first 3 days of life (BF/EF group) and BF supplemented with cow's milk formula from the first day of life to 5 months of age (BF plus CMF group). Blood sampling was available at 5 (309 of 312 [99.0%]) and 24 (286 of 302 [94.7%]) months of age. B, As a post hoc analysis, infants in the BF/EF group are further divided into the following 3 subgroups: (1) continued BF/EF; (2) switched from

EF to CMF on day 15 of life and after; and (3) switched from EF to CMF on day 14 of life or before. Subgroups were compared with the BF plus CMF group as adding CMF from the first day of life. Levels of CM-IgE are compared among these 4 groups at 5 and 24 months of age. *P* values were computed by regression analysis. Error bars indicate 95% CIs. BF indicates breastfeeding; CMF, cow's milk formula; EF, elemental formula; and U_A , allergen units.

hanced, but risks of clinical food allergies (ie, secondary outcomes) were not increased at all compared with 39 infants.

Adverse Events

We obtained the following information from an interview at the outpatient clinic just after the 2-year-old birthday: admission for any reason, emergency department visit for any reason, growth retardation, and height and weight at the second birthday. There was no evidence of a difference between infants in the BF/EF and BF plus CMF groups for safety outcomes (eTable in Supplement 2).

Discussion

In this RCT, avoiding exposure to CMF for at least the first 3 days of life decreased risks of sensitization to cow's milk and CMA compared with supplementing with CMF from the first

day of life. Although the previous cohort study⁶ showed that early exposure to CMF within 14 days after birth reduced the risk of CMA, exposure to small quantities of CMF for the first 3 days of life was not monitored. Thus, the results of that observational study are not necessarily in contrast to those of the present trial. The RCT by Saarinen et al⁷ showed that CMA was detected in 2.4% of infants supplemented with CMF and 1.5% of those supplemented with extensively hydrolyzed whey formula at maternity hospitals, where CMF was started as a supplement at a median of 15 hours. On the other hand, in the present trial, 2.6% in the BF plus CMF group vs 0 in the BF/EF group developed CMA. Thus, the results of both RCTs are considered similar, but amino acid-based EF may be less allergenic than the whey hydrolysate formula, even if extensively hydrolyzed. Moreover, a prospective cohort study⁸ showed that all 39 CMAs developed in 1539 infants who received CMF supplementation during the first 3 days of life, whereas no CMAs occurred in 210 infants

Table 3. Effects of Avoiding CMF at Birth on Immediate and Anaphylactic Types of Food Allergy

Effects	Study Group, No. (%) of Infants ^a			RR (95% CI) ^b	P Value for Interaction ^c
	Total (n = 302)	BF/EF (n = 151)	BF Plus CMF (n = 151)		
Immediate Type of Food Allergy					
Cumulative incidence by the second birthday	67 (22.2)	22 (14.6)	45 (29.8)	0.49 (0.31-0.77)	NA
Prevalence at the second birthday	24 (7.9)	4 (2.6)	20 (13.2)	0.20 (0.07-0.57)	NA
Subgroups stratified by 25(OH)D levels ^d					
Lower tertile (n = 99)	8 (8.1)	2 (4.2)	6 (11.8)	0.35 (0.08-1.67)	.64
Middle tertile (n = 96)	8 (8.3)	0	8 (16.7)	0 (NA)	
Higher tertile (n = 98)	8 (8.2)	2 (4.1)	6 (12.2)	0.33 (0.07-1.57)	
Anaphylactic Type of Food Allergy					
Cumulative incidence by second birthday	14 (4.6)	1 (0.7)	13 (8.6)	0.08 (0.01-0.58)	NA
Prevalence at second birthday	14 (4.6)	1 (0.7)	13 (8.6)	0.08 (0.01-0.58)	NA
Subgroups stratified by 25(OH)D levels ^d					
Lower tertile (n = 99)	5 (5.1)	1 (2.1)	4 (7.8)	0.27 (0.03-2.29)	.55
Middle tertile (n = 96)	6 (6.3)	0	6 (12.5)	0 (NA)	
Higher tertile (n = 98)	3 (3.1)	0	3 (6.1)	0 (NA)	
OFC-Positive Immediate Type of Food Allergy					
Cumulative incidence by second birthday	12 (4.0)	1 (0.7)	11 (7.3)	0.09 (0.01-0.70)	NA
Prevalence at second birthday	9 (3.0)	0	9 (6.0)	0 (NA)	NA
Subgroups stratified by 25(OH)D levels ^d					
Lower tertile (n = 99)	3 (3.0)	0	3 (5.9)	0 (NA)	>.99
Middle tertile (n = 96)	1 (1.0)	0	1 (2.1)	0 (NA)	
Higher tertile (n = 98)	5 (5.1)	0	5 (10.2)	0 (NA)	
Cow's Milk Allergy					
Cumulative incidence by second birthday	11 (3.6)	1 (0.7)	10 (6.6)	0.10 (0.01-0.77)	NA
Prevalence at second birthday	4 (1.3)	0	4 (2.6)	0 (NA)	NA
Subgroups stratified by 25(OH)D levels ^d					
Lower tertile (n = 99)	2 (2.0)	0	2 (3.9)	0 (NA)	>.99
Middle tertile (n = 96)	2 (2.1)	0	2 (4.2)	0 (NA)	
Higher tertile (n = 98)	0	0	0	0 (NA)	
Egg Allergy					
Cumulative incidence by second birthday	47 (15.6)	17 (11.3)	30 (19.9)	0.57 (0.33-0.98)	NA
Prevalence at second birthday	9 (3.0)	1 (0.7)	8 (5.3)	0.13 (0.02-0.99)	NA
Subgroups stratified by 25(OH)D levels ^d					
Lower tertile (n = 99)	4 (4.0)	1 (2.1)	3 (5.9)	0.35 (0.04-3.29)	.68
Middle tertile (n = 96)	2 (2.1)	0	2 (4.2)	0 (NA)	
Higher tertile (n = 98)	3 (3.1)	0	3 (6.1)	0 (NA)	
Wheat Allergy					
Cumulative incidence by second birthday	8 (2.6)	1 (0.7)	7 (4.6)	0.14 (0.02-1.15)	NA
Prevalence at second birthday	5 (1.7)	0	5 (3.3)	0 (NA)	NA
Subgroups stratified by 25(OH)D levels ^d					
Lower tertile (n = 99)	0	0	0	0 (NA)	>.99
Middle tertile (n = 96)	3 (3.1)	0	3 (6.3)	0 (NA)	
Higher tertile (n = 98)	2 (2.0)	0	2 (4.1)	0 (NA)	

Abbreviations: BF, breastfeeding; CMF, cow's milk formula; EF, elemental formula; NA, not applicable; OFC, oral food challenge; RR, risk ratio; 25(OH)D, 25-hydroxyvitamin D.

^a Cumulative incidence and prevalence are calculated using column totals; 25(OH)D subgroups, using row totals.

^b Estimated as the effects of intervention (ie, avoiding supplementation with CMF at birth) on risk of outcomes.

^c To clarify whether the intervention significantly affected these subgroups, *P* values for interaction were analyzed using the Mantel-Haenszel test.

^d Blood samples for measuring 25(OH)D levels were available in 303 infants at 5 months of age, but the number of infants with data at 5 months and followed up until 24 months of age was reduced to 293.

who did not receive CMF supplementation, findings considered to be consistent with this trial.

Of interest, avoiding CMF for at least the first 3 days of life reduced not only CMA risk but also other food allergy risks in the present trial. Germ-free mice treated with antibiotics or devoid of any bacterial colonization were demonstrated to be highly susceptible to the anaphylactic type of food allergy.^{19,20} On the other hand, gut bacterial flora (eg, *Anaerostipes caccae*) were suggested to protect against food allergy in a murine model by transferring microbiota from healthy and allergic infants.²⁰ Cesarean delivery is a well-known risk factor for food allergy, which may be explained by the evidence that infants born by cesarean delivery acquire nonmaternal vaginal bacteria from the hospital environment.²¹ In addition, a longer duration of BF was associated with a lower prevalence of *Clostridium difficile*,²² colonization of which at 1 month of age was associated with wheeze, eczema, and asthma later in life.²³ Necrotizing enterocolitis, an inflammatory bowel necrosis most commonly affecting preterm infants, can be triggered by feeding of CMF and prevented by BF, which may be further influenced by an altered intestinal microbiome that activates an uncontrolled proinflammatory response.²⁴ Based on these findings, we generated the hypothesis that exposure to a high volume of cow's milk protein with scarce growth of the gut microbiome just after birth may trigger enterocolitis, enhance permeability of gut membrane, facilitate absorption of food allergens, and increase risks of food allergy later in infancy.

In the present trial, the immediate and anaphylactic types of food allergy independent of food type (ie, not only cow's milk but also egg, wheat, and others) were shown to be primarily preventable by avoiding exposure to CMF at birth, although the mechanisms are unknown. Avoiding CMF is easily and immediately applicable to clinical practice throughout the world without incurring the cost and time of therapy. Recent RCTs demonstrated that early introduction of peanuts and heated egg powder among infants with eczema produced marked risk reductions of peanut²⁵ and egg allergy.²⁶ However, avoiding CMF at birth may be safer.

In subgroup analysis, avoiding CMF at birth was effective only in the subgroup of middle 25(OH)D levels. A U-shaped or inverted U-shaped association was reported between 25(OH)D levels and aeroallergen sensitization²⁷ or eczema,²⁸ respectively. These outcomes may suggest not linear but bimodal function of 25(OH)D in the serum on immune functions. However, vitamin D hypotheses in food allergy are still far from conclusions.

Limitations

This trial has several limitations. First, this trial was not double-blinded. For this reason, the primary outcome was set as CM-IgE level, an objective variable, and the OFC tests were performed by pediatricians who were blinded to allocation group. The secondary outcomes were further determined by the end

point committee, which was blinded to the intervention arms. Second, the OFC test was not administered to all participants. The OFC test is the criterion standard for the diagnosis of food allergy, but it is a resource-intensive procedure with some level of risk involved; thus, it was reserved for the equivocal cases predefined in this trial.²⁹ Third, the follow-up period was to the second birthday, which may be too short to evaluate the risks of food allergy because some infants with food allergy can outgrow their allergy after the second birthday, or some infants may show the immediate and anaphylactic types of allergy to certain foods (eg, nuts) after the second birthday. Fourth, the prevalence of each food allergy is different in different countries (eg, egg allergy is highest in Japan, probably because Japanese prefer to eat raw eggs, whereas CMA is highest in other countries).³⁰ Fifth, we cannot differentiate which intervention—avoiding CMF or exposing to EF for the first 3 days—was effective in preventing development of food allergy. However, CMF and EF are fundamentally the same except whether cow's milk protein or amino acids without cow's milk protein is included. Randomization into the BF plus CMF group from the first day of life, BF alone for the first 3 days while avoiding CMF and EF, and BF plus EF for the first 3 days while avoiding CMF is ideal. However, if mothers do not have enough breast milk, it is ethically hard to keep an intervention of BF alone even for 72 hours at birth. Sixth, amounts of CMF or EF added to BF were highly variable, ranging from anywhere to near-exclusive BF or near-exclusive CMF with different duration based on subjective impression of adequate infant intake. Regardless of these variabilities in the intervention, the difference was obvious between the BF/EF and BF plus CMF groups, suggesting that the critical factor may not be the amount of CMF exposure or its duration but timing of the start of adding CMF to BF. Seventh, after 3 days of life, most infants in the BF/EF group were switched from BF/EF to BF plus CMF, whereas all infants in the BF plus CMF group remained in the same group, which may cause differential misclassification. Thus, adding CMF to BF after 3 days of life may have protective effects on the development of food allergy, including CMA. Eighth, cow's milk sensitization as the primary outcome does not always lead to CMA, although CMA develops among infants with cow's milk sensitization. If we focused on OFC-proven CMA as one of the secondary outcomes, statistical power is too small.

Conclusions

This study found that not only sensitization to cow's milk but also clinical food allergies are primarily preventable by avoiding CMF supplementation at birth. This prevention is easily and immediately applicable to clinical practice throughout the world without the cost and time of therapy.

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Concept and design: M. Urashima, Mezawa, T. Urashima, Hirano, Tachimoto.
Acquisition, analysis, or interpretation of data: All authors.

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Critical revision of the manuscript for important intellectual content: Mezawa, Okuyama, T. Urashima, Hirano, Gocho, Tachimoto.

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Obtained funding: M. Urashima, T. Urashima, Tachimoto.

Administrative, technical, or material support: Okuyama, T. Urashima, Gocho, Tachimoto. **Supervision:** T. Urashima, Tachimoto.

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Trial Protocol and Statistical Analysis Plan (SAP)

Original Trial Protocol and Statistical Analysis Plan (SAP) in English p2 ~ p25

Final Trial Protocol and Statistical Analysis Plan (SAP) in English p26 ~ p50

References p51 ~ p52

Appendix 1: Table 1. Oral Food Challenge (OFC) test p53

Appendix 2: Table 2. Permutated blocks of five p54

Appendix 3: Table 2. Questionnaires of food intake p55 ~ p58

Summary of changes to the protocol approved by the ethics committee p59 ~ p61

***The statistical analysis plan is contained within the protocol files.**

Trial Protocol and Statistical Analysis Plan (SAP)

(original)

Title: Randomized clinical trial in preventing food allergy Breast feeding vs. breast feeding plus formula milk

ABC (Atopy prevention by Breastfeeding or Cow's milk formula)

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Ver. 1. 2013.8.1

Protocol synopsis

Official title of the study

Randomized clinical trial in preventing food allergy Breast feeding vs. breast feeding plus formula milk

Brief title

Food allergy prevention trial

Region

Japan

Objectives

Compare atopic sensitization at 5-6 months and 2 years of age between breastfeeding with or without amino acid milk (=avoiding supplementation with cow's milk formula (CMF) for at least the first 3 days of life) and breastfeeding with CMF (=adhering to BF supplemented with CMF ≥ 5 ml/day from the first day of life to 1 month and ≥ 40 ml/day after 1 month until weaning) (Efficacy)

Primary outcomes

Atopic sensitization

Cow's milk sensitization, i.e., Cow's milk-specific IgE at 24 months of age, which will be compared with other allergens sensitization, e.g., egg-white.

Key secondary outcomes

- I. Immediate type of food allergy (FA) including anaphylaxis
- II. Worsening of atopic dermatitis
- III. Desensitization of FA
- IV. Atopic sensitization alone
- V. Asthmatic bronchitis
- VI. Allergic rhinitis

Study design

Basic design: Parallel

Randomization: YES

Randomization unit: Individual

Blinding: Prospective, randomized, open, blinded-endpoint (PROBE) design

Control: Active

Stratification: NO

Dynamic allocation: NO

Institution consideration: NOT considered as an adjustment factor

Intervention

No. of arms: 2

Purpose of intervention: Primary prevention of food allergy (FA)

Type of intervention: Food

1. **Intervention 1:** Breastfeeding (BF) with or without amino acid-based elemental formula (EF), called the BF±EF group (if the amount of breast milk is not sufficient, EF will be added), until 5 months of age. When the mother needs to add EF of more than 150 ml/day for 3 consecutive days to BF because of a low breast milk amount, EF is switched to cow's milk formula (CMF). Thus, the offspring allocated to BF±EF can avoid CMF for at least the first 3 days of life.
2. **Intervention 2:** BF plus CMF (≥ 5 ml/day from the first day of life to 1 month and ≥ 40 ml/day after 1 month until weaning), called the BF+CMF group, until 5 months of age or starting solid food.

Participants

Age: Neonates at birth

Gender: Male and female

Key inclusion criteria

1. Be in a high-risk group for atopic sensitization: at least one of the father, mother, brothers,

or sisters of infants has current and/or past atopic diseases (asthma, atopic dermatitis, food allergy, allergic rhinitis, hay fever)

2. Obtain written informed consent.

a. Participants who understand randomization into two groups: BF with or without amino acid milk and BF with cow's formula milk.

b. Blood examination at 5-6 months and 2 years of age is required.

3. Be available to visit the outpatient clinic at 1 month, 2 months, 3 months, 4 months, 5 months, 7~8 months, 9~10 months, 12 months, 14~15 months, 18 months, and 24 months of age.

Key exclusion criteria

1. Less than 36 weeks of gestational age at birth or birth weight less than 2000 g.

2. Having serious congenital anomalies.

3. Being a triplet or more; twins may participate.

4. Abnormal delivery such as asphyxia (Apgar score 6 or less)

Target sample size

Total, n = 300;

BF±EF group, n = 150;

BF+CMF group, n = 150.

Location of the trial

Tokyo, Japan

a. Patient care: Outpatient clinic of Jikei University Hospital

b. Data monitoring and analysis: Division of Molecular Epidemiology, Jikei University School of Medicine

Study period

Accrual, if the number of randomized participants reaches >300, then entry is stopped.

The trial ends when the last participant is followed up after the 2-year-old birthday.

Follow-up

Participating parent(s) are interviewed, and their children are examined from 1 to 24 months of age at the outpatient clinic of Jikei University Hospital. All infants undergo blood examinations to measure serum levels of total IgE and antigen-specific IgE at 5 and 24 months of age. Unscheduled visits and blood examinations are performed at the clinic when a participating infant has atopic symptoms, particularly when FA is suspected.

Statistical analysis

I. Primary outcome: Cow's milk-specific IgE

- A) Antigen-specific IgE ≥ 0.35 U_A/ml at 24 months of age as a dichotomous variable, risk ratio (RR) and 95% confidence intervals (95% CI)
- B) Serum levels of cow's milk-specific IgE as a continuous variable at 5 and 24 months of age: Mann-Whitney test between the comparative groups.

II. Secondary outcomes

Clinical food allergy: RR, and 95% CI are used to evaluate the secondary outcomes.

Ethics

1. Private information is carefully protected.
2. All personal information about the participants is rendered innominate in a linkable fashion at Jikei University Hospital.
3. Liability insurance is obtained to compensate participants for side effects of interventions.

Funding

The Jikei University School of Medicine

Background

The prevalence of food allergy (FA) has been growing over the last 25 years. Especially, the frequency of anaphylaxis, which may be fatal, has increased 5 times since 1990 in developed countries.¹⁻³ In 2000, the American Academy of Pediatrics (AAP) made the following recommendations: Infants at high risk for developing allergy, identified by a strong (one or both parents, sibling) family history of allergy, may benefit from the following: (1) Breastfeeding (BF) mothers should continue BF for the first year of life or longer. During this time, for infants at risk, hypoallergenic formulas can be used to supplement BF; (2) Mothers should eliminate peanuts and tree nuts (e.g., almonds, walnuts, etc.) and consider eliminating eggs, cow's milk, fish, and perhaps other foods from their diets while nursing; (3) Solid foods should not be introduced into the diet of high-risk infants until 6 months of age, with dairy products delayed until 1 year, eggs until 2 years, and peanuts, nuts, and fish until 3 years of age.⁴ However, in 2008, AAP revised the recommendations as follows: (1) For infants at high risk of developing atopic disease, there is evidence that exclusive BF for at least 4 months compared with feeding intact CMF decreases the cumulative incidence of atopic dermatitis and cow's milk allergy (CMA) in the first 2 years of life. Comparative studies of the various hydrolyzed formulas have also indicated that not all formulas have the same protective benefit. Extensively hydrolyzed formulas may be more effective than partially hydrolyzed formulas for the prevention of atopic disease; (2) At the present time, antigen avoidance during lactation does not prevent atopic disease, although more data are needed to substantiate this conclusion; (3) Although solid foods should not be introduced before 4 to 6 months of age, there is no current convincing evidence that delaying their introduction beyond this period has a significant protective effect on the development of atopic disease regardless of whether infants are fed cow's milk protein formula or human milk.⁵

Although WHO recommends BF for the first 6 months of life to improve childhood morbidity and mortality,⁶ supplementation of BF with cow's milk formula (CMF) is quite common in many countries including Japan. In contrast to WHO's recommendation, a population-based prospective cohort study in Israel demonstrated that the frequency of IgE-mediated CMA can be dramatically lowered if infants start receiving regular CMF within the first 14 days of life (0.05%) compared with beginning CMF between 105 and 194 days (1.75%). They thus

recommended CMF supplementation at birth to promote oral tolerance to cow's milk.⁷ Although such studies do not eliminate the possibility of reverse causality as an explanation for this finding (high-risk infants with eczema are deliberately breast-fed longer) due to the nature of observational studies, they raise the question as to whether exposure to CMF as a supplement to BF within 14 days of life may play a role in preventing allergic disease. Indeed, the German Infant Nutritional Intervention Study Group disagreed because they could not reproduce the risk reduction by early exposure to CMF in post-hoc analysis of their previous randomized controlled trial.⁸

Sometimes during the first 3 days of life, the amounts of breast milk are small or postpartum mothers are too tired to breast feed. Consequently, newborn infants are quite often supplied CMF in addition to BF during hospital admission. Indeed, many Japanese maternity wards basically recommend BF, but allow mothers or nurses to help by supplying CMF as a supplement to BF after feeding colostrum, e.g., 6 to 10 hours after birth. In addition to the controversy regarding CMF supplementation before or after day 14, whether supplementary feeding with CMF during the first 3 days of life is protective,⁹ neutral,¹⁰ or rather enhances¹¹ risks of atopic diseases is also controversial, as shown in randomized controlled trials. In 1988, a cohort study in Denmark showed that all 39 CMAs developed in 1,539 infants who had been supplied CMF during the first 3 days of life, whether breast fed or not, after discharge from the maternity hospital, whereas no CMAs were observed in 210 infants who had not been supplied CMF.¹² In the discussion of the cohort study in Israel, the authors stated: "Because of the way our data were collected, we cannot exclude neonatal exposure to small quantities of CMP formula in the newborn nursery either forgotten by the mother or done without her knowledge. However, the role of a brief intermittent early exposure to milk in the neonatal unit is controversial^{10,11} and might have a low effect, if any, on the development of atopy. Accordingly, we found it appropriate not to consider such intermittent exposures to CMP in this study".

Hypotheses

1. Does avoiding exposure to CMF for the first 3 days of life prevent development of FA

later?

This is the main hypothesis of this study and was explained in the 'Background'.

Many Japanese maternity wards encourage BF, but allow mothers or nurses to supplement BF with CMF, e.g., approximately 6 to 10 hours after birth or even earlier, based on maternal preferences, but not based on clinical evidence. However, more than 20 to 30 years ago, sugar water was given instead of CMF supplement at birth. Thus, we hypothesized that early exposure to CMF at birth may be associated with the recent increase in children with FA. Therefore, a randomized clinical trial (RCT) was conducted to assess whether the risk of CMF sensitization and FA is decreased by either avoiding or supplementing CMF at birth.

2. Vitamin D hypotheses

Refer to the review article by Dr. G. Lack.¹³

The vitamin D hypothesis takes two forms: the vitamin D excess hypothesis argues that increases in vitamin D levels have led to increased allergies. The vitamin D deficiency hypothesis argues the opposite.

Objectives

Specific Aim 1: To compare atopic sensitization at 5-6 months and 2 years of age between BF with or without amino acid milk and BF with CMF.

Specific Aim 2: Subgroup analysis based on serum levels of 25-hydroxyvitamin D (25OHD) at 5 months, e.g., tertile, may be performed.

Study design

Randomized, controlled, parallel two-group trial at a single institution

Pregnant women during the third trimester are randomly assigned to either BF with or without amino acid-based EF, called the BF±EF group, or BF plus CMF (≥5 ml/day from the first day of life to 1 month and ≥40 ml/day after 1 month until weaning), called the BF+CMF group, in a 1:1 ratio. Infants are followed until they are at least 2 years of age. Follow-up is extended 1 year if participating infants have FA. When the mother needs to add more than 150 ml/day EF for 3 consecutive days to BF because of a low amount of breast milk, EF is switched to CMF. Thus, the offspring allocated to BF±EF can avoid CMF for at least the first 3 days of life.

Subgroup

1. Tertiles of 25(OH)D levels at 5 months of age

Measure 25OHD levels in the serum obtained at 5 months of age.

Research Implementation System

I. Clinical examination

- a. Pediatrician: Dr. Mitsuyoshi Urashima: Enrollment and participants' clinical examination before allergic signs. If participating infants have any allergic signs, i.e., food-specific IgE ≥0.35 U_A/ml blood sampling at 5 months of age, then they are referred to Dr. Tachimoto to diagnose atopic diseases and for follow-up.

At the outpatient clinic of The Maternal and Child Center in Jikei University Hospital

- b. Pediatrician: Dr. Hiroshi Tachimoto: Clinical examination after allergic signs and diagnosis of atopic diseases with or without the oral food challenge (OFC) test, management, and follow-up.

At the outpatient clinic of Pediatrics in Jikei University Hospital

II. Endpoint committee

Among the following five members who do not know the allocation group and will

not examine these participating infants at outpatient clinics, if ≥ 2 recognize the outcome as positive, the outcomes are finally determined to be positive.

1. Hidetoshi Mezawa, M.D., Ph.D.
2. Takashi Urashima, M.D., Ph. D.
3. Daishi Hirano, M.D., Ph.D.

Department of Pediatrics

The Jikei University School of Medicine

Tokyo, Japan

What the committee is expected to determine.

These five members determine whether the infant has had an episode of FA or not.

0. No FA

1. Immediate allergic reaction
2. Anaphylaxis

For 1 (and 2) above, they determine the status at the second birthday by reviewing detailed episodes, results of the OFC test, and levels of food-specific IgE.

1. Sustained unresponsiveness = outgrow
Cow's milk allergy, the infant can drink raw milk ≥ 50 ml
Egg allergy, the infant can eat baked egg ≥ 30 g,
Wheat allergy, the infant can eat udon noodle ≥ 15 g
2. Probable unresponsiveness, e.g., in the process of increasing dose of allergenic food, but not yet reach to predefined dose of unresponsiveness mentioned above.
3. Not yet unresponsive, when the infant eats, he or she still reacts to the allergenic food.
4. Not sure because the infant has not consumed the food allergen

The infants are still state of either 2, 3, or 4 above, they are considered not yet outgrow = remain food allergy.

III. Data monitoring

A) Randomization using a block of four by Dr. Akio Nakashima who does not interview participating families and does not examine their infants.

B) Data monitoring

If the frequency of adverse events deviates in either intervention group, the committee reports the data to the Safety and Data Monitoring Committee as well as the lead investigator.

a. Akio Nakashima, M.D., Ph.D. (physician and nephrologist)

b. Ms. Haruka Wada (secretary at Molecular Epidemiology)

Division of Molecular Epidemiology

The Jikei University School of Medicine

Tokyo, Japan

IV. Safety and Data Monitoring Committee

a. Judging from data of interim analysis, this committee may recommend stopping or continuing the trial.

b. Responding to a report of adverse events, the committee may recommend stopping the trial or allowing the trial to continue.

① Motohiro Ebisawa, M.D., Ph.D.

② Sakura Sato, M.D., Ph.D.

③ Kiyotake Ogura, M.D., Ph.D.

Department of Pediatrics

Sagamihara National Hospital

Kanagawa, Japan

Participants

Inclusion criteria

1. Be in a high-risk group for atopic sensitization: at least one of the father, mother, brothers, or sisters of infants has atopic diseases (asthma, atopic dermatitis, FA, allergic rhinitis, hay fever)
2. Obtained written informed consent.

- a. Participants who understand randomization into two groups: BF with or without amino acid milk and BF with CMF.
- b. Blood examinations at 5-6 months and 2 years of age are required.
3. Be available to visit the outpatient clinic at 1 month, 2 months, 3 months, 4 months, 5 months, 7~8 months, 9~10 months, 12 months, 14~15 months, 18 months, and 24 months of age.

Exclusion criteria

1. Less than 36 weeks of gestational age at birth or birth weight less than 2000 g.
2. Having serious congenital anomalies.
3. Being a triplet or more: twins may participate.
4. Abnormal delivery such as asphyxia (Apgar score 6 or less at 5 minutes after birth)

Interventions

1. BF±EF

Mothers in this group are asked to maintain exclusive BF. However, if the amount of breast milk is not sufficient, then EF may be added until starting solid food. If the mother needs to supply EF more than 150 ml/day for 3 consecutive days in addition to BF because of a low amount of breast milk, EF is switched to CMF. Thus, the offspring allocated to BF±EF can avoid CMF for at least the first 3 days of life.

2. BF+CMF

Mothers in this group are asked to maintain exclusive BF, but are also asked to simultaneously supply a small amount of CMF ≥ 5 ml/day from the first day of life until 1 month, and ≥ 40 ml/day after 1 month until weaning. When infants are born after 18:00, CMF may be added on the 2nd day.

Both CMF (Hohoemi®) and EF (Meiji Elemental formula®) are purchased from Meiji Holdings Co. Ltd. (Chuo-ku, Tokyo, Japan).

EF (Meiji Elemental formula®)

For infants with FA, total proteins are replaced with refined amino acids. However, other

fats, carbohydrates, vitamins, and minerals are basically the same as in CMF. Energy is 66 kcal/100 ml (17%) and 391 kcal/100 g powder.

Blinding to allocation group

- a. Participating family: Not blinded
- b. Dr. M.U.: Not blinded, because he needs to confirm compliance with the intervention by interviewing participating families at the outpatient clinic in the Maternal and Child Center of Jikei University Hospital. When an infant has food-specific IgE ≥ 0.10 U_A/ml at 5 months blood test, the infant is referred to Dr. H.T. at the outpatient clinic of Pediatrics without informing Dr. H.T. about the allocation group.
- c. Dr. H.T.: Blinded, who is a specialist in pediatric allergic diseases, diagnoses FA, without knowing the allocation group.
- d. Endpoint committee: Blinded and no contact with the participants.

Outcome Measures

Primary outcome

1. Atopic sensitization:

The primary outcome was atopic sensitization to cow's milk protein, i.e., serum levels of cow's milk-specific IgE (CM-IgE) ≥ 0.35 UA/ml at 24 months of age. Serum levels of CM-IgE at 5 and 24 months of age as a continuous variable were used as reference values for primary outcome. Other atopic sensitization to egg white and wheat ≥ 0.35 UA/ml at 24 months of age were also evaluated to be compared with cow's milk sensitization. Outsourced for measuring with ImmunoCAP Specific IgE® (Thermo Fisher Diagnostics K.K., Minato-ku, Tokyo, Japan) to SRL Inc. and compared as both continuous and dichotomous (CM-IgE: ≥ 0.35 UA/ml) variable between BF±EF and BF+CMF group.

Secondary outcomes

1. Immediate type of FA

Presenting with one organ symptom of skin (urticaria, angioedema, erythema, pruritus, periorbital edema, etc.), respiratory (dry staccato cough, hoarseness,

wheezing, etc.), gastrointestinal (nausea, vomiting, colicky abdominal pain, etc.), or circulatory (tachycardia, hypotension, loss of consciousness, etc.) problems triggered by the food ingestion that appeared within a few hours, in combination with blood examination measuring serum levels of the suspected food-specific IgE: ≥ 0.35 U_A/ml by means of a fluorescence enzyme immunoassay.

In this trial, symptoms triggered by skin contact directly with food or indirectly with food-contaminated hands are not included.

The OFC test (Appendix 1) is performed around 1 year of age for infants who are sensitized to certain foods but for whom clinical reactivity after ingestion of the food allergen is unknown. Thus, immediate allergic reaction is a food-specific IgE: ≥ 0.35 U_A/ml plus either a FA episode or positive OFC test. FA is re-evaluated regarding whether the infant could eat the causative food allergen without an allergic reaction at the 2-year-old birthday.

2. Anaphylactic type of FA

When symptoms are derived from multiple organs, e.g., urticaria and wheezing, the condition is defined as an anaphylactic reaction.

3. Worsening of atopic dermatitis

Check the SCORAD index¹⁴ at the 1- and 3-month check-ups.

4. Desensitization of food allergy (= unresponsiveness)

If infants, who had been once diagnosed as immediate type of FA especially with cow's milk allergy, egg allergy, and wheat allergy, can eat the allergenic food (raw milk ≥ 50 ml, baked egg 30g, udon noodle 15g) without allergic signs by 2nd birthday, then they are defined as 'sustained unresponsiveness = outgrow' to the food allergen in this trial.

5. Atopic sensitization alone

Although serum levels of food-specific IgE are positive (≥ 0.35 U_A/ml), the infant never had allergic signs and symptoms even by eating the allergenic food.

6. Asthmatic bronchitis

A stringent index that includes frequent wheezing during the first 3 years of life and either one major risk factor (parental history of asthma or eczema) or two of three minor risk factors (eosinophilia, wheezing without colds, and allergic rhinitis).¹⁵

Major Criteria

1. Parental asthma†
2. Eczema diagnosed by a medical doctor‡
3. Sensitization with elevated mite- or house dust-specific IgE ≥ 0.10 U_A/ml

Minor Criteria

1. Allergic rhinitis diagnosed by a medical doctor§
2. Wheezing apart from colds
3. Eosinophilia ($\geq 4\%$)

7. Allergic rhinitis

- a. Hay fever, e.g., Sugi pollen allergy: sugi pollen-specific IgE ≥ 0.10 U_A/ml
- b. Allergic rhinitis: Rhinitis signs and symptoms for 1 month and mite- or house dust-specific IgE ≥ 0.10 U_A/ml

Safety outcomes

The following are obtained from an interview at the outpatient clinic just after the 2-year-old birthday.

1. Admission for any reason
2. Emergency room visit for any reason
3. Growth retardation: Height and weight at 2-year-old birthday

Flow of participants

1. Informed consent and registration

- a. Pregnant women and their husbands are informed about the trial in detail at a

childbirth class during late pregnancy through a talk by Dr. M. Urashima and the informed consent document. Written, informed consent is sent to the data monitoring center at the Molecular Epidemiology Division of Jikei University School of Medicine.

- b. Current or previous family history of atopic diseases (asthma, atopic dermatitis, FA, allergic rhinitis, hay fever) is obtained through questionnaires as well as parental demographic information such as maternal age and body weight/height. Only pregnant women who are confirmed to have a family history of atopic diseases are included.
- c. Food questionnaires of mothers (Appendix 3) are taken during the third trimester of pregnancy and at 1, 3, and 5 months of age after birth.

2. At the maternity ward of Jikei University Hospital

- a. Some included pregnant women are excluded after delivery, due to, e.g., cleft palate.
- b. Perinatal information, e.g., gestational weeks and birth weight, are taken from charts.
- c. Nursing staff is informed of the participants' names and to which group they are allocated.
- d. Mothers are informed to which group they belong after delivery; thus, mothers are not blinded to the allocation group.
- e. Mothers belonging to the BF+CMF group are asked to supplement BF with CMF at least 5 ml from the first day of delivery. However, if the delivery time is after 18:00, they are allowed to supply CMF in addition to BF starting at midnight of the 2nd day.
- f. Mothers belonging to the BF±EF group may add amino acid-based EF when they feel that BF is not enough. If the mother adds more than 150 ml/day EF to BF for 3 consecutive days, EF is switched to CMF. Thus, offspring allocated to BF±EF can avoid CMF for at least the first 3 days of life.
- g. Nurses at the maternity ward are allowed to feed babies instead of their mothers.

3. At the outpatient clinic in the Maternal and Child Center of Jikei University Hospital

- a. Participating parent(s) are interviewed, and their children are examined at least seven times from 1, (2,) 3, (4,) 5-6, (7-8,) 9-10, 12, (14-15,) 18, and 24 months of

age at the outpatient clinic of Jikei University Hospital by a study pediatrician (M.U.) who is not blinded to the allocation group.

- b. If infants have exanthema, the pediatrician provides their parents clear guidance about aggressive skin care such as how to wash their face and body using soap and a shower by showing them a video.
- c. Blood is sampled from all infants at 5-6 and 24 months.

4. At the outpatient clinic in Pediatrics of Jikei University Hospital

In case of at least one antigen-specific IgE is 0.10 IU_A/ml or more, the infant is referred to a study specialist of pediatric allergy (H.T.) at the same Jikei University Hospital who does not know the infant's allocation group.

5. Pediatrics Ward of Jikei University Hospital

The OFC test is performed at admission (Appendix 1) by pediatricians who are blinded to the allocation group.

6. Efforts to reduce loss to follow-up

- a. Using email, we try to contact participants who move to distant places regarding whether their infants have FA and make efforts to reduce the number of infants lost to follow-up, from whom IgE data are not obtained at 5 or 24 months.
- b. If participants do not visit the outpatient clinic after their 2-year-old birthday and we receive no responses to the email, they are considered lost to follow-up.

7. Blood sampling

All infants undergo blood examinations to measure serum total and antigen-specific IgE.

- a. At the 5- to 6-month check-up prior to first solid food introduction:
Egg white, cow's milk, wheat, mites.
- b. At 2 years of age:
Egg white, cow's milk, wheat, mites, Japanese cedar pollen, dog dandruff, cat dandruff.
- c. Unscheduled visits are made to the clinic when participating infants have atopic

symptoms, particularly suspicious of FA.
e.g., exanthema just after eating an egg dish.

8. Randomization

a. EF and CMF

Both CMF (Hohoemi[®]) and EF (Meiji Elemental formula[®]) are purchased from Meiji Holdings Co. Ltd. (Chuo-ku, Tokyo, Japan).

b. Randomization

Pregnant women that meet the inclusion criteria are randomly assigned to either the BF+CMF or BF±EF group according to an identification number generated by computer based on permuted blocks of four that are determined beforehand (Appendix 2). Staff members at the maternity ward are informed of which intervention applies to each pregnant participant before hospitalization for childbirth.

c. Blinding

According to the nature of the intervention, we do not use double blinding. However, we try to minimize bias using the following strategies:

Primary outcome: Cow's milk-specific IgE as an objective indicator.

Secondary outcomes: determine by prospective, randomized, open, blinded-endpoint (PROBE), determined by the endpoint committee members who do not examine the infants or are blinded to the allocation group'.

9. Compliance with CMF or EF

1. At the maternity ward, every day's CMF or EF intake is monitored by each participant (mother). The nurse may help with the intervention and monitoring.
2. At the outpatient clinic, mothers are asked whether they are using CMF, EF, or exclusive BF.

10. Monitoring of secondary outcomes

a. Scheduled

At either the Maternal and Child Center or Pediatrics of Jikei University Hospital, participating infants are examined frequently.

b. Unscheduled

If the infants have any allergic signs, e.g., urticaria after eating allergenic food, they are advised to call Jikei University Hospital. H.T. or other pediatricians try to examine the infants on the day or next day and perform a blood examination.

Statistical analysis

1. Sample size

300

2. Sample size calculation

300 pregnant women and their offspring

We hypothesized that cow's milk sensitization at 2 years of age, i.e., cow's milk-specific IgE ≥ 0.35 U_A/ml, would be present in 10% of one group of infants and 25% of the other group, with a type I error (two-sided) of 5% and a power of 90%, on the assumption of 3% loss to follow-up. Therefore, 1:1 divided samples of approximately 300 participants were calculated as sufficient to detect a significant difference.

. sampsi .1 .25

Estimated sample size for two-sample comparison of proportions

Test Ho: $p_1 = p_2$, where p_1 is the proportion in population 1
and p_2 is the proportion in population 2

Assumptions:

alpha = 0.0500 (two-sided)

power = 0.9000

p_1 = 0.1000

$$p2 = 0.2500$$

$$n2/n1 = 1.00$$

Estimated required sample sizes:

$$n1 = 146$$

$$n2 = 146$$

$$n = 292$$

Assuming 3% loss to follow-up, 300 are needed to enroll.

3. Study period

We assume that 100 pregnant women per year will participate in this trial. Therefore, the accrual period will be 3 years. The last infant is followed up 2 years later. Thus, a total of 5 years is predicted to be needed to accomplish this trial.

4. Interim analysis

Interim analyses are planned two times at reaching 100 and 200 participating infants who had their second birthday. The P value for significance at the interim analysis is set as <0.001 according to the Peto's stopping boundaries.

5. Planned methods of analysis

Outcomes are analyzed by intention-to-treat analyses between the two comparative groups.

- a. Serum levels of cow's milk-specific IgE at 5 months and 24 months of age as a continuous variable are compared using Mann-Whitney test.
- b. Risk of cow's milk-specific IgE ≥ 0.35 U_A/ml will be analyzed with a 2x2 table with the chi-square test and represented by the risk ratio (RR), risk difference (RD) and 95% confidence interval (95% CI).
- c. Secondary outcomes will be compared with RR, RD, and 95% CI.
- d. Safety outcomes will be compared with RR, RD and 95% CI or Student's t test.
- e. Subgroup analysis will be done according to levels of 25(OH)D.

- f. All reported P values will be two-sided.
- g. Values with $P < 0.05$ will be considered statistically significant.
- h. All data will be statistically analyzed using Stata 14.0 (StataCorp LP., College Station, TX, USA).

6. Subgroup analyses

To clarify whether BF±EF or BF+CMF significantly affects the subgroups of 25(OH)D (tertile), P for interaction ($P_{\text{interaction}}$) is computed by creating multiplicative variables. The results of these analyses are not corrected for multiple comparisons.

Safety

Many Japanese maternity wards recommend BF, but allow mothers or nurses to help by supplying CMF to supplement BF after feeding colostrum, e.g., 6 to 10 hours after birth. This is a food intervention trial. Thus, basically no safety concerns exist. However, the following are compared.

- a. Admission due to any disease
- b. Emergency visit or admission for any reason
- c. Body height and weight at 24 months of age

Early withdrawal

Unknown serious adverse events.

Compensation

Mitsui Sumitomo Insurance Fire provides liability insurance (NB41027726) for the principal investigator, to compensate for costs of treating disability as a result of side effects caused by consuming milk.

The maximum amount of compensation is 100,000,000 yen per person and 300,000,000 yen per trial.

Consideration concerning the protection of human rights and privacy

1. Written informed consent is obtained after sufficient explanation.
2. Participants can withdraw from the trial after providing written, informed consent.
3. Withdrawal is not considered a disadvantage for participants.
4. Private information is exchanged with study ID at the Jikei University School of Medicine; therefore, private information cannot leak from there (linkable anonymizing).

Publication of research results

We aim to publish the results of this trial in a scientific journal or present them at academic conferences. Personal information will be carefully concealed when we present the results.

Research funds

- 1) The Jikei University School of Medicine

Attribution of intellectual property rights

If intellectual property rights such as patent rights become relevant, such rights will be attributable to the investigator.

2017.12.25

Trial Protocol and Statistical Analysis Plan (SAP)

(final)

Title: Randomized clinical trial in preventing food allergy Breast feeding vs. breast feeding plus formula milk

ABC (Atopy prevention by Breastfeeding or Cow's milk formula)

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- d. Mai Okuyama, M.D
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- a. Akio Nakashima, MD, PhD
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Ver. 1. 2013.8.1

Ver. 2. 2016.7.22

Ver. 3. 2017.12.25

Protocol synopsis

Official title of the study

Randomized clinical trial in preventing food allergy Breast feeding vs. breast feeding plus formula milk

Brief title

Food allergy prevention trial

Region

Japan

Objectives

Compare atopic sensitization at 5-6 months and 2 years of age between breastfeeding with or without amino acid milk (=avoiding supplementation with cow's milk formula (CMF) for at least the first 3 days of life) and breastfeeding with CMF (=adhering to BF supplemented with CMF ≥ 5 ml/day from the first day of life to 1 month and ≥ 40 ml/day after 1 month until weaning) (Efficacy)

Primary outcomes

Atopic sensitization

Cow's milk sensitization, i.e., Cow's milk-specific IgE at 24 months of age, which will be compared with other allergens sensitization, e.g., egg-white.

Secondary outcomes

- I. Immediate type of food allergy (FA) including anaphylaxis
- II. Desensitization of FA (= sustained unresponsiveness, or outgrow)

Exploratory outcomes

- I. Atopic dermatitis
- II. Asthmatic bronchitis

III. Sensitization to Japanese cedar pollen

IV. Allergic rhinitis

Study design

Basic design: Parallel

Randomization: YES

Randomization unit: Individual

Blinding: Prospective, randomized, open, blinded-endpoint (PROBE) design

Control: Active

Stratification: NO

Dynamic allocation: NO

Institution consideration: NOT considered as an adjustment factor

Intervention

No. of arms: 2

Purpose of intervention: Primary prevention of FA

Type of intervention: Food

1. **Intervention 1:** Breastfeeding (BF) with or without amino acid-based elemental formula (EF), called the BF±EF group (if the amount of breast milk is not sufficient, EF will be added), until starting solid food. When the mother needs to add EF of more than 150 ml/day for 3 consecutive days to BF because of a low breast milk amount, EF is switched to cow's milk formula (CMF). Thus, the offspring allocated to BF±EF can avoid CMF for at least the first 3 days of life.
3. **Intervention 2:** BF plus CMF (≥ 5 ml/day from the first day of life to 1 month and ≥ 40 ml/day after 1 month until weaning), called the BF+CMF group, until 5 months of age or starting solid food.

Participants

Age: Neonates at birth

Gender: Male and female

Key inclusion criteria

1. Be in a high-risk group for atopic sensitization: at least one of the father, mother, brothers, or sisters of infants has current and/or past atopic diseases (asthma, atopic dermatitis, food allergy, allergic rhinitis, hay fever)
2. Obtain written informed consent.
 - a. Participants who understand randomization into two groups: BF with or without amino acid milk and BF with cow's formula milk.
 - b. Blood examination at 5-6 months and 2 years of age is required.
3. Be available to visit the outpatient clinic at 1 month, 2 months, 3 months, 4 months, 5 months, 7~8 months, 9~10 months, 12 months, 14~15 months, 18 months, and 24 months of age.

Key exclusion criteria

1. Less than 36 weeks of gestational age at birth or birth weight less than 2000 g.
2. Having serious congenital anomalies.
3. Being a triplet or more; twins may participate.
4. Abnormal delivery such as asphyxia (Apgar score 6 or less)

Target sample size

Total, n = 300;

BF±EF group, n = 150;

BF+CMF group, n = 150.

Location of the trial

Tokyo, Japan

- a. Patient care: Outpatient clinic of Jikei University Hospital
- b. Data monitoring and analysis: Division of Molecular Epidemiology, Jikei University School of Medicine

Study period

Accrual, if the number of randomized participants reaches >300, then entry is stopped.

The trial ends when the last participant is followed up after the 2-year-old birthday.

Follow-up

Participating parent(s) are interviewed, and their children are examined from 1 to 24 months of age at the outpatient clinic of Jikei University Hospital. All infants undergo blood examinations to measure serum levels of total IgE and antigen-specific IgE at 5 and 24 months of age. Unscheduled visits and blood examinations are performed at the clinic when a participating infant has atopic symptoms, particularly when FA is suspected.

Statistical analysis

I. Primary outcome: Cow's milk-specific IgE

- A) Antigen-specific IgE ≥ 0.35 U_A/ml at 24 months of age as a dichotomous variable, risk ratio (RR) and 95% confidence intervals (95% CI)
- B) Serum levels of cow's milk-specific IgE as a continuous variable at 5 and 24 months of age: Mann-Whitney test between the comparative groups.

II. Secondary outcomes

Clinical food allergy: RR, and 95% CI are used to evaluate the secondary outcomes.

Ethics

1. Private information is carefully protected.
2. All personal information about the participants is rendered innominate in a linkable fashion at Jikei University Hospital.
3. Liability insurance is obtained to compensate participants for side effects of interventions.

Funding

1. JSPS KAKENHI Grant Number JP16K09074
2. The Practical Research Project for Allergic Disease and Immunology of the Japan Agency for Medical Research and Development, AMED (15ek0410019h0101)
3. Dairy Products Health Science Council and Japan Dairy Association

Background

The prevalence of food allergy (FA) has been growing over the last 25 years. Especially, the frequency of anaphylaxis, which may be fatal, has increased 5 times since 1990 in developed countries.¹⁻³ In 2000, the American Academy of Pediatrics (AAP) made the following recommendations: Infants at high risk for developing allergy, identified by a strong (one or both parents, sibling) family history of allergy, may benefit from the following: (1) Breastfeeding (BF) mothers should continue BF for the first year of life or longer. During this time, for infants at risk, hypoallergenic formulas can be used to supplement BF; (2) Mothers should eliminate peanuts and tree nuts (e.g., almonds, walnuts, etc.) and consider eliminating eggs, cow's milk, fish, and perhaps other foods from their diets while nursing; (3) Solid foods should not be introduced into the diet of high-risk infants until 6 months of age, with dairy products delayed until 1 year, eggs until 2 years, and peanuts, nuts, and fish until 3 years of age.⁴ However, in 2008, AAP revised the recommendations as follows: (1) For infants at high risk of developing atopic disease, there is evidence that exclusive BF for at least 4 months compared with feeding intact CMF decreases the cumulative incidence of atopic dermatitis and cow's milk allergy (CMA) in the first 2 years of life. Comparative studies of the various hydrolyzed formulas have also indicated that not all formulas have the same protective benefit. Extensively hydrolyzed formulas may be more effective than partially hydrolyzed formulas for the prevention of atopic disease; (2) At the present time, antigen avoidance during lactation does not prevent atopic disease, although more data are needed to substantiate this conclusion; (3) Although solid foods should not be introduced before 4 to 6 months of age, there is no current convincing evidence that delaying their introduction beyond this period has a significant protective effect on the development of atopic disease regardless of whether infants are fed cow's milk protein formula or human milk.⁵

Although WHO recommends BF for the first 6 months of life to improve childhood morbidity and mortality,⁶ supplementation of BF with cow's milk formula (CMF) is quite common in many countries including Japan. In contrast to WHO's recommendation, a population-based prospective cohort study in Israel demonstrated that the frequency of IgE-mediated CMA can be dramatically lowered if infants start receiving regular CMF within the first 14 days of life (0.05%) compared with beginning CMF between 105 and 194 days (1.75%). They thus

recommended CMF supplementation at birth to promote oral tolerance to cow's milk.⁷ Although such studies do not eliminate the possibility of reverse causality as an explanation for this finding (high-risk infants with eczema are deliberately breast-fed longer) due to the nature of observational studies, they raise the question as to whether exposure to CMF as a supplement to BF within 14 days of life may play a role in preventing allergic disease. Indeed, the German Infant Nutritional Intervention Study Group disagreed because they could not reproduce the risk reduction by early exposure to CMF in post-hoc analysis of their previous randomized controlled trial.⁸

Sometimes during the first 3 days of life, the amounts of breast milk are small or postpartum mothers are too tired to breast feed. Consequently, newborn infants are quite often supplied CMF in addition to BF during hospital admission. Indeed, many Japanese maternity wards basically recommend BF, but allow mothers or nurses to help by supplying CMF as a supplement to BF after feeding colostrum, e.g., 6 to 10 hours after birth. In addition to the controversy regarding CMF supplementation before or after day 14, whether supplementary feeding with CMF during the first 3 days of life is protective,⁹ neutral,¹⁰ or rather enhances¹¹ risks of atopic diseases is also controversial, as shown in randomized controlled trials. In 1988, a cohort study in Denmark showed that all 39 CMAs developed in 1,539 infants who had been supplied CMF during the first 3 days of life, whether breast fed or not, after discharge from the maternity hospital, whereas no CMAs were observed in 210 infants who had not been supplied CMF.¹² In the discussion of the cohort study in Israel, the authors stated: "Because of the way our data were collected, we cannot exclude neonatal exposure to small quantities of CMP formula in the newborn nursery either forgotten by the mother or done without her knowledge. However, the role of a brief intermittent early exposure to milk in the neonatal unit is controversial^{10,11} and might have a low effect, if any, on the development of atopy. Accordingly, we found it appropriate not to consider such intermittent exposures to CMP in this study".

Hypotheses

1. Does avoiding exposure to CMF for the first 3 days of life prevent development of FA

later?

This is the main hypothesis of this study and was explained in the 'Background'.

Many Japanese maternity wards encourage BF, but allow mothers or nurses to supplement BF with CMF, e.g., approximately 6 to 10 hours after birth or even earlier, based on maternal preferences, but not based on clinical evidence. However, more than 20 to 30 years ago, sugar water was given instead of CMF supplement at birth. Thus, we hypothesized that early exposure to CMF at birth may be associated with the recent increase in children with FA. Therefore, a randomized clinical trial (RCT) was conducted to assess whether the risk of CMF sensitization and FA is decreased by either avoiding or supplementing CMF at birth.

3. Vitamin D hypotheses

Refer to the review article by Dr. G. Lack.¹³

The vitamin D hypothesis takes two forms: the vitamin D excess hypothesis argues that increases in vitamin D levels have led to increased allergies. The vitamin D deficiency hypothesis argues the opposite.

4. Dietary fat hypothesis

Refer to the review article by Dr. G. Lack.¹³

The dietary fat hypothesis argues that reduction in consumption of animal fats and a corresponding increase in the use of margarine and vegetable oils have led to the increase in allergies. The argument is that there has been an increase in the consumption of ω -6 polyunsaturated fatty acids, such as linoleic acid, and similarly that through reduced consumption of oily fish, there has been a reduction in ω -3 polyunsaturated fatty acids, such as eicosapentaenoic acid. ω -6 fatty acids lead to the

production of prostaglandin E2 (PGE2), whereas ω -3 fatty acids inhibit synthesis of PGE2. PGE2 reduces interferon- γ production by T lymphocytes, thus resulting in increased IgE production by B lymphocytes. This has been proposed to explain the increase in the prevalence of asthma, eczema, and allergic rhinitis.

Objectives

Specific Aim 1: To compare atopic sensitization at 5-6 months and 2 years of age between BF with or without amino acid milk and BF with CMF.

Specific Aim 2: Subgroup analysis based on tertiles of serum levels of 25-hydroxyvitamin D (25OHD) at 5 months will be performed.

Exploratory Aim 1: To determine the relationships between serum levels of free fatty acids and FA and other atopic diseases.

Study design

Randomized, controlled, parallel two-group trial at a single institution

Pregnant women during the third trimester are randomly assigned to either BF with or without amino acid-based EF, called the BF $\pm$ EF group, or BF plus CMF ($\geq$ 5 ml/day from the first day of life to 1 month and $\geq$ 40 ml/day after 1 month until weaning), called the BF+CMF group, in a 1:1 ratio. Infants are followed until they are at least 2 years of age. Follow-up is extended 1 year if participating infants have FA. When the mother needs to add more than 150 ml/day EF for 3 consecutive days to BF because of a low amount of breast milk, EF is switched to CMF. Thus, the offspring allocated to BF $\pm$ EF can avoid CMF for at least the first 3 days of life.

Subgroup

1. Tertiles of 25(OH)D levels at 5 months of age

Measure 25OHD levels in the serum obtained at 5 months of age.

Exploratory variables

1. Fatty acid levels in the serum, which are planned to report as another clinical study.

Measure fatty acid levels in the serum obtained at 5 months of age.

Twenty-four kinds of fatty acids such as arachidonic acid will be measured by high-performance liquid chromatography, which may be outsourced to SRL, using residual serum samples obtained at 5 months of age.

Research Implementation System

I. Clinical examination

- a. Pediatrician: Dr. Mitsuyoshi Urashima: Enrollment and participants' clinical examination before allergic signs. If participating infants have any allergic signs, e.g., food-specific IgE ≥ 0.35 UA/ml blood sampling at 5 months of age, then they are referred to Dr. Tachimoto to diagnose atopic diseases and for follow-up.

At the outpatient clinic of The Maternal and Child Center in Jikei University Hospital

- b. Pediatrician: Dr. Hiroshi Tachimoto: Clinical examination after allergic signs and diagnosis of atopic diseases with or without the oral food challenge (OFC) test, management, and follow-up.

At the outpatient clinic of Pediatrics in Jikei University Hospital

II. Endpoint committee

Among the following five members who do not know the allocation group and will not examine these participating infants at outpatient clinics, if ≥ 3 recognize the outcome as positive, the outcomes are finally determined to be positive.

1. Hidetoshi Mezawa, M.D., Ph.D.
2. Takashi Urashima, M.D., Ph. D.
3. Daishi Hirano, M.D., Ph.D.
4. Mai Okuyama, M.D.

5. Noriko Gocho, M.D.
Department of Pediatrics
The Jikei University School of Medicine
Tokyo, Japan

What the committee is expected to determine.

These five members determine whether the infant has had an episode of FA or not.

0. No FA

1. Immediate allergic reaction

2. Anaphylaxis

For 1 (and 2) above, they determine the status at the second birthday by reviewing detailed episodes, results of the OFC test, and levels of food-specific IgE.

1. Sustained unresponsiveness

Cow's milk allergy, the infant can drink raw milk ≥ 50 ml

Egg allergy, the infant can eat baked egg ≥ 30 g,

Wheat allergy, the infant can eat udon noodle ≥ 15 g

2. Probable unresponsiveness, e.g., in the process of increasing dose of allergenic food, but not yet reach to predefined dose of unresponsiveness mentioned above.

3. Not yet unresponsive, when the infant eats, he or she still reacts to the allergenic food.

4. Not sure because the infant has not consumed the food allergen

The infants are still state of either 2, 3, or 4 above, they are considered not yet outgrow = remain food allergy.

III. Data monitoring

A) Randomization using a block of four by Dr. Akio Nakashima who does not interview participating families and does not examine their infants.

B) Data monitoring

If the frequency of adverse events deviates in either intervention group, the committee reports the data to the Safety and Data Monitoring Committee as well as the lead investigator.

- a. Akio Nakashima, M.D., Ph.D. (physician and nephrologist)
- b. Ms. Haruka Wada (secretary at Molecular Epidemiology)

Division of Molecular Epidemiology
The Jikei University School of Medicine
Tokyo, Japan

IV. Safety and Data Monitoring Committee

- a. Judging from data of interim analysis, this committee may recommend stopping or continuing the trial.
- b. Responding to a report of adverse events, the committee may recommend stopping the trial or allowing the trial to continue.

- ① Motohiro Ebisawa, M.D., Ph.D.
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Participants

Inclusion criteria

- 1. Be in a high-risk group for atopic sensitization: at least one of the father, mother, brothers, or sisters of infants has atopic diseases (asthma, atopic dermatitis, FA, allergic rhinitis, hay fever)
- 2. Obtained written informed consent.
 - a. Participants who understand randomization into two groups: BF with or without amino acid milk and BF with CMF.
 - b. Blood examinations at 5-6 months and 2 years of age are required.
- 3. Be available to visit the outpatient clinic at 1 month, 2 months, 3 months, 4 months, 5

months, 7~8 months, 9~10 months, 12 months, 14~15 months, 18 months, and 24 months of age.

Exclusion criteria

1. Less than 36 weeks of gestational age at birth or birth weight less than 2000 g.
2. Having serious congenital anomalies.
3. Being a triplet or more: twins may participate.
4. Abnormal delivery such as asphyxia (Apgar score 6 or less at 5 minutes after birth)

Interventions

1. BF±EF

Mothers in this group are asked to maintain exclusive BF. However, if the amount of breast milk is not sufficient, then EF may be added until starting solid food. If the mother needs to supply EF more than 150 ml/day for 3 consecutive days in addition to BF because of a low amount of breast milk, EF is switched to CMF. Thus, the offspring allocated to BF±EF can avoid CMF for at least the first 3 days of life.

2. BF+CMF

Mothers in this group are asked to maintain exclusive BF, but are also asked to simultaneously supply a small amount of CMF ≥ 5 ml/day from the first day of life until 1 month, and ≥ 40 ml/day after 1 month until weaning. When infants are born after 18:00, CMF may be added on the 2nd day.

Both CMF (Hohoemi[®]) and EF (Meiji Elemental formula[®]) are purchased from Meiji Holdings Co. Ltd. (Chuo-ku, Tokyo, Japan).

EF (Meiji Elemental formula[®])

For infants with FA, total proteins are replaced with refined amino acids. However, other fats, carbohydrates, vitamins, and minerals are basically the same as in CMF. Energy is 66 kcal/100 ml (17%) and 391 kcal/100 g powder.

Blinding to allocation group

- a. Participating family: Not blinded
- b. Dr. M.U.: Not blinded, because he needs to confirm compliance with the intervention by interviewing participating families at the outpatient clinic in the Maternal and Child Center of Jikei University Hospital. When an infant has food-specific IgE ≥ 0.10 U_A/ml at 5 months blood test, the infant is referred to Dr. H.T. at the outpatient clinic of Pediatrics without informing Dr. H.T. about the allocation group.
- c. Dr. H.T.: Blinded, who is a specialist in pediatric allergic diseases, diagnoses FA, without knowing the allocation group.
- d. Endpoint committee: Blinded and no contact with the participants.

Outcome Measures

Primary outcome

1. Atopic sensitization:

The primary outcome was atopic sensitization to cow's milk protein, i.e., serum levels of cow's milk-specific IgE (CM-IgE) ≥ 0.35 UA/ml at 24 months of age. Serum levels of CM-IgE at 5 and 24 months of age as a continuous variable were used as reference values for primary outcome. Other atopic sensitization to egg white and wheat ≥ 0.35 UA/ml at 24 months of age were also evaluated to be compared with cow's milk sensitization. Outsourced for measuring with ImmunoCAP Specific IgE® (Thermo Fisher Diagnostics K.K., Minato-ku, Tokyo, Japan) to SRL Inc. and compared as both continuous and dichotomous (CM-IgE: ≥ 0.35 UA/ml) variable between BF±EF and BF+CMF group.

Secondary outcomes

1. Immediate type of FA

Presenting with at least one organ symptom of skin (urticaria, angioedema, erythema, pruritus, periorbital edema, etc.), respiratory (dry staccato cough, hoarseness, wheezing, etc.), gastrointestinal (nausea, vomiting, colicky abdominal pain, etc.), or circulatory (tachycardia, hypotension, loss of consciousness, etc.) problems triggered by the food ingestion that appeared within a few hours, in combination with blood examination measuring serum levels of the suspected food-specific IgE: ≥ 0.35

U_A/ml by means of a fluorescence enzyme immunoassay.

In this trial, symptoms triggered by skin contact directly with food or indirectly with food-contaminated hands are not included.

The OFC test (Appendix 1) is performed around 1 year of age for infants who are sensitized to certain foods but for whom clinical reactivity after ingestion of the food allergen is unknown. Thus, immediate allergic reaction is a food-specific IgE: ≥ 0.35 U_A/ml plus either a FA episode or positive OFC test. FA is re-evaluated regarding whether the infant could eat the causative food allergen without an allergic reaction at the 2-year-old birthday.

2. Anaphylactic type of FA

When symptoms are derived from multiple (at least two of above) organs, e.g., urticaria and wheezing, the condition is defined as an anaphylactic reaction.

3. Desensitization of FA (= Sustained unresponsiveness or outgrow)

If infants, who had been once diagnosed as immediate type of FA especially with cow's milk allergy, egg allergy, and wheat allergy, can eat the allergenic food (raw milk ≥ 50 ml, baked egg 30g, udon noodle 15g) without allergic signs by 2nd birthday, then they are defined as 'sustained unresponsiveness = outgrow' to the food allergen in this trial.

4. Each food allergy

- a. Cow's milk allergy was defined allergic reaction by OFC-test or cow's milk intake with or without heated or processed with cow's milk-specific IgE are positive (≥ 0.35 U_A/ml).
- b. Egg allergy was defined allergic reaction by OFC-test or egg intake with or without heated or processed with egg white-specific IgE are positive (≥ 0.35 U_A/ml).

- c. Wheat allergy was defined allergic reaction by OFC-test or wheat intake with or without heated or processed with wheat-specific IgE are positive (≥ 0.35 U_A/ml).
- d. Other food allergy was defined allergic reaction by OFC-test or a certain food intake with or without heated or processed with the food-specific IgE are positive (≥ 0.35 U_A/ml).

Exploratory outcomes: which are planned to report in another opportunity.

1. Atopic dermatitis

Check the SCORAD index¹⁴ at the 1- and 3-month check-ups.

2. Asthmatic bronchitis

A stringent index that includes frequent wheezing during the first 3 years of life and either one major risk factor (parental history of asthma or eczema) or two of three minor risk factors (eosinophilia, wheezing without colds, and allergic rhinitis).¹⁵

Major Criteria

- 1. Parental asthma†
- 2. Eczema diagnosed by a medical doctor‡
- 3. Sensitization with elevated mite- or house dust-specific IgE ≥ 0.10 U_A/ml

Minor Criteria

- 1. Allergic rhinitis diagnosed by a medical doctor§
- 2. Wheezing apart from colds
- 3. Eosinophilia ($\geq 4\%$)

3. Sensitization to Japanese cedar pollen

Japanese cedar pollen-specific IgE ≥ 0.10 U_A/ml

4. Allergic rhinitis

Allergic rhinitis: Rhinitis signs and symptoms for 1 month and mite-specific IgE ≥ 0.10 U_A/ml

Safety outcomes

The following are obtained from an interview at the outpatient clinic just after the 2-year-old birthday.

1. Admission for any reason
2. Emergency room visit for any reason
3. Growth retardation: Height and weight at 2-year-old birthday

Flow of participants

1. Informed consent and registration

- a. Pregnant women and their husbands are informed about the trial in detail at a childbirth class during late pregnancy through a talk by Dr. M. Urashima and the informed consent document. Written, informed consent is sent to the data monitoring center at the Molecular Epidemiology Division of Jikei University School of Medicine.
- b. Current or previous family history of atopic diseases (asthma, atopic dermatitis, FA, allergic rhinitis, hay fever) is obtained through questionnaires as well as parental demographic information such as maternal age and body weight/height. Only pregnant women who are confirmed to have a family history of atopic diseases are included.
- c. Food questionnaires of mothers (Appendix 3) are taken during the third trimester of pregnancy and at 1, 3, and 5 months of age after birth.

2. At the maternity ward of Jikei University Hospital

- a. Some included pregnant women are excluded after delivery, due to, e.g., cleft palate.
- b. Perinatal information, e.g., gestational weeks and birth weight, are taken from charts.
- c. Nursing staff is informed of the participants' names and to which group they are allocated.
- d. Mothers are informed to which group they belong after delivery; thus, mothers are not blinded to the allocation group.
- e. Mothers belonging to the BF+CMF group are asked to supplement BF with CMF at least 5 ml from the first day of delivery. However, if the delivery time is after 18:00,

they are allowed to supply CMF in addition to BF starting at midnight of the 2nd day.

- f. Mothers belonging to the BF±EF group may add amino acid-based EF when they feel that BF is not enough. If the mother adds more than 150 ml/day EF to BF for 3 consecutive days, EF is switched to CMF. Thus, offspring allocated to BF±EF can avoid CMF for at least the first 3 days of life.
- g. Nurses at the maternity ward are allowed to feed babies instead of their mothers.

3. At the outpatient clinic in the Maternal and Child Center of Jikei University Hospital

- a. Participating parent(s) are interviewed, and their children are examined at least seven times from 1, (2,) 3, (4,) 5-6, (7-8,) 9-10, 12, (14-15,) 18, and 24 months of age at the outpatient clinic of Jikei University Hospital by a study pediatrician (M.U.) who is not blinded to the allocation group.
- b. If infants have exanthema, the pediatrician provides their parents clear guidance about aggressive skin care such as how to wash their face and body using soap and a shower by showing them a video.
- c. Blood is sampled from all infants at 5-6 and 24 months.

4. At the outpatient clinic in Pediatrics of Jikei University Hospital

In case of at least one antigen-specific IgE is 0.1 IU_A/ml or more, the infant is referred to a study specialist of pediatric allergy (H.T.) at the same Jikei University Hospital who does not know the infant's allocation group.

5. Pediatrics Ward of Jikei University Hospital

The OFC test is performed at admission (Appendix 1) by pediatricians who are blinded to the allocation group.

6. Efforts to reduce loss to follow-up

- a. Using email, we try to contact participants who move to distant places regarding whether their infants have FA and make efforts to reduce the number of infants lost to follow-up, from whom IgE data are not obtained at 5 or 24 months.
- b. If participants do not visit the outpatient clinic after their 2-year-old birthday and

we receive no responses to the email, they are considered lost to follow-up.

7. Blood sampling

All infants undergo blood examinations to measure serum total and antigen-specific IgE.

- a. At the 5- to 6-month check-up prior to first solid food introduction:
Egg white, cow's milk, wheat, mites.
- b. At 2 years of age:
Egg white, cow's milk, wheat, mites, Japanese cedar pollen, dog dandruff, cat dandruff.
- c. Unscheduled visits are made to the clinic when participating infants have atopic symptoms, particularly suspicious of FA.
e.g., exanthema just after eating an egg dish.

8. Randomization

a. EF and CMF

Both CMF (Hohoemi[®]) and EF (Meiji Elemental formula[®]) are purchased from Meiji Holdings Co. Ltd. (Chuo-ku, Tokyo, Japan).

b. Randomization

Pregnant women that meet the inclusion criteria are randomly assigned to either the BF+CMF or BF±EF group according to an identification number generated by computer based on permuted blocks of four that are determined beforehand (Appendix 2). Staff members at the maternity ward are informed of which intervention applies to each pregnant participant before hospitalization for childbirth.

c. Blinding

According to the nature of the intervention, we do not use double blinding. However, we try to minimize bias using the following strategies:

Primary outcome: Cow's milk-specific IgE as an objective indicator.

Secondary outcomes: determine by prospective, randomized, open, blinded-endpoint (PROBE), determined by the endpoint committee members who do not examine the

infants or are blinded to the allocation group'.

9. Compliance with CMF or EF

1. At the maternity ward, every day's CMF or EF intake is monitored by each participant (mother). The nurse may help with the intervention and monitoring.
2. At the outpatient clinic, mothers are asked whether they are using CMF, EF, or exclusive BF.

10. Monitoring of secondary outcomes

a. Scheduled

At either the Maternal and Child Center or Pediatrics of Jikei University Hospital, participating infants are examined frequently.

b. Unscheduled

If the infants have any allergic signs, e.g., urticaria after eating allergenic food, they are advised to call Jikei University Hospital. H.T. or other pediatricians try to examine the infants on the day or next day and perform a blood examination.

Statistical analysis

1. Sample size

300

2. Sample size calculation

300 pregnant women and their offspring

We hypothesized that cow's milk sensitization at 2 years of age, i.e., cow's milk-specific IgE ≥ 0.35 U_A/ml, would be present in 10% of one group of infants and 25% of the other group, with a type I error (two-sided) of 5% and a power of 90%, on the assumption of 3% loss to follow-up. Therefore, 1:1 divided samples of approximately 300 participants were calculated as sufficient to detect a significant difference.

. sampsi .1 .25

Estimated sample size for two-sample comparison of proportions

Test Ho: $p_1 = p_2$, where p_1 is the proportion in population 1
and p_2 is the proportion in population 2

Assumptions:

alpha = 0.0500 (two-sided)
power = 0.9000
 $p_1 = 0.1000$
 $p_2 = 0.2500$
 $n_2/n_1 = 1.00$

Estimated required sample sizes:

$n_1 = 146$
 $n_2 = 146$

$n = 292$

Assuming 3% loss to follow-up, 300 are needed to enroll.

3. Study period

We assume that 100 pregnant women per year will participate in this trial. Therefore, the accrual period will be 3 years. The last infant is followed up 2 years later. Thus, a total of 5 years is predicted to be needed to accomplish this trial.

4. Interim analysis

Interim analyses are planned two times at reaching 100 and 200 participating infants who had their second birthday. The P value for significance at the interim analysis is set as <0.001 according to the Peto's stopping boundaries.

5. Planned methods of analysis

Outcomes are analyzed by intention-to-treat analyses between the two comparative groups.

- a. Serum levels of cow's milk-specific IgE at 5 months and 24 months of age as a continuous variable are compared using Mann-Whitney test.
- b. Risk of cow's milk-specific IgE ≥ 0.35 U_A/ml will be analyzed with a 2x2 table with the chi-square test and represented by the risk ratio (RR), and 95% confidence interval (95% CI).
- c. Secondary outcomes will be compared with RR, and 95% CI.
- d. Safety outcomes will be compared with RR, RD and 95% CI or Student's t test.
- e. Subgroup analysis will be done according to tertiles of 25(OH)D.
- f. All reported P values will be two-sided.
- g. Values with $P < 0.05$ will be considered statistically significant.
- h. All data will be statistically analyzed using Stata 14.0 (StataCorp LP., College Station, TX, USA).

6. Subgroup analyses

To clarify whether BF $\pm$ EF or BF+CMF significantly affects the subgroups of 25(OH)D (e.g., tertile, quartile, quintile), P for interaction ($P_{\text{interaction}}$) is computed by creating multiplicative variables. The results of these analyses are not corrected for multiple comparisons.

Safety

Many Japanese maternity wards recommend BF, but allow mothers or nurses to help by supplying CMF to supplement BF after feeding colostrum, e.g., 6 to 10 hours after birth. This is a food intervention trial. Thus, basically no safety concerns exist. However, the following are compared.

- a. Admission due to any disease
- b. Emergency visit or admission for any reason
- c. Body height and weight at 24 months of age

Early withdrawal

Unknown serious adverse events.

Compensation

Mitsui Sumitomo Insurance Fire provides liability insurance (NB41027726) for the principal investigator, to compensate for costs of treating disability as a result of side effects caused by consuming milk.

The maximum amount of compensation is 100,000,000 yen per person and 300,000,000 yen per trial.

Consideration concerning the protection of human rights and privacy

1. Written informed consent is obtained after sufficient explanation.
2. Participants can withdraw from the trial after providing written, informed consent.
3. Withdrawal is not considered a disadvantage for participants.
4. Private information is exchanged with study ID at the Jikei University School of Medicine; therefore, private information cannot leak from there (linkable anonymizing).

Publication of research results

We aim to publish the results of this trial in a scientific journal or present them at academic conferences. Personal information will be carefully concealed when we present the results.

Research funds

1. JSPS KAKENHI Grant Number JP16K09074
2. the Practical Research Project for Allergic Disease and Immunology of the Japan Agency for Medical Research and Development, AMED (15ek0410019h0101)
3. Dairy Products Health Science Council and Japan Dairy Association

Attribution of intellectual property rights

If intellectual property rights such as patent rights become relevant, such rights will be attributable to the investigator.

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Appendix 1

Table 1. Oral Food Challenge (OFC) test

Oral Food Challenge (OFC) test			
Hen's egg	STEP1	egg yolk x 1 (As pumpkin cake)	70 g
	STEP2	whole egg x 1/2 (As pumpkin cake)	70 g
	STEP3	whole egg x 1 (As scrambled egg)	45 g
Milk and its products	STEP1	Heated milk (As pumpkin cake)	1 ml
	STEP2	Heated milk (As pumpkin cake)	10 ml
	STEP3	Heated milk (As potage soup)	50 ml
	STEP4	Yogurt	48 ml
Wheat	STEP1	Flour (As pumpkin cake)	1 g
	STEP2	Flour (As pumpkin cake)	5 g
	STEP3	Flour (As pumpkin cake)	15 g
	STEP4	Flour (As pumpkin cake)	30 g
		Udon dried noodles 3g = Flour 1g = wheat protein 0.08g	
		Udon dried noodles 15g = Flour 5g = wheat protein 0.4g	
		Udon dried noodles 50g = Flour 15g = wheat protein 1.3g	
		Udon dried noodles 100g = Flour 30g = wheat protein 2.6g	
Soy bean	STEP1	Tofu	100 g
Peanuts	STEP1	Peanuts	2 g
	STEP2	Peanuts	10 g
Almond	STEP1	Almond	2 g
	STEP2	Almond	10 g
Walnuts	STEP1	Walnuts	2 g
	STEP2	Walnuts	10 g
Cashew Nuts	STEP1	Cashew Nuts	2 g
	STEP2	Cashew Nuts	10 g
Sesame (other kinds of nuts)	STEP1	Sesame	2 g
	STEP2	Sesame	10 g
Soba	STEP1	Soba	15 g
	STEP2	Soba	50 g
	STEP3	Soba	100 g
Meat		Boiled meat	50 g
Fish		Boiled Fish	40 g
Crustacean Soft body		Shrimp, Crab, Squid, Octopus (served as Hamburger)	15 g
Shellfish		Clams, Scallop, etc. (served as hamburger)	20 g
Fish egg		Baked Tarraco	20 g
		Salmon roe	10 g
Potatoes		Boiled Potato Soup	100 g
Corn		Corn compote	30 g
Fruits		Strawberry Puree	120 ml

Appendix 2

Table 2. Permuted blocks of four

1	2	3	4	5	6
A	A	P	P	A	P
A	P	A	P	P	A
P	P	A	A	A	P
P	A	P	A	P	A

Appendix 3

Questionnaires about food intake

If you obtained written informed consent from the participant, please send the following information by FAX.

Date: _____ year, _____ month, _____ day

Person who filled out the form:

✎ During the last month, how often did you eat each kind of food per week? Please check (☑).

	Rarely	Once per week	Several times per week	Once per day	Twice per day	Three times per day
Example	☐	☐	☒	☐	☐	☐
Place to eat meal						
Restaurant (eating out)	☐	☐	☐	☐	☐	☐
Ramen shop (eating out)	☐	☐	☐	☐	☐	☐
Fast food (hamburger)	☐	☐	☐	☐	☐	☐
Fast food (rice bowl)	☐	☐	☐	☐	☐	☐
Fast food (soba, udon)	☐	☐	☐	☐	☐	☐
Lunch box bought at convenience store	☐	☐	☐	☐	☐	☐
Cafeteria at work place	☐	☐	☐	☐	☐	☐
At home	☐	☐	☐	☐	☐	☐
Foods						

Butter	☐	☐	☐	☐	☐	☐
Margarine	☐	☐	☐	☐	☐	☐
Vegetable oil	☐	☐	☐	☐	☐	☐
Vegetable oil Please check the most appropriate answer	Safflower oil	Canola oil	Sesame oil Soy oil Sunflower oil	Peanut oil, Coconut oil, Corn oil, Palm oil	others	
Olive oil	☐	☐	☐	☐	☐	☐
Biscuits ^{*1}	☐	☐	☐	☐	☐	☐
Junk food (potato chips, etc.)	☐	☐	☐	☐	☐	☐
Chocolate	☐	☐	☐	☐	☐	☐
Mayonnaise ^{*2}	☐	☐	☐	☐	☐	☐
Plain bread	☐	☐	☐	☐	☐	☐
Pastry	☐	☐	☐	☐	☐	☐
Croissant	☐	☐	☐	☐	☐	☐
Other kinds of bread						
Instant noodles	☐	☐	☐	☐	☐	☐
Fried food at home (tempura, fried chicken, fried potatoes, etc.)	☐	☐	☐	☐	☐	☐
Fried food outside (tempura, fried chicken, fried potatoes, etc.)	☐	☐	☐	☐	☐	☐
Beef (including hamburger) ^{*3}	☐	☐	☐	☐	☐	☐
Processed meat (sausage, salami, hot dogs, bacon, etc.)	☐	☐	☐	☐	☐	☐

Cow's milk	☐	☐	☐	☐	☐	☐
Cheese	☐	☐	☐	☐	☐	☐
Creams (include cream for coffee)	☐	☐	☐	☐	☐	☐
Ice cream	☐	☐	☐	☐	☐	☐
Pork	☐	☐	☐	☐	☐	☐
Chicken	☐	☐	☐	☐	☐	☐
Heated eggs	☐	☐	☐	☐	☐	☐
Raw eggs	☐	☐	☐	☐	☐	☐
Fish eggs (salmon roe, flying fish roe, roe, sea urchin, dried mullet roe, caviar, etc.)	☐	☐	☐	☐	☐	☐
Soy (including soy food, natto, tofu, etc.)	☐	☐	☐	☐	☐	☐
Soba	☐	☐	☐	☐	☐	☐
Tuna (canned tuna, sashimi, etc.)	☐	☐	☐	☐	☐	☐
Swordfish	☐	☐	☐	☐	☐	☐
Salmon (including canned salmon)	☐	☐	☐	☐	☐	☐
Mackerel, sardine, horse mackerel, Pacific saury (including canned)	☐	☐	☐	☐	☐	☐
White fish (red snapper, etc.)	☐	☐	☐	☐	☐	☐
Peanuts (including peanut butter)	☐	☐	☐	☐	☐	☐
Walnuts	☐	☐	☐	☐	☐	☐
Almonds	☐	☐	☐	☐	☐	☐
Cashew nuts	☐	☐	☐	☐	☐	☐
Macadamia nuts	☐	☐	☐	☐	☐	☐

Hazelnuts	☐	☐	☐	☐	☐	☐
Coconut (including processed food)	☐	☐	☐	☐	☐	☐
Other kinds of nuts						
Supplement						

*1. Biscuits include cream puffs, sponge cake, donuts, etc.

*2. Mayonnaise includes creamy salad dressing.

*3. Beef includes heart, liver, diaphragm, and stomach.

Summary of changes to the Trial Protocol and Statistical Analysis Plan (SAP)

Ver. 1. 2013.8.1

Ver. 2. 2016.7.22

Ver.3. 2018.4.1

Ver. 2: certified on 2016.7.22 by the Jikei ethics committee.

1. Measurement of serum levels of free fatty acids

As exploratory analyses, we decided to analyze serum levels of free fatty acids using the residual serum samples stocked in deep freezers (-80°C).

Associations among food intake questionnaires, serum levels of free fatty acids, and FA are analyzed, as exploratory study.

We added newly followings in SAP.

- a. Associations between free fatty acids and FA are analyzed.
- b. Associations between answers to questionnaires on food intake and FA risks are explored.
- c. Associations between answers to questionnaires on food intake and free fatty acids are explored.
- d. Associations between 25(OH)D levels and growth at the 2-year-old birthday will be compared.

2. Key secondary outcomes were rearranged.

Definition was not changed but separated into secondary outcomes and exploratory outcomes. In addition, each food allergy was made clear.

From;

Key secondary outcomes

1. immediate type of food allergy including anaphylaxis
2. worsening of atopic dermatitis
3. desensitization of food allergy
4. atopic sensitization alone
5. asthmatic bronchitis

To;

Secondary outcomes: food allergy related

- I. Immediate type of food allergy (FA)
- II. Anaphylactic type of FA
- II. Desensitization of FA (= Sustained unresponsiveness or outgrow)
- III. Atopic sensitization alone
- III. Each food allergy
 - a. Cow's milk allergy
 - b. Egg allergy
 - c. Wheat allergy
 - d. Other food allergy

Exploratory outcomes: atopic conditions other than food allergy

- I. Atopic dermatitis
- II. Asthmatic bronchitis
- III. Sensitization to Japanese cedar pollen
- IV. Allergic rhinitis

Ver. 3: certified on 2018.4.1, to increase the credibility of the secondary outcomes.

1. New members in the endpoint committee

We added one general pediatrician and one specialist in pediatric allergies to the endpoint committee.

From;

- a. Hidetoshi Mezawa, M.D., Ph.D.,
- b. Takashi Urashima, M.D., Ph.D.,
- c. Daishi Hirano, M.D., Ph.D

To;

- a. Hidetoshi Mezawa, M.D., Ph.D.,
- b. Takashi Urashima, M.D
- c. Daishi Hirano, M.D., Ph.D
- d. Mai Okuyama, M.D
- e. Noriko Gocho, M.D.

Supplementary Online Content

Urashima M, Mezawa H, Okuyama M, et al. Primary prevention of cow's milk sensitization and food allergy by avoiding supplementation with cow's milk formula at birth: a randomized clinical trial. *JAMA Pediatr*. Published online October 21, 2019. doi:10.1001/jamapediatrics.2019.3544

eTable. Safety Outcomes

This supplementary material has been provided by the authors to give readers additional information about their work.

eTable. Safety outcomes

Outcomes	No. (%) of participants			
	BF±EF n = 156	BF+CMF n = 156	RR	95%CI
Admission due to any cause	21 (14)	16 (11)	1.31	0.71-2.42
Bronchopneumonia	7 (4.6)	4 (2.6)	1.75	0.52-5.85
MCLS ^a	4 (2.6)	3 (2.0)	1.33	0.30-5.86
Dehydration	2 (1.3)	4 (2.6)	0.50	0.09-2.69
Congenital anomalies	3 (2.0)	0 (0.0)	-	
Urinary tract infection	2 (1.3)	1 (0.7)	2.00	0.18-21.82
Inguinal hernia	1 (0.7)	1 (0.7)	1.00	0.06-15.84
Asthmatic bronchitis	1 (0.7)	1 (0.7)	1.00	0.06-15.84
Aseptic meningitis	1 (0.7)	1 (0.7)	1.00	0.06-15.84
Bone fracture	0 (0.0)	1 (0.7)	0.00	-
Emergency room visit only	29 (19)	26 (23)	0.81	0.52-1.25
Either admission or emergency room visit	50 (32)	51 (33)	0.95	0.71-1.35
Height at 24 months of age, mean (SD), cm	84.8 (2.8)	84.6 (2.7)		
Weight at 24 months of age, mean (SD), kg	11.6 (1.1)	11.5 (1.2)		

a. MCLS, mucocutaneous lymph node syndrome

Data Sharing Statement

Urashima. Primary Prevention of Cow's Milk Sensitization and Food Allergy by Avoiding Supplementation With Cow's Milk Formula at Birth. *JAMA Pediatr*. Published October 21, 2019. 10.1001/jamapediatrics.2019.3544

Data

Data available: Yes

Data types: Deidentified participant data

How to access data: https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000013538

When available: With publication

Supporting Documents

Document types: None

Additional Information

Who can access the data: Mitsuyoshi Urashima

Types of analyses: for a specified purpose

Mechanisms of data availability: after approval of a proposal