Title: Pediatric Eosinophilic esophagitis (pedEoE): effect of allergen heat denaturation on EoE remission: a pilot trial

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State of the art:

Eosinophilic esophagitis is a chronic, antigen (food), driven immune-mediated disease characterized by : 1) symptoms of esophageal dysfunction , 2) histologic evidence of eosinophilic inflammation of the mucosa and is typically 3) affecting local esophageal tissue (1). It is a relatively newly defined disease for which pediatric guidelines were first described in 2014 (figure 1).

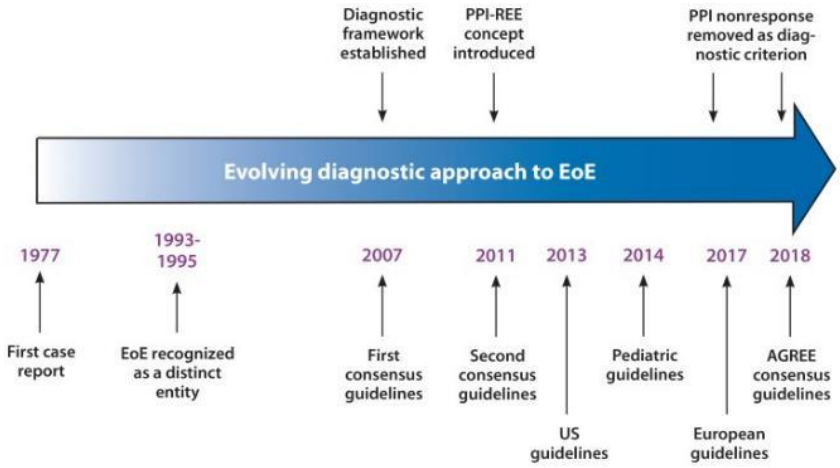


Figure 1 (1)

The **incidence** of EoE is estimated to be 3,7/100.000/year and its prevalence is steadily increasing and current rates are similar as eg in a related disease inflammatory bowel disease with rates 1:1,000 (2). The disease shows a familial pattern in 2% of the subjects, raising the possibility of either a genetic predisposition (presentation in monozygotic twins 41% and in dizygotic twins 22%) or exposure to an unknown but common environmental factor (80%). In children is the average age between 6 and 10 years with a Male predominance (70% males) as well as Caucasian predominance. This disease is certainly associated with atopy as 26-50% of children with EoE (pedEoE) also have asthma; 30-90% of them have allergic rhinitis, 19-55 % have atopic dermatitis and 9-24 % have a well-defined IgE mediated food allergy (2). However, our own personal data retrospectively obtained in 81 children with pedEoE (s64441) indicates an even higher rate of associated IgE-mediated food allergy and IgE-mediated comorbidities in over 80% of the children (PhD thesis Lisa Nuyttens-manama thesis Toon Dominicus).

Presenting **symptoms** in children are different depending on the age of manifestation and are listed in figure 2A and 2B and Figure 3 (3, 4). Typical food impaction, as observed in adults with EoE, can be the presenting symptom in adolescents, but is seldom a symptom in young

children in whom vomiting, chronic nausea and feeding problems might be more frequent. Children develop spontaneous coping mechanisms to live with the disease, such as drinking a lot of fluids during meals, being unable to eat unless a drink is available, chew the food for a long time and/of cut the food in small pieces (3).

A

Common symptoms of eosinophilic esophagitis	
Younger Children	**Older Children and Adolescents**
Vomiting	Epigastric pain
Chronic nausea	Dysphagia
Regurgitation	Nighttime cough
Irritability/feeding difficulties	Food impaction

B

Less common symptoms of eosinophilic esophagitis
Growth failure
Hematemesis
Esophageal dysmotility
Failure to thrive
Malnutrition

Figure 2 A and B: Typical symptoms in children with EoE depending on age of presentation (3)

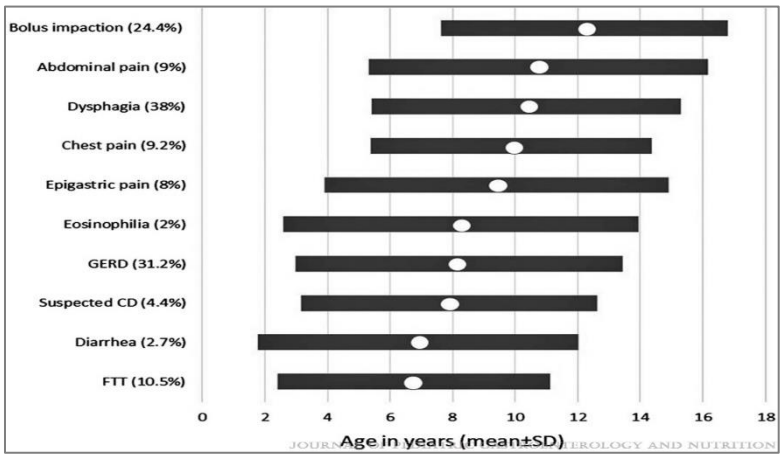


Figure 3: Spread of typical symptoms in children with EoE depending on age of presentation (4)

The disease can be **diagnosed** if in presence of typical signs and symptoms, a gastro-duodenoscopy with multiple biopsies in esophagus is performed. EoE can present macroscopically with furrowing, white mucosal plaques, esophageal trachealization, esophageal narrowing, stricture, and mucosal tearing (5). EoE is suspected when at least 15 eosinophils per high-powered field (hpf) (or 60 eos/mm^2) are documented and non-EoE disorders that cause or potentially contribute to Esophageal Eosinophilia are ruled out (1, 5). Sensitivity of 100% and specificity of 96% can be obtained if the number of proximal and distal biopsies in the esophagus is increased to 2-4 biopsies proximal and distal. Therefore this number is currently recommended for successful diagnosis (5). Similarly, remission is defined as < 15 eosinophils per high-powered field and also requires 2-4 biopsies both in proximal and distal esophagus (5)

Risk factors for the disease are represented in figure 4.

Risk factor	Comment
Aeroallergens	Might cause EoE or increase disease activity; can cross react with food allergens; may explain seasonal variation in diagnosis
Food allergens	Directly trigger EoE; elimination can lead to disease remission
Helicobacter pylori	Inversely associated with EoE; decrease in H. Pylori prevalence has accompanied increase in EoE prevalence over the last 20 years; mechanistic data lacking
Infections (herpex simplex virus; mycoplasma)	Associated with EoE; mechanistic data lacking
Oral or sublingual immunotherapy	Causes or induces EoE in certain patients; baseline EoE status for reported cases usually not know prior to immunotherapy
Proton pump inhibitors	Reported to induce IgE antibodies to certain foods
Cold or arid climates	Increased odds of EoE in these climate zones, but not in temperate or tropical zones
Population density	Odds of EoE increase as population density decreases
Early life factors	Antibiotic use, Cesaeran section, and preterm delivery increase the odds of pediatric EoE
Connective tissue disorders	Ehlers-Danlos, Marfan syndrome, and Loeys-Dietz syndrome have been associated with EoE
Celiac disease	Associated with EoE; EoE is more common in patients with celiac disease than would be expected
Autoimmune conditions	Inflammatory bowel disease , rheumatoid arthritis, igA deficiency, multiple sclerosis, and Hashimoto's thyroiditis associated with EoE

Figure 4: Typical associated diseases in patients with EoE depicted from Gastroenterol 2018 (5)

Unfortunately, EoE tends to become chronic and might lead to irreversible esophageal damage and esophageal strictures when untreated (6).

Treatment is needed to improve the quality of life of patients with EoE, to stop the deposition of subepithelial fibrous tissue in the esophageal wall and to reduce the risk of severe esophageal injury by preventing long-lasting food impactions. Whether treatment goals should be focusing on improvement of symptoms, on reducing the esosinophilic inflammation and/or prevention of remodeling and reversal of fibrosis, is subject of intense debate.

Treatment guidelines now uniformly advise the use of high-dose proton-pump inhibitors (PPI) as first treatment line for 8 weeks, after which a new biopsy is recommended to search for remission (7). If the patient is in remission (eosinophils <5/hpf), long-term PPI (1 mg/kg/d) treatment is recommended and continued as long as yearly biopsies remain negative (and hence might be lifelong). On the other hand, if a patient is not yet in remission (eosinophils > 15/hpf), or only has partial remission (eosinophils 5-15/hpf), a second treatment line is initiated. For this second treatment line, parents and children in accordance with their treating physician have two different options: 1) to start a treatment with local corticosteroids or 2) to start a treatment with food elimination diet.

1) Option 1: use of local corticosteroids:
    a) Swallowed corticosteroids which are highly effective (response rate (RR) 50-87 %): Fluticasone (normally used as inhaler) (110 microgram , 2-4 times/day)
    b) Budesonide (resolved in Xanthaan gum) <10 years : 1 mg; >10 years : 2 mg
    c) (in adults orodispersible Budesonide Jorveza® has recently been approved)

    Patients are recommended to avoid drinking and eating for at least half an hour after administration; high RR but risk of side effects such as Infection : Candida albicans (10-15%), adrenal suppression, epithelial atrophy, growth delay (8).

In UZ Leuven, if parents/children and treating physician agree on the use of option 1, treatment with local corticosteroids is initiated as Budesonide in Xanthaangum with subsequent biopsy to prove remission after 8 weeks treatment. If the patient is in remission, treatment is continued for at least a year till new biopsy, after which, if the patient remains in remission, sometimes a dose-reduction can be discussed. If the patient is not in remission, either an increase in the Budesonide dose or switch to option 2 will be discussed. Alternatively, the continuation of Budesonide with the start of a restriction diet in parallel can be discussed.

2) Option 2: use of diet (We recently published the remission rates for each of these diets based on meta-analysis (10).)
    a) Elementary diet (amino-acid (AA)) diet. First publication by Kelly *et al* in 1995); RR in children 90%-95%; duration minimum 6 weeks; clinical improvement observed after 8,5 ± 3,8 days, histological remission in 2 weeks (9). However, the diet is highly restrictive and almost impossible to

implement in older children even for a six week period. It moreover delays speech and has palatability issues (10). In real-life, the option of an elementary diet in Belgium after weaning is seldom discussed, as EoE unfortunately still forms no basis for reimbursement of the very expensive AA diet (although a reimbursement procedure has recently been started)(10).

b) Empiric elimination of common dietary antigens. This diet is usually referred to as "6-food, 4-food, 2-food or 1-food" diet :
6 food : cow's milk, soy, eggs, wheat, nuts and fish; 4 food : cow's milk, eggs, wheat, soy; 2 food : cow's milk and wheat & 1 food : cow's milk (9, 10). A step-down or step-up approach for these empiric eliminations can be used. When a step-down treatment is chosen, the patient will first eliminate all foods, after which (if remission is obtained), new food products will be gradually introduced and 8 weeks after the introduction of each new food product, a gastroscopy with biopsy will be done to check for the maintenance of remission. If EoE re-occurs, the newly introduced food product is causal and will be permanently eliminated. If the step-up approach is chosen, a single food product is eliminated and if no remission is visualized on biopsy 8 weeks after elimination, an additional food product will be eliminated until remission is obtained and those foods will be eliminated permanently. More specifically:
    a. Step-down treatment starts as an 8 weeks 6-food diet after which a new biopsy is needed to prove remission. After this initial remission the diet is reduced with one specific food rationally chosen by the caregiver, the parents and the child for 8 weeks after which a new biopsy needs to prove whether or not the newly introduced food can stay in the diet or has to be removed again. This has to be repeated for each food product that will be reintroduced. As such, the diet starts with a high level of restrictions, has long diagnostic procedures and requires a high number of endoscopies. A new guideline tries to reduce the initial diet by starting from a 4 food diet instead, as nuts/peanuts and fish/shell-fish is only responsible for a limited number of cases (5)

b. Step-up treatment starts as an 8 week diet on cow's milk alone after which a new biopsy is taken to study remission. If remission is obtained (RR 33% in children), the patients remains on a cow's milk free diet. However, if there is no remission, a second food product needs to be eliminated. This in general might reduce the number of biopsies needed, but in children in whom after multiple weeks of limited and later-on very limited food product allowed, still no remission is obtained, the psychological barrier to add another food product to the forbidden list, which the chance of (again) failure to obtain remission, is an extreme burden.

c) Allergy-driven Food Elimination

Using allergy testing-guided food elimination in children results in a success rate in children that is slightly higher than the success rate of a 2-food elimination diet, but is lower than its rate with a 4-food elimination diet (2, 10). Its use is certainly more recommended in children when compared to adults in whom allergy-driven food elimination has a low success rate.

In UZ Leuven if parents, children and treating physician agree on option 2 (diet-driven treatment), in infants and pre-school children it is adviced to start with a 1-food diet (milk) and if unsuccessful (after 8 weeks diet new biopsy shows no remission) a step-up protocol to a 4-food diet (milk, eggs, wheat and soy) is adviced. As in Belgium soy intake in infants is usually limited, for the large majority of young infants and pre-school children this de facto means a 3-food diet (milk, eggs and wheat). In school-children and adolescents, children are advised to start with a 2-food or 4-food diet (depending on their willingness to adhere to the protocol) and to step-up to a 6-food diet immediately when after 8 weeks of initial diet no remission was obtained. Allergy testing usually occurs to rule out (associated) typical IgE mediated food allergy and/or pollen-associated risk factors with the chance of increased number of eosinophils in the esophagus during the pollen season and in this case, biopsies will not be taken during the pollen season.

However, whereas in IgE-mediated pediatric food allergy recent evidence exists that heat-denaturation of proteins might reduce their allergenicity and could lead to symptomfree introduction of certains recipes, nothing is known about the impact of heat denaturation on food proteins in pedEoE. Indeed, for several decades, it is known that food allergic

individuals tolerate heat-denaturated food proteins more easily than raw proteins. Moreover, the potential use of heat-denaturated food proteins to speed up tolerance towards allergens has recently been suggested. Hence, the hypothesis was generated that ingestion of small amounts of denaturated proteins might lead to higher rates of tolerance induction (11). We were able to demonstrate the change in food protein components upon heating of hen's egg allergen in specific recipes, and furthermore reported that by gradual introduction of progressively less heated hen's egg containing products, tolerance in infants was observed after 24-30 weeks in baked-egg tolerant subjects (12). In parallel, heat inactivation of cow's milk proteins has also been reported (13) and results in gradual tolerance in cow's milk allergic subjects (14). Moreover, the psychological impact of introduction of some heat denaturated food proteins in the diet of children with strict allergen avoidance, has been demonstrated to be of utmost importance (15). In pedEoE however, the effect of heat denaturation on the eosinophilic inflammation in the esophagus, has not yet been studied. We have several indications that allow us to hypothesize that heat denaturation in pedEoE could allow protein introduction while allowing remission rates, as observed in typical IgE-mediated food allergy. Indeed, pediatric EoE (pedEoE) (in contrast to EoE in adults) is linked in over 80% of cases to IgE mediated food and/or environmental allergy (PhD thesis Lisa Nuyttens, Manama thesis Toon Dominicus). Moreover, the same short list of 6 food products as described in food allergy, namely milk, hen's egg, wheat, soy, peanut/tree nuts, fish/shellfish is involved in more than 60% of the pedEoE cases (10). In an almost similar percentage (58%) when compared to 6-food diet, remission in pedEoE patients is possible using an allergy-test directed elimination diet (10) also pointing to involvement of IgE mediated allergy in pedEoE (2). Moreover, allergen oral immunotherapy protocols in IgE mediated food allergic patients on the opposite, can induce pedEoE in small numbers between 0.5-1% (16). We furthermore demonstrated in a patient with eosinophilic gastro-enteritis that plant-food pollen-associated eosinophilic increase can be the driving force of the disease (17). All of these point to the involvement of IgE mediated allergy in pedEoE opposed to EoE in adults. Finally, recently a report in a boy with cow's milk protein induced EoE, demonstrated that heat-denaturation of the cow's milk proteins induced remission, in a cow's milk driven pedEoE (18). We therefore will study, similarly as in children with IgE mediated food allergy, the possibility to introduce heat-denaturated cow's milk or hen's egg protein instead of complete avoidance in order to induce remission in pedEoE. To that aim, we intend to provoke 8 pedEoE children on a hen's egg and/or cow's milk free diet, with heat denaturated hen's egg and/or cow's milk proteins and plan a biopsy to study remission after 8 weeks of introduction at home. If the (re)introduction

was succesfull, we will introduce recipes with gradually less heated proteins after which sequentially a new biopsy will be performed after 8 weeks of introduction. Although it can be anticipated that introduction of the least heated step (raw egg and/or uncooked (UHT-)treated cow's milk) will induce recidive of the disease, we will discuss with parents whether or not they would accept an 8-weeks reintroduction protocol of the final step and afterwards observation of its effect on the esophagus in their children. One might indeed even hope for tolerance induction while using this graded protocol, similar as in food protein allergic subjects, although we have no indication of such a tolerance induction in EoE patients, who today are taught to avoid these allergens "live-long".

In contrast to the results related to allergen sIgE, allergen-specific IgG4 (sIgG4) is more consistently associated with oral tolerance (18bis). Allergen sIgG4 antibodies can compete with IgE for allergen binding to type II IgE receptor-expressing cells, such as basophils and mast cells, leading to the blockage of this receptor. The production of both IgE and IgG4 antibodies is known to be up-regulated by interleukin-4 (IL-4) produced from activated Th2 cells. However, IL-10 secreted by Treg cells can suppress IgE production and simultaneously increase IgG4 production (Figure 5). Therefore, IgE and IgG4 production are not always similarly increased. Not only are IgE and IgG4 important to predict reactivity to an allergen or to confirm food tolerance, also the ratio of IgE to IgG4 seems to play a key role (19). Okamoto *et al.* studied the concentrations of hen's egg sIgE and IgG4 antibodies during oral food challenges (OFC) with egg white in children sensitized to hen's egg. Hen's egg sIgE concentrations were higher in children with a positive OFC than in children with a negative OFC. On the other hand, the concentration of hen's egg sIgG4 antibodies was higher in the negative OFC than in the positive food challenge. However, the ratio of IgE to IgG4 seemed to be an even more useful parameter than IgE or IgG4 alone to predict clinical reactivity during hen's egg challenge as it also had a more accurate sensitivity and specificity (20, 21). Similarly for cow's milk, Savilahti et al. showed higher levels of cow's milk sIgE in cow's milk allergic children compared with those who became tolerant by the age of 3 years, but cow's milk sIgG4 serum concentrations were lower among patients with persistent cow's milk allergy than among patients tolerating cow's milk by the age of 3 years (20, 21).

Regulatory B (Breg) cells are immunosuppressive B cells, characterized by the production of IL-10, IL-35 and transforming growth factor beta (TGF-β). IL-10 secreting Breg (Br1) cells, are known for being the main producers of IgG4 (19). Through the secretion of IL-10, IL-35 and TGF-β, Breg cells are known to suppress the activation and proliferation of effector T

cells, and also induce the differentiation of Foxp3+ regulatory T cells and type 1 regulatory (Tr1) cells (Figure 5).

Some indication exist that systemic as well as local (in the esophagal lesion) food specific IgG4 could be linked to the EoE as well (22). In that sense, we might predict that if our hypothesis stands, serum food specific IgG4 could be used as biomarker for the disease (and/or for tolerance towards a certain food protein). Moreover, *in vitro* activation of PBMC from those pedEoE children by (gradually less heat-denaturated) food proteins (+/- the B cell activating cytokine IL-21 (23)) and afterwards flow cytometric B cell staining with B (and plasma) cell activation markers, could be developped as an *in vitro* diagnostic test to search for B cell activation in those children.

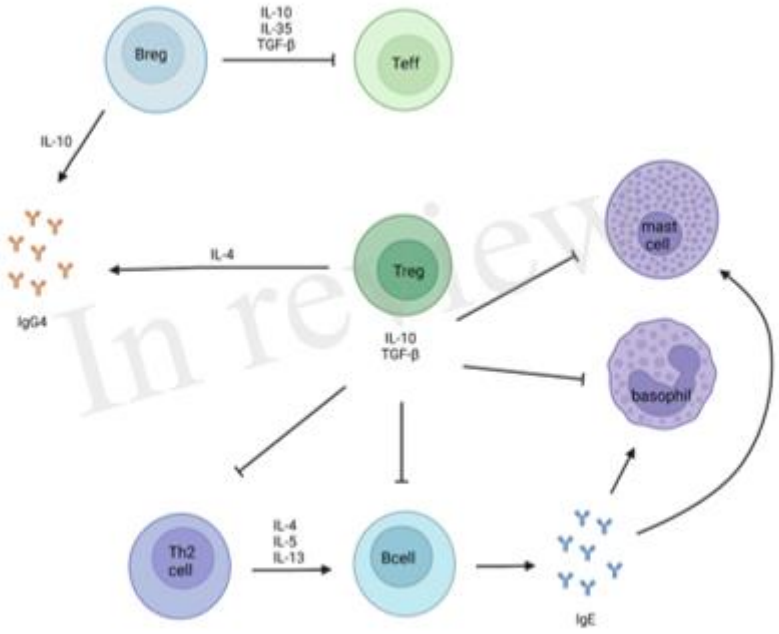


Figure 5: Figure by Nuyttens L *et al, in revision* (19)

Study objectives:

The objective of the study is to study whether the introduction of heated food products (more specifically heated hen's egg and/or cow's milk) in children with EoE would be possible without re-occurrence of the eosinophilic inflammation, while the intake of less heated products might cause disease recidive. Moreover, we would like to study whether the gradual re-introduction of less heated products after the most heated form is tolerated, could lead to tolerance induction in EoE.

Primary end-point:

- Primary end-point of the study is to study the number of ped EoE children by inclusion on hen's egg and/or cow's milk free diet with potential remaining remission (<15 eosinophils/hpf on esophagal biopsies) after 8 weeks (re)introduction of heat-denaturated hen's egg and/or cow's milk proteins (introduced sequentially as on the one hand baked egg (cake), hard-boiled egg, omelet and soft-boiled egg and on the other hand 20' cooked cow's milk, 15', 10' and 5' cooked cow's milk).

Secondary end-points:

- The number of pedEoE children in whom remission remain with introduction of cake and/or 20'cooked cow's milk (step 1), but no longer with less heated proteins
- To study whether remission is associated with specific circulating and/or local food protein IgG4 reduction (when baseline levels (serum) would be (stored) available) (and increase upon unsuccessful introduction of less heated proteins)
- To study whether remission could be associated with changes in local IgG4 production (on biopsy staining)
- To study whether we could develop an *in vitro* mimicking B cell activation test by stimulating PBMC with food proteins obtained after different heat-denaturating steps
- Can we observe a correlation between Pediatric EoE PEESS v2.0 and Peds-QL scores (obtained in all ped EoEchildren participating in study s64441) and histologic remission?
- To study the presence of associated atopy (tree-, grass- or weed pollen or fungi allergy)
- To study the presence of associated typical IgE mediated food allergy (sensitization and documented symptoms)

Material and methods

- *Inclusion criterion*: All children (aged 12 months and older) presenting (since 1-1-2014: moment of diagnostic guideline standardization for pedEoE) with diagnosed pedEoE at UZ Leuven eliminating either hen's egg or cow's milk or both and in complete remission after their latest biopsy (no longer than 12 months earlier, but preferentially as short as possible after their latest biopsy) are eligible for the study. If the last biopsy has been performed more than 12 months earlier, a new gastro-duodenoscopy will be performed to verify remission and rule out e.g. active gastritis. If they are on PPI and/or local budesonide treatment, this should be stable for at least 3 months and will remain untouched during the entire study. We will include 18 pedEoE subjects in total.
- *Exclusion criteria*:
    - Children younger than 12 months
    - Children with active pedEoE
    - Children who refuse to adhere to the protocol
    - Children with associated IgE mediated hen's egg and/or cow's milk allergy with specific IgE antibody titers that predict active (baked egg and/or baked milk) food allergy with cut-off titers as used at the consultation of allergy (KLL). Those children will become eligible however if their titers decrease while the study is still open.

Study design:

Study subjects and study procedure

If inclusion criteria are met, after obtaining informed consent from the parents (and when old enough, assent from the children) 18 children will be included in this pilot pedEoE trial. Children will be provoked in-house (UZ Leuven petit-cru), based on the written procedure available for heated egg (wheat or rice flour based) or cow's milk provocation. If on diet for both, parents and children will decide which allergen they will start with, but both food products will be introduced sequentially in time (this is after complete termination of the first food introduction). Afterwards, they will introduce heated egg by cake or 20' boiled cow's milk at least three times a week for 8 weeks (as for the TETI study (10)) and will return to the hospital for a gastroscopy with biopsies. EoE remission will be studied on 2X3 biopsies

(upper and lower esophagus) by eosinophil staining within the routine anatomopathological laboratory within our hospital.

If remission remains, subjects will be invited to introduce 10' hard-boiled egg (consecutively hard-boiled egg white; hard-boiled egg yolk) or 15'boiled cow's milk at home (2-3 times a week) for 8 weeks, after which they will return to the hospital for the second gastroscopy with similar procedure.

If remission is lost, they will return to the strict avoidance diet for at least 10 weeks (allowing 2 weeks of additional healing) after which they will return to the hospital for their second gastroscopy. Upon remission after avoidance, the children will remain eligible for a second heat denaturated food challenge (when on diet for both). When they are not on diet for both hen's egg and cow's milk, the study for them will end (final visit).

In children in whom remission remained after introduction of either hard-boiled egg or 15'boiled cow's milk, regular intake of 7' baked omelet or equivalent (waffle, pancake) or 10'boiled cow's milk will be introduced for 8 weeks after which they will return to the hospital for gastroscopy. Similarly as above, subjects will return to complete avoidance or (upon parental decision in agreement with treating physicians) they can return to introduction of the tolerated step (step 1) for 10 weeks, after which a new gastroscopy will be performed. When they are not on diet for both hen's egg and cow's milk, the study for them will end (final visit).

Similarly as above introduction of step 4, 4' soft-boiled egg or 5' boiled cow's milk will be introduced at home for 8 weeks afterwards upon remaining remission. Gastroscopy with biopsies is planned afterwards. If introduction of step 3 (omelet or 10' boiled cow's milk) was no longer associated with remission, children will return to complete avoidance or (upon parental decision together with treating physician) to step 1 or step 1+2.

Children who were able to tolerate step 4 for either hen's egg or cow's milk, will then be offered the possibility to choose for either termination of the study or (optionally) introduce either raw egg (tiramisu or chocolate mousse) or uncooked UHT-treated cow's milk for 8 weeks after which gastroscopy with biopsies will be performed.

For all steps if symptoms would (re)occur, gastroscopy with biopsy will be planned earlier and the introduction of the step will be on hold, until the biopsy would have proven remaining of remission. Upon EoE recidive, the patient will return to complete avoidance.

Number of study visits (n=9+4 telephone visits):

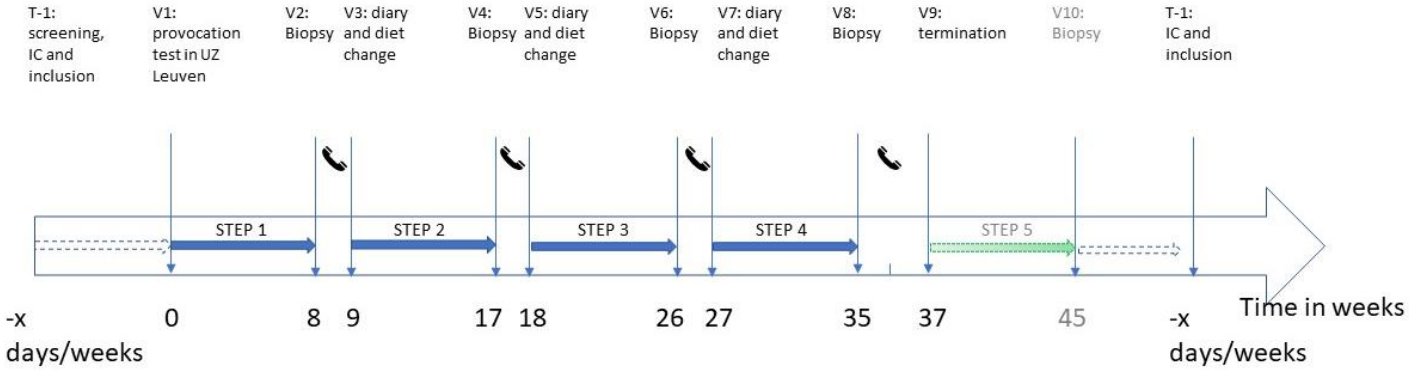
T-1 V0: screening visit: within SOC; informed consent and assent

T0 V1: visit 1: step 1 in hospital provocation and biomarker

T8 V2: 8 weeks later visit 2: gastroscopy with biopsy and biomarker

V2T: telephone guidance discussing biopsy results (Go/no go V3; if no go: V8 and V9 termination visit)

T9 V3: visit 3: clinical examination and dietary advice step 2



T17 V4: 8 weeks later visit 4: gastroscopy with biopsy and biomarker

V4T: telephone guidance discussing biopsy results (Go/no go V5; if no go V8 and V9 termination visit)

T18 V5: visit 5: clinical examination and dietary advice step 3

T26 V6: 8 weeks later visit 6: gastroscopy with biopsy and biomarker

V6T: telephone guidance discussing biopsy results

T27 V7: visit 7: clinical examination and dietary advice step 4

T35 V8: 8 weeks later visit 8: gastroscopy with biopsy and biomarker

V8T: telephone guidance discussing biopsy results

T37 V9: visit 9: termination visit

T45 V10 optional: new biopsy after step 5 (unlimited, unheated egg or UHT milk)

After at least 6 weeks post V9 (or V10 if applicable), inclusion for the second food allergen can occur (cross-over).

Blood and esophagus biomarker study

IgG4 staining on the biopsies will be performed by prof de Hertogh. Baseline blood (2X10 mL) will be obtained before the in-house provocation procedure and serum will be stored for specific IgG4 analysis later on. In parallel, PBMC will be isolated at the Allergy and Clinical

immunology Research Laboratory (LACI) and stimulated *in vitro* with heat denaturated proteins, as routinely performed within LACI. B (and plasma) cells will be stained by 4-laserflow cytometry (BD *Fortessa).* IgG4 production in supernatants will be studied by ELISA (specific IgG4 by Immunocap 100) and membrane bound IgG(4) (on biopsy material) by flow cytometry. This procedure will be repeated during each gastroscopy (when IV line is placed).
Parents will be offered a parking ticket for the visits in UZ Leuven and a lunch ticket during the day of the study visits with gastroscopy. After the study is finished, study participants (and their parents) will be offered a ticket for an attraction park (choice out of 4 parks).

Statistical analysis

The study is part of a funded FWO-TBM project that will start in October 2023. We will start the inclusion of the study trial from July 2023 on using internal funding for the first inclusion when needed. We hypothesize that the majority of children will start with hen's egg introduction, given the fact that more recipes containing hen's egg are existing and might change the lives of parents and children drastically when allowed. We hence do not anticipate or intend equal distribution of hen's egg and cow's milk inclusion. If in all 18 children step 1 induces reoccurrence of the disease, the study will be stopped and no cross-over to the second food allergen will any longer be possible.
Data reporting will be mainly descriptive. No statistical testing is foreseen.

Data policy

Ethics and regulatory approvals

The Participating Site and Investigator will perform the Study fully in accordance with the terms of the Protocol, this Agreement including all its appendices and Applicable Laws and Regulations (as defined as follows: the current version of the World Medical Association's Declaration of Helsinki, the guidelines and guidance documents specifying Good Clinical Practice ("GCP") and guidelines of competent authorities; all applicable laws, rules and legislation in relation to clinical trials, use of human biological material, data protection and the processing of personal data, and patient's rights, including but not limited to the General Data Protection Regulation 2016/679 ("GDPR") and the Belgian law of 30 July 2018 on the protection of natural persons with regard to the processing of personal data). The Participating Site will ensure that the Investigator and all employees, agents, contractors and/or the

representatives involved in the performance of the Study (jointly "Staff") are properly trained and sufficiently qualified. The Participating Site shall provide Staff with all necessary documentation to allow the proper performance of the Study, which shall be conducted in compliance with the Protocol. The Participating Site shall be responsible to ensure that all work performed by Staff is done in compliance with the Protocol, this Agreement and all Applicable laws and Regulations. The Participating Site undertakes to impose on all employees, agents, contractors and/ or representatives the terms and conditions not less strict than those set out in this Agreement.

Insurance/Indemnity

In accordance with the Belgian law relating to experiments in humans dated May 7, 2004, and/or with the Belgian law relating to clinical trials dated May 7, 2017 (as applicable) Sponsor shall be liable, even without fault for any damages incurred by the Study Subject and linked directly or indirectly to the participation to the Study, and shall provide compensation therefore through its insurance program.

Before commencing the Study, the Sponsor shall enter into an insurance contract which covers this liability, and the liability of every individual intervening in the Study, irrespective of the nature of the affiliation between the intervening individual, the Sponsor and the Study Subject. Every contractual provision aiming at limiting this liability is considered null and void.

Investigator and Sponsor shall have and maintain in full force and effect during the term of this Agreement (and following termination or completion of the Study to cover any claims arising from the Study) adequate insurance coverage for: (i) medical professional and/or medical malpractice liability, and (ii) general liability resulting from the Study at the Participating Site required by Belgian law

Confidentiality and privacy

Data will be stored for 25 years on a password-access limited and secured UZ Leuven server. The pseudonymization key will be stored separately from the data and only be accessible for the principal investigators of the study. The Sponsor, Participating Site and Investigators shall treat all information and data relating to the Study as confidential and with respect to the Participant's privacy. The Sponsor, Participating Site and Investigators shall not disclose

such information to any third parties or use such information for any purpose other than the performance of the Study.

Publication Policy

It is anticipated that the results of the overall Study shall be published in a publication, involving the data of all participants in the Study.
Publications will be coordinated by the Investigator of Sponsor. Authorship to publications will be determined in accordance with the requirements published by the International Committee of Medical Journal Editors and in accordance with the requirements of the respective medical jour

Adverse Event Reporting

1. Definitions

1.1. Adverse Event (AE)

An AE is any untoward medical occurrence in a patient or subject during an experiment, and which does not necessarily have a causal relationship with this treatment.

An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the use of a product, whether or not considered related to the product. Any worsening (i.e., any clinically significant adverse change in the frequency or intensity of a pre-existing condition) should be considered an AE.

1.2 Adverse Reaction (AR)

An AR is any untoward and unintended responses to an investigational medicinal product or to an experiment and, when an investigational product is concerned, related to any dose administered.

1.3 Serious Adverse Event (SAE)

An SAE is any untoward medical occurrence that results in any of the following:

- Death
- A life-threatening[a] experience
- In-patient hospitalisation or prolongation of existing hospitalisation
- A persistent or significant disability or incapacity
- A congenital anomaly or birth defect
- Important medical events that may be considered an SAE when - based on appropriate medical judgement - they may jeopardise the subject and may require medical or surgical intervention to prevent one of the above outcomes

[a] The term “life threatening” in the definition of SAE refers to an event in which the subject was at risk of death at the time of the event. It does not refer to an event which hypothetically might have caused death if it was more severe.

1.4 Adverse Events of Special Interest (AESI)

Define here any AEs that are of special interest (e.g. specific for the intervention) and must follow the same reporting timelines as SAEs.

The following events should be reported within the same timelines as SAEs:

- none

2 Adverse Events that do not require reporting

Define here any AEs or SAEs that are expected and do not require reporting for this trial. The following should not be reported as AEs:

- Pre-existing conditions, including those found as a result of screening (these should be reported as medical history or concomitant illness).
- Pre-planned procedures unless the condition for which the procedure was planned has worsened from the first trial-related activity after the subject has signed the informed consent.

The following events are commonly observed in the study population and are therefore not considered as adverse events for the purpose of the trial:

- (seasonal) allergic rhinoconjunctivitis, (seasonal) asthma attacks, atopic dermatitis

Although these events should not be reported to the Sponsor, these should be recorded in the patient's medical notes according to routine practice.

The following events not to be considered as SAEs are:

- Pre-planned hospitalisations unless the condition for which the hospitalisation was planned has worsened from the first trial-related activity after the subject has signed the informed consent.
- Hospitalisation as part of a standard procedure for protocol therapy administration. However, hospitalisation or prolonged hospitalisation for a complication of therapy administration will be reported as an SAE.
- Hospitalisation or prolongation of hospitalisation for technical, practical, or social reasons, in absence of an AE.

3 Recording and reporting of Adverse Events

See also: Safety Reporting ; Documents.

The information listed below that should be recorded for each AE can be supplemented with additional information. These data points should be incorporated in the (e)CRF.

Specific timelines for reporting AEs in the (e)CRF can be added, e.g. within 7 days after becoming aware.

Investigators will seek information on AEs during each patient contact. All events, whether reported by the patient or noted by trial staff, will be recorded in the patient's medical record and in the (e)CRF within a reasonable time after becoming aware. If available, the diagnosis should be reported on the AE page, rather than the individual signs or symptoms. If no diagnosis is available, the Investigator should record each sign and symptom as individual AEs.

The following minimum information should be recorded for each AE:

- AE description
- start and stop date of the AE
- severity
- seriousness
- causality assessment to the study interventions
- outcome

3.1 Assessment

If specific assessment references are to be used for events reported in the trial, this should be detailed in the below section. The below guidances can be modified according to the specifications of the trial.

All AEs must be evaluated by an Investigator as to:

- Seriousness: whether the AE is an SAE. See above for the seriousness criteria.
- Severity: Severity must be evaluated by an Investigator according to the following definitions:
    - Mild – no or transient symptoms, no interference with the subject's daily activities

- Moderate – marked symptoms, moderate interference with the subject's daily activities
- Severe – considerable interference with the subject's daily activities, unacceptable

- Causality:
  - None – An AE which is not related to the study-related interventions
  - Unlikely – An AE for which an alternative explanation is more likely (e.g. concomitant medication(s), concomitant disease(s)), and/or the relationship in time suggests that a causal relationship is unlikely
  - Possible – An AE which might be due to the study-related interventions. An alternative explanation is inconclusive. The relationship in time is reasonable; therefore the causal relationship cannot be ruled out.
  - Probable - An AE which might be due to the study-related intervention. The relationship in time is suggestive (e.g. confirmed by dechallenge). An alternative explanation is less likely.
  - Definitely – An AE which is known as a possible adverse reaction and cannot be reasonably explained by an alternative explanation. The relationship in time is very suggestive (e.g. it is confirmed by dechallenge and rechallenge).

3.2 Timelines for reporting

The below timelines for adverse event reporting are to be made study-specific. The safety reporting flow should be described in detail in the below section (eg. through eCRF or paper forms).

- After informed consent has been obtained and after initiation of study-related interventions:
  - All AEs, SAEs and AESIs causally related to a study-related intervention will be reported until 30 days after the last study-related intervention or until last follow-up visit (whichever occurs first).

All SAEs and AESI as defined in the protocol must be reported to the Sponsor within 24 hours of the trial staff becoming aware of the event. The immediate report shall be

followed by detailed, written reports. The immediate and follow-up reports shall identify subjects by Trial identification.

SAE details will be reported by the Investigator to the Sponsor:

- By completing the SAE form in the (e)CRF OR

If an authorised Investigator from the reporting site is unavailable, initial reports without causality and expectedness assessment should be submitted to the Sponsor by a healthcare professional within 24 hours of becoming aware of the SAE, but must be followed-up by medical assessment as soon as possible thereafter.

3.3 Follow-up

The Investigator must record follow-up information by updating the medical records and the appropriate forms in the (e)CRF. The worst case severity and seriousness of an event must be kept throughout the trial.

SAE follow-up information should only include new (e.g. corrections or additional) information and must be reported within 24 hours of the Investigator's first knowledge of the information. This is also the case for previously non-serious AEs which subsequently become SAEs.

- All SAEs must be followed up until the outcome of the event is 'recovered', 'recovered with sequelae', 'not recovered' (in case of death due to another cause) or 'death' (due to the SAE) and until all related queries have been resolved, or until end of trial (whichever occurs first).
- Non-serious AEs must be followed up until the patient's last study visit, and until all related queries have been resolved.

SAEs after the end of the trial: If the Investigator becomes aware of an SAE with suspected causal relationship to the study-related interventions after the subject has ended the trial, the Investigator should report this SAE within the same timelines as for SAEs during the trial.

3.4 Pregnancy

Female subjects must be instructed to notify the Investigator immediately if they become pregnant during the trial. The Investigator must report any pregnancy in subjects during the trial to the Sponsor.

3.5 Death

All deaths will be reported without delay to the Sponsor (irrespective of whether the death is related to disease progression, study procedure or is an unrelated event). The sponsor will notify all deaths, as soon as possible after becoming aware, to the Central EC and the EC of the concerned site and provide additional information if requested.

4 Reporting requirements to Ethics Committee's (EC's)

The Investigator is responsible for ensuring that all safety events are recorded in the (e)CRF and reported to the Sponsor in accordance with instructions provided below. The Sponsor will promptly evaluate all SAEs and AESIs against medical experience to identify and expeditiously communicate possible new safety findings to Investigators and based on applicable legislation.

4.1 Annual reporting

The sponsor has the obligation to, once a year throughout the clinical trial (or on request), submit a progress report to the EC's containing an overview of all SARs occurred during the reporting period and taking into account all new available safety information received during the reporting period.

4.2 Overview reporting requirements

	WHAT	HOW	TO	TIMELINES
Investigator	AE	AE form	sponsor	as defined in protocol
	SAE	SAE form	sponsor	Immediately (within 24 hours of becoming aware of the event) Exceptions: as defined in protocol
	death	SAE form	sponsor	asap
Sponsor	death	SAE form + narrative	Ethics Committees	asap
	Annual Progress Report	APR template	Ethics Committees	annually

5 Pharmacovigilance / Materiovigilance

The below section is applicable for experiments where a medicinal product will be used by the participants according to the standard of care.

Any adverse reactions related to a medicinal product used by the participants in the study, can be reported to the Compentent Authorities according to the pharmacovigilance guidelines.

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