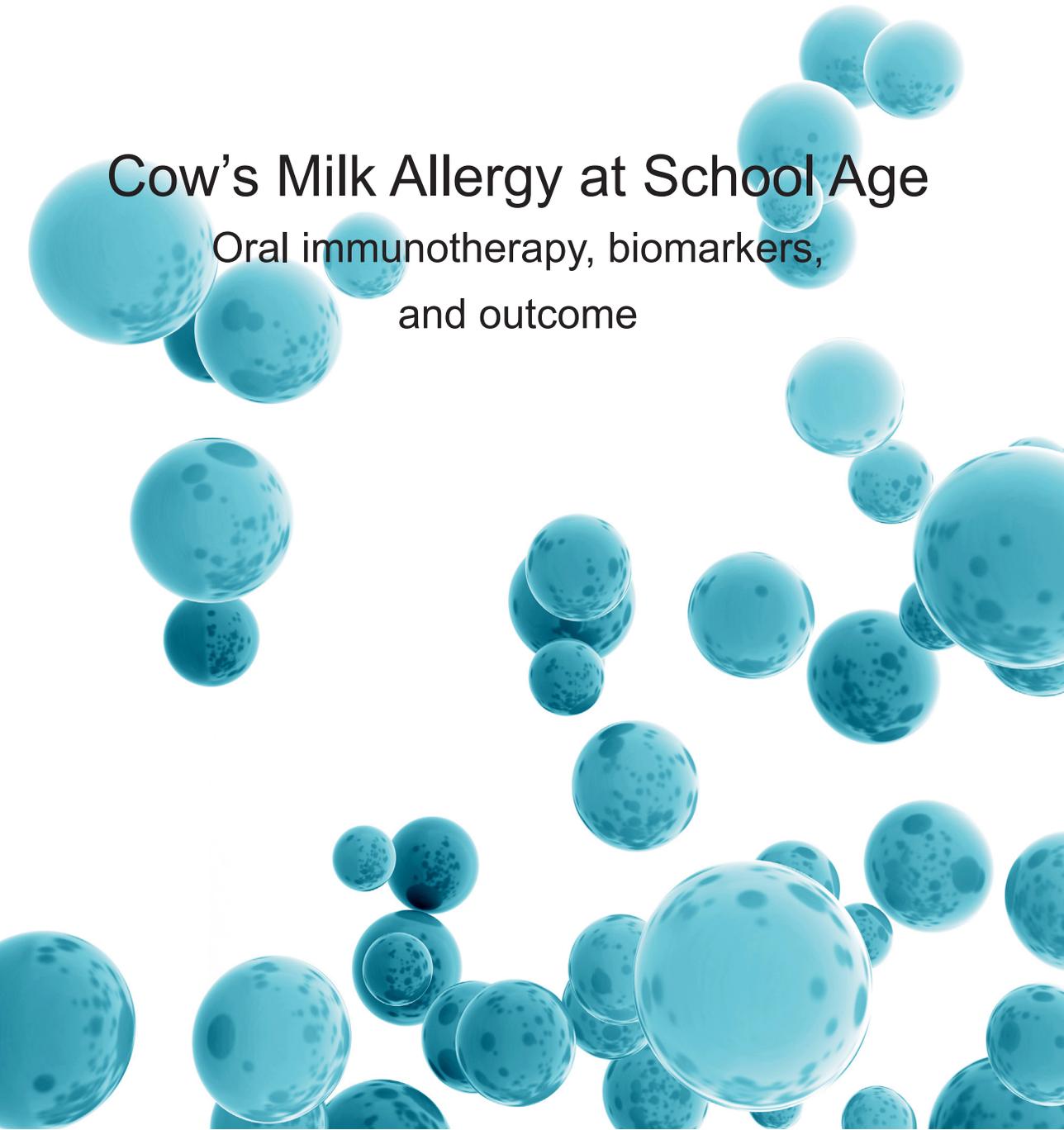


SUSANNA SALMIVESI

Cow's Milk Allergy at School Age

Oral immunotherapy, biomarkers,
and outcome





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ACADEMIC DISSERTATION

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UNIVERSITY OF TAMPERE

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To my family

Abstract

Background: Cow's milk allergy (CMA) is one of the most common food allergies in early childhood. The prevalence in infancy is around 2-3%. Cow's milk protein (CMP) is usually the first allergen introduced into the diet of an infant as a breast-milk substitute. The current management has been a strict avoidance, especially in severe CMA cases. Since milk is a ubiquitous food, the elimination diet may cause a remarkable burden for families with little children. The prevalence has remained unchanged in the 21st century, but it offers a good prognosis, as most milk-allergic children recover by ages 3-5. Still, a notable portion of children with IgE-mediated CMA will not recover until school age. Thus, there is a need for treatments other than an avoidance diet. The most-studied treatment option has been oral immunotherapy (OIT). However, its immunologic basis still is mostly obscure.

Aims: The main aim of the present study was to find out how double-blind, placebo-controlled OIT is accomplished for school-age children with IgE-mediated CMA. The specific research questions were to find out: 1) whether OIT protocol is effective and safe in school-age children with persistent and even severe IgE-mediated CMA 2) what immunological changes occur during milk OIT 3) whether there is long-term effectiveness and safety in successful OIT; and 4) whether there are any immunological biomarkers in allergy or inflammatory parameters to predict successful OIT.

Subjects and methods: Twenty-eight school-age children, 6-14 years old, who have been treated for CMA since they were under 2 years old at the Department of Pediatrics at Tampere University Hospital in Finland, were recruited for the OIT study in May 2008. They all had a medical history of challenge-proven IgE-mediated CMA, determined either with skin-prick tests (SPT) ≥ 3 (with at least half in relation to histamine control) or CM-specific IgE $> 3.5\text{kU/l}$. The patients were randomized using permutations by the chief pharmacist of the hospital with a 2:1 ratio in the blocks of six (4+2) into an active treatment group of 18 children and a placebo group of 10 children. The double-blind, placebo-controlled, OIT study lasted for six months until the final check-up at the outpatient clinic, with the target daily dose

was 200 mL of milk (which equals 6,400 mg of milk protein). Serum samples were drawn during the first study visit and in six months at the last study visit. Immediately after the blind OIT, all 10 children in the control group received open-label OIT according to the same protocol. Blood samples were collected from the control group at 12 months, when the open-label OIT ended. The blood samples were analyzed for immunological aspects, i.e., allergic and inflammatory biomarkers. After the OIT protocol, patients were followed annually through phone calls for the first three years to track their milk consumption and milk-related symptoms. A follow-up was done annually through a postal questionnaire until the final phone call seven years after the initiation of milk OIT.

Results: Twenty-four children (out of 28, or 86 %) completed the double-blind, placebo-controlled study. Two children in the active group discontinued the study because of abdominal pain. One child in the control group discontinued because of accidental milk-protein-induced anaphylaxis, and another child dropped out due to motivational problems. Among the participants completing the OIT protocol, all 16 children in the active group and five of eight in the placebo group suffered mild symptoms from milk or placebo consumption. After a six-month blind OIT, all 10 children in the control group were successfully desensitized with the open-label OIT. One year after the end of the study, 13 (81%) children in the active group used milk or milk products corresponding 6,400 mg of milk protein a day. Approximately three-and-a-half years later, one more child had discontinued daily milk consumption. Thus, there were 22 of 28 (79%) daily milk users in the follow-up phase of the study. Seven years after the study began, 24 participants were reached by phone and 14 were consuming milk or milk products, corresponding to at least 6,400 mg milk protein used daily. Thus, the long-term follow-up result was 14 of 24 (58%). At the end of the six-month, double-blind, placebo-controlled study, serum IL-6 and IL-10 levels increased in the active group compared with the placebo group. When combining the changes in biomarkers of the treatment group in the blind phase and the placebo group in the open-label OIT, blood eosinophils and serum total IgE levels decreased and milk-specific IgG and IgG4, serum IL-4 and IL-6, leptin and resistin levels increased. At the beginning of the study, high milk-specific IgE referred to discontinuing milk OIT. No other biomarker at the baseline predicted milk consumption at the seven-year mark. Only high post-OIT serum adiponin levels correlated with poor long-term compliance with milk consumption.

Conclusions: Our randomized, double-blind, placebo-controlled study confirmed that milk OIT is effective in desensitizing school-age children with IgE-mediated CMA. In the treatment group, 16 of 18 (89%) children successfully completed the OIT protocol. Seven years later, half the patients were consuming milk and their milk-related symptoms were less frequent than shortly after the end of OIT. Due to milk OIT, there were changes in allergy markers in the peripheral blood eosinophils, total IgE levels decreased, and milk-specific IgG and IgG4 levels increased. As a novel finding, serum adipokines, leptin, and resistin levels increased during milk OIT.

Tiivistelmä

Tausta: Lehmänmaitoallergia on varhaislapsuuden yleisin ruoka-allergia. Sen esiintyvyys imeväisikäisillä on noin 2-3 %. Lehmänmaidon proteiini on yleensä ensimmäinen kosketus allergeeneihin varhaislapsuudessa korvikemaidon kautta. Nykysuosituksena on ollut maitoproteiinin tarkka välttäminen erityisesti vaikeassa maitoallergiassa. Koska maito on yleisesti käytössä oleva ruoka-aine, välttämisdieetistä aiheutuu huomattavaa räsitusta lapsiperheille. Ennuste on hyvä, sillä suurin osa maitoallergikoista paranee 3-5 ikävuoteen mennessä. Kuitenkin huomattava osa IgE-välitteisistä maitoallergikoista sairastaa tautia edelleen kouluiässä. Täten tarve perinteisen välttämisdieetin vaihtoehdolle on ilmeinen. Tutkituin kokeellinen maitoallergian hoito on suun kautta suurenevin annoksin tapahtuva siedätyshoito, jonka immunologinen tausta on vielä pääosin tuntematon.

Tutkimuksen tarkoitus: Pääasiallisena tarkoituksena oli selvittää maitosiedätyshoidon tehoa kaksoissokkoasetelmassa kouluikäisillä lapsilla, jotka eivät ole toipuneet IgE-välitteisestä maitoallergiastaan. Tarkemmat tutkimuskysymykset olivat: 1) onko maitosiedätyshoito tehokas ja turvallinen kouluikäisten lasten vaikeassa IgE-välitteisessä maitoallergiassa 2) mitä immunologisia muutoksia maitosiedätyksen aikana tapahtuu 3) voiko maitosiedätyshoito olla tehokasta ja turvallista hoitoa myös pitkällä aikavälillä 4) onko immunologisista mittareista apua ennustamaan mitkä potilaat hyötyvät maitosiedätyshoidosta pitkäaikaisesti.

Aineisto ja menetelmät: Tutkimus käynnistyi toukokuussa 2008 Tampereen yliopistollisessa sairaalassa (Tays), lastentautien yksikössä. Siihen rekrytoitiin mukaan 28 iältään 6-14 -vuotiasta lasta, joiden IgE-välitteistä maitoallergiaa oli aiemmin hoidettu Tays:ssa. Sisäänottokriteereinä oli ikä vähintään 6 vuotta, positiivinen ihostesti maidolle (Prick \geq 3mm ja vähintään puolet histamiinipaukaman kokoon nähden) tai positiivinen seerumin maito-spesifinen IgE $>$ 3.5kU/l ja joko positiivinen avoin maitoaltistus tai anamnestisesti luotettava reaktio maidolle vahinkoaltistuksessa 6 kk aikana ennen tutkimuksen käynnistymistä. Sairaala-apteekkari satunnaisti potilaat suhteessa 2:1 (4+2) lohkoutusta apuna käyttäen joko

aktiiviryhmään (18 lasta) tai plaseboryhmään (10 lasta). Kaksoissokko, plasebo-kontrolloitu tutkimus kesti noin kuusi kuukautta loppukontrolliin saakka ja sen tavoitteena oli 200 ml maitoa (tai 6400 mg maitoproteiinia) päivittäin. Tämän jälkeen verrokkiryhmään päätyneet lapset saivat saman protokollan mukaisen avoimen maitosiedätyshoidon heti perään. Kaksoissokkosiedätyshoidon ensimmäisellä ja viimeisellä käynnillä sekä avoimen siedätyshoidon lopetuskäynnillä otettiin verinäytteitä, joista tutkittiin allergiaan, tulehdukseen ja immuniteettiin liittyviä tekijöitä. Tutkimuspotilaiden maidon käyttöä ja siihen liittyneitä oireita seurattiin ensin vuosittaisilla puhelinsoitoilla ensimmäiset kolme vuotta ja sen jälkeen postissa lähetettävillä kyselylomakkeilla kunnes lopullinen seurantatulokset saatiin puhelimitse seitsemän vuoden kohdalla tutkimuksen alusta lähtien.

Tulokset: Kaksoissokko maitosiedätyshoito-protokollan läpäisi 24/28 (86 %) lasta. Kaksi lasta aktiiviryhmästä keskeytti hoidon vatsaoireiden takia. Yksi lapsi verrokkiryhmästä keskeytti protokollan maitoproteiiniin liittyneen vahingon ja sen aiheuttaman anafylaksian vuoksi. Toinen lapsi verrokkiryhmästä keskeytti motivaatio-ongelmien takia. Kaikilla aktiiviryhmän ja 2/3 kontrolliryhmän lapsilla oli lieviä oireita maitoon tai plaseboon liittyen. Kaikki 10 verrokkiryhmän lasta siedätettiin onnistuneesti maidolle välittömästi kaksoissokkotutkimuksen päätyttyä. Vuoden kuluttua siedätyshoidon päättymisestä aktiiviryhmän lapsista 13 (81 %) käytti päivittäin maitoa tai maitotuotteita 6400 mg maitoproteiinia vastaavan määrän. Kolmen ja puolen vuoden kohdalla yksi lapsi lisää oli keskeyttänyt hoidon, joten seurantatulokset olivat 22/28 (79 %). Seitsemän vuoden kuluttua tutkimuksen alusta lähtien puhelimitse tavoitetusta 24 tutkittavasta 14 käytti onnistuneesti maitoa. Täten pitkäaikaisseurantatulokset olivat 14/24 (58 %). Kaksoissokkosiedätyshoidon lopussa hoitoryhmän lapsilla seerumin interleukiinit IL-6 ja IL-10 olivat merkittävästi korkeammalla tasolla kuin verrokkiryhmällä. Kun sokkotutkimuksen ja avoimen hoitoryhmän osalta lähtövaiheen ja aktiivihoidon lopetusvaiheen biomarkerit yhdistettiin, havaittiin muutoksina veren eosinofiilien ja kokonais-IgE-tason lasku sekä maito-spesifisen IgG:n ja IgG4:n, seerumin IL-4:n ja IL-6:n, leptiinin ja resistiinin nousu merkittävästi. Tutkimuksen alussa korkea maito-IgE-taso viittasi OIT-protokollan keskeytymiseen. Sen sijaan lähtövaiheen muista markkereista mikään ei ennustanut maidon käyttöä seitsemän vuoden kuluttua tutkimuksen alusta lähtien. Ainoastaan siedätyshoidon jälkeinen korkea adipsiini-taso korreloi epäonnistuneeseen pitkäaikaiseen maidon käyttöön.

Johtopäätökset: Kaksoissokko, plasebokontrolloitu tutkimuksemme osoitti, että maitosiedätyshoito on tehokasta hoitoa kouluikäisillä IgE-välitteistä maitoallergiaa sairastavilla lapsilla. Siedätyshoitoprotokollan läpäisi hoitoryhmän lapsista 16/18 (89 %). Hoidon teho säilyi vielä seitsemän vuoden kuluttua tutkimuksen alusta 50 %:lla potilaista ja maidon käyttöön liittyneet oireet vähenivät vuosien kuluessa. Hoidon aikana perifeerisen veren allergiamittarit kuten eosinofiili- ja kokonais-IgE-pitoisuus laskivat ja maito-spesifinen IgG- ja IgG4-pitoisuus nousivat. Uutena löydöksenä seerumin adipokiinit, leptiini ja resistiini, nousivat hoidon aikana.

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LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

- I. Salmivesi, S., Korppi, M., Mäkelä, M.J., Paassilta, M. (2012). Milk oral immunotherapy is effective in school-aged children. *Acta Paediatrica*, 102, 172 – 176.
- II. Salmivesi, S., Paassilta, M., Huhtala, H., Nieminen, R., Moilanen, E., Korppi, M. (2016). Changes in biomarkers during a six-month oral immunotherapy intervention for cow's milk allergy. *Acta Paediatrica*, 105, 1349 – 1354.
- III. Paassilta, M., Salmivesi, S., Mäki, T., Helminen, M., Korppi, M. (2015). Children who were treated with oral immunotherapy for cows' milk allergy showed long-term desensitization seven years later. *Acta Paediatrica*, 105, 215 – 219.
- IV. Salmivesi, S., Paassilta, M., Huhtala, H., Nieminen, R., Moilanen, E., Korppi, M. (2017). Elevated serum adipsin may predict unsuccessful treatment for cows' milk allergy but other biomarkers do not. *Acta Paediatrica*, 107, 328 – 332.

ABBREVIATIONS

AE	Adverse event
AIT	Allergen immunotherapy
APT	Atopy patch test
CM	Cow's milk
CMA	Cow's milk allergy
CM-IgE	Cow's milk Immunoglobulin-E
CMP	Cow's milk protein
CRD	Component resolved diagnostics
DBPCFC	Double-blind, placebo-controlled food challenge
EAACI	European Academy of Allergy and Clinical Immunology
EAT	"Enquiring About Tolerance"
EoE	Eosinophilic esophagitis
Eos	Eosinophils
EPIT	Epicutaneous immunotherapy
IgE	Immunoglobulin-E
IgG	Immunoglobulin-G
kU/l	kiloUnits per liter
LEAP	"Learning Early About Peanut allergy"
mL	milliliter
OFC	Oral food challenge
QoL	Quality of life
RCT	Randomized controlled trial
SCIT	Subcutaneous immunotherapy
sIgE	Specific Immunoglobulin-E
SIT	Specific immunotherapy
SLIT	Sublingual immunotherapy
SOTI	Specific oral-tolerance induction
SPT	Skin-prick test
SU	Sustained unresponsiveness
Treg	Regulatory T-cell

1 INTRODUCTION

Cow's milk (CM) allergy (CMA) is an immune mediated disease, originating either from an immunoglobulin E (IgE) or non-IgE pathway. This thesis will concentrate on IgE-mediated CMA, which affects 0.5 to 3% of children under 2 years old (Chafen et al. 2010, Schoemaker et al. 2015). Thus, CMA is a common, chronic disease in childhood.

On one hand, CMA's natural history generally is favorable, since most patients recover by the time they enter school. On the other hand, an increasing number of IgE-mediated CM allergic children have, during recent decades, presented with a chronic disease, persisting until 16 years of age in 21% of cases (Hoest, Halken 1990, Saarinen et al. 2005, Skripak et al. 2007). In general, the more atopic diseases a patient has and the higher the specific IgE, the lower the likelihood of developing tolerance to foods that have induced allergic symptoms earlier. Likewise, the higher the CM-specific IgE, the worse the prognosis of CMA (Ahrens et al. 2012).

The definite diagnosis of CMA relies on a clinical-provocation test (Fiocchi et al. 2010). Even though the gold standard for making a diagnosis is a double-blind, placebo-controlled food challenge, an open challenge is usually performed in clinical practice. When the diagnosis has been set, the only universally approved treatment is an elimination diet. This type of treatment has its own downsides. Food allergies generally can have a significant negative effect on quality of life (QoL), as reported by food-allergic children and their parents (Bollinger et al. 2006, Flokstra-de Blok, B M J et al. 2010). The fear of anaphylaxis due to accidental ingestion plays a crucial role since allergic reactions due to accidental exposure for milk, eggs, or peanuts are common (Fleischer et al. 2012).

New, effective treatment options for CMA are highly desirable and are being explored. Current knowledge suggests that tolerance induction through allergen-specific immunotherapy is possible. Subcutaneous immunotherapy (SCIT) utilized successfully for pollen allergies, however, has been shown to be unsafe when treating food-allergic patients (Nelson et al. 1997). Obviously, oral immunotherapy (OIT) has been the most intriguing way to treat food-allergic patients because it is the most natural and studied route in the 21st century. The first large, but not controlled, milk OIT clinical trial was conducted in an Italian study (Meglio et al. 2004). It showed

promising results in desensitizing severe IgE-mediated CMA children. There were 21 patients older than 5 years who were treated starting with diluted milk and ending with raw milk. The first dose consisted of 0.06 mg of CM protein, and the final dose consisted of 200 mL of undiluted milk. In this study, 71.4% of the children achieved the target amount and 14.3% tolerated partial amounts (40-80 mL) after six months. During the protocol of milk OIT, the children were on a co-treatment of daily antihistamine. Most patients underwent a double-blind oral food challenge before the study, but the desensitization was not measured by a challenge test at the end. Overall, in a Cochrane review on milk OIT, it was concluded that oral immunotherapy appeared to desensitize 62% of treated patients (Yeung et al. 2012). Side events are frequent, but only 9% of patients required adrenaline during milk OIT.

Despite intensive research around OIT, the immunologic basis is still mainly obscure. The first double-blind, placebo-controlled, milk OIT study was published in the U.S. (Skripak et al. 2008). This study showed that milk-specific IgG4 levels increased in the active group, which was confirmed by the next RCT (Pajno et al. 2010). In an RCT for CMA, 2-year-old children's skin-prick test reactivity and specific IgE to milk and casein decreased (Martorell et al. 2011).

Only a few published long-term follow-up studies have been done on milk OIT (Meglio et al. 2008, Keet et al. 2013, Luyt, Bravin & Luyt 2014). These studies suggest that desensitization in most patients is carried out by consuming milk daily and that the long-term effectiveness of milk OIT seem to decrease over years. Long-term safety is one concern, especially with the data indicating that up to 2.7% of either milk, egg, or peanut OIT-treated patients may develop eosinophilic esophagitis (Lucendo, Arias & Tenias 2014), and IgE-mediated food allergies themselves are a risk factor for developing eosinophilic esophagitis (Hill, Dudley & Spergel 2017).

Our study was designed mainly to find out whether we can desensitize school-age children with severe IgE-mediated CMA in a double-blind, placebo-controlled fashion. The protocol was modified from the already-published Italian one (Meglio et al. 2004). Also, clarifying the potential immunological changes associated with milk OIT was another purpose of this study.

2 REVIEW OF THE LITERATURE

2.1 Cow's milk allergy in early childhood

2.1.1 Definition of allergy and food allergy

Allergy has been defined as the presence of hypersensitivity reactions, which are initiated by specific immunologic mechanisms (Johansson et al. 2001). An allergy can be antibody-mediated (usually by IgE), cell-mediated, or mediated by both immune pathways.

Food allergy is defined as the presence of adverse reactions to foods from immunologic mechanisms. Particularly in primary care and community settings, this term includes both IgE- and non-IgE-mediated food allergies (Sackeyfio et al. 2011). However, an appropriate term for all other-than-IgE-mediated reactions would be non-allergic food hypersensitivity (Johansson et al. 2004). This definition suggests that the term *IgE-mediated food allergy* should be used only if IgE is involved, even though the general term *food allergy* commonly covers both IgE-mediated and non-IgE-mediated reactions.

In European children, food allergies are most commonly triggered by proteins in cow's milk, eggs, wheat, soy, peanuts, tree nuts, fish, and shellfish (Nwaru et al. 2014b). Although local dietary preferences may elicit variations in prevalence rates in food allergies when comparing nations (Prescott et al. 2013), cow's milk and hen's eggs seem to be the most common causes of food allergies in children worldwide (Rona et al. 2007). These are among the first complementary foods to be introduced into infants' diets.

2.1.2 Prevalence of food allergy

The prevalence rates of CMA depend on the criteria of the diagnosis, i.e., whether it is based on self-reported symptoms, specific serum IgE or specific skin prick-test (SPT) positivity, food challenge test results, or a combination of tests. In the U.S. population, food allergies affected more than 1%, but less than 10%, when any of

these methods was used, with cow's milk allergy (CMA) affecting from 0.6% to 0.9% when any method other than self-reporting was used (Chafen et al. 2010). A large U.S. self-reporting survey reported that 1.7% of children suffered from CMA (Gupta et al. 2011). Globally, the self-reported prevalence of CMA varied from 1.2% to 17% (Rona et al. 2007). The same study estimated that the lowest prevalence rates of CMA were from 0% to 3% based on the challenge-proved method. The studies based on questionnaires when compared with standardized methods such as food challenges seem to overestimate prevalence. In the EuroPrevall study from nine European countries, the diagnosis of CMA is based on a double-blind, placebo-controlled food challenge (DBPCFC), with incidence ranging from <0.3% to 3% (Schoemaker et al. 2015). Likewise, a review of food allergies in Europe showed that the overall pooled point prevalence of challenge-verified food allergies was 0.9%, but interestingly, some evidence suggested that food-allergy prevalence may be increasing (Nwaru et al. 2014a).

In southeastern Finland, the overall lifetime prevalence of physician-diagnosed food allergies among 1- to 4-year-old children was 9%, with milk the most commonly reported essential food item, causing symptoms in 13% (Pyrhönen et al. 2009). The study provided evidence, in contrast to European evidence, of a stable prevalence of CMA in children. Interestingly, the self-reported prevalence rate for CMA in children starting elementary school at 6 to 7 years old in western Finland was 1.5% (Kallio et al. 2011). An identical study was performed four years later, and the CMA prevalence rate was still 1.3% (Järvenpää et al. 2014).

2.1.3 Diagnosis

The diagnosis of food allergy is based on a detailed clinical history focused on allergy, skin prick tests (SPTs), serum food-specific IgE (sIgE) measurements, and a food challenge test (Lack 2008). However, there is no clear consensus on whether the challenge test should be under double-blind or open conditions (Chafen et al. 2010). The reactions from food can be triggered by ingestion, inhalation, or skin contact (Muraro et al. 2014). A careful dietary history refers to the likelihood of the diagnosis and the mechanism of symptoms, whether they are IgE- or non-IgE-mediated. In addition, such history can identify the potential foods triggering the symptoms. It is important for food allergies to be diagnosed correctly since an avoidance diet decreases patients' quality of life and can even be dangerous (Flinterman et al. 2006). When CM-sensitized children with atopic eczema were on a long elimination period,

they developed severe acute allergic reactions even though they previously tolerated milk.

2.1.3.1 Symptoms

Often a child presents persistent signs and symptoms of food allergies in more than one organ system (Sackeyfio et al. 2011). Symptoms can appear on the skin (urticaria, angioedema, erythema, worsening of atopic eczema), or in the gastrointestinal (vomiting, diarrhea, abdominal pain, cramping) or respiratory systems (rhinitis, persistent cough, wheeze, stridor, asthma) (Lack 2008, Burks et al. 2012b). Non-IgE-mediated symptoms typically include worsening of atopic eczema or an insidious onset of gastrointestinal symptoms (NIAID-Sponsored Expert Panel 2010). Severe atopic eczema in infancy is frequently associated with food allergies (Hill, Hosking 2004).

Symptoms of IgE-mediated food allergies develop within a few minutes to two hours after eating the food in question. In severe food allergies, an allergic reaction can be serious, rapid in onset and life-threatening. Such a systemic reaction is called anaphylaxis (Johansson et al. 2004, Sampson et al. 2006). A French study showed that a severe allergic reaction to milk may occur even at a dose of less than 1 mL (Morisset et al. 2003). However, an expert panel of Australia and New Zealand decided that the lowest eliciting dose for CMA children was 0.1 mg of milk protein (Taylor et al. 2014).

2.1.3.2 Testing

SPTs and CMA-specific IgE measurements with CM extracts containing crude allergens are routinely used when IgE-mediated CMA is suspected, but the responses only reveal sensitization, not the allergic disease itself. In addition, SPTs overestimate the likelihood of food allergies, as their sensitivity is around 90%, but their specificity is only about 50% (Lack 2008). The pooled sensitivities of SPT and sIgE for CMA were 88% and 87%, and specificities 68% and 48%, respectively (Soares-Weiser et al. 2014). This is why these tests are better for excluding than confirming diagnoses of IgE-mediated CMA.

The less commonly used test in assessing food allergy is atopy-patch testing (APT). It may be useful in non-IgE-mediated immunologic reactions (Burks et al. 2012b). In an earlier study, it tended to correlate better with IgE-mediated CMA, but

the challenge test was essential to confirm the diagnosis (Saarinen, Suomalainen & Savilahti 2001). However, APT is not recommended for routine clinical use (Muraro et al. 2014). The pooled sensitivity of this test was 53% in CMA, and the specificity was 88% (Soares-Weiser et al. 2014).

The component-resolved diagnostics (CRD), also called molecular diagnostics, is a fairly new methodology in food-allergy testing. It utilizes native purified or recombinant allergens and can be performed either in microarray or in single-test formats (Muraro et al. 2014). One study consisting of children and adults did not find these tests superior to conventional testing for predicting clinical reactivity in CMA (Hochwallner et al. 2010). Other studies indicated that CRD could be useful when monitoring CMA patients and predicting resolution of the disease (Ahrens et al. 2012, Savilahti et al. 2010). One study showed that CRD is more useful than the oral food challenge (OFC) when predicting the likelihood of reactions (Tuano, Davis 2015). In bovine milk, there are many milk proteins in the casein and whey fractions that can cause an allergic reaction (Wal 2004). Most CMA patients are sensitized to the major allergens (caseins, β -lactoglobulins, and α -lactalbumin) (Matricardi et al. 2016). Thus, high levels of casein-specific IgE, which is very heat-resistant, seem to correlate with reactivity to baked milk (Caubet et al. 2013) and severe allergic reactions due to accidental exposure (Boyano-Martínez et al. 2009).

2.1.3.3 Food-provocation tests

The conclusive diagnosis of CMA requires a precise clinical history and an elimination diet followed by an oral food-challenge test. The blind challenge test, DBPCFC, is considered the gold standard for diagnosing food allergies since both the patient and observer biases are removed (Sampson et al. 2012). Because DBPCFC is a resource-intensive and time-consuming approach, it is mostly replaced by an open OFC in clinical practice. This is especially the case when a patient has objective evidence of an allergy and not just subjective symptoms (Lack 2008). In OFC, the patient is given incremental doses of milk at 30-minute intervals and monitored for symptoms and objective signs.

2.2 Management of cow's milk allergy

The only established treatment for CMA is an elimination diet. Traditionally, management relies on elimination of CM protein from the diet and the use of rescue

medications if needed in the event of accidental reactions (Muraro et al. 2014, NIAID-Sponsored Expert Panel 2010). This is challenging considering how ubiquitous milk is in foods.

2.2.1 Prophylaxis

Recent studies indicate promising allergy prevention in infancy. These studies suggest introducing the most common allergenic foods as early as 3 months old, instead of exclusive breast-feeding for the first six months of life (Du Toit et al. 2015, Perkin et al. 2016). The British randomized, controlled trial (RCT) “Learning Early About Peanut Allergy” (LEAP) found that peanut consumption for high-risk infants at 4 to 11 months old decreased the risk of developing peanut allergies by the time they are 5 years old (Du Toit et al. 2015). LEAP findings already have elicited new recommendations in the U.S. – a high-risk country – to introduce peanuts into the diets of high-risk infants (Togias et al. 2017). The LEAP study led to a similar recommendation to introduce peanuts early in other countries, such as the United Kingdom and Australia, where peanut allergies are prevalent (Fleischer et al. 2015). Another British study “Enquiring About Tolerance” (EAT) suggested that introducing eggs and peanuts early is safe and could support tolerance induction (Perkin et al. 2016). However, adverse events may be age-, adherence- and dose-dependent. In the case of CMA, no randomized, controlled trials have been published so far. Preliminary findings in a prospective study from Israel were that, like with CMA, the timing of exposure to CM protein may be crucial (Katz et al. 2010). This study suggested CM protein supplementation introduced at birth might promote oral tolerance. Thus, these studies have begun a new era after the allergen-avoidance strategy failed to prevent food allergies.

2.2.2 Elimination diet and milk substitutes

When the diagnosis is confirmed, the traditional approach in the therapy for common food allergies has been avoiding culprit allergens that trigger symptoms (Chafen et al. 2010). Such avoidance is not a curative treatment and is not without risks. With severe cow’s milk allergies, in which even traces of milk protein may cause anaphylaxis, strict elimination and carrying an epinephrine auto-injector all the time are necessary. Despite adult supervision, accidental milk-induced anaphylaxis may occur in children (De Schryver et al. 2017). Food allergies can have a significant

effect on quality of life, both for the caregiver and the child (Bollinger et al. 2006, Flokstra-de Blok, B M J et al. 2010, Springston et al. 2010). Social life, including eating outside the home, can be stressful for both children and parents (Begen et al. 2017).

Elimination diets and CM allergy generally can affect nutrition and growth in food-allergic children (Mehta, Groetch & Wang 2013, Agostoni et al. 2007). CM elimination, in particular, influenced both weight and height development of CM-allergic children in a population-wide study (Robbins, Wood & Keet 2014). In a smaller study, decreased growth was associated with low calcium and vitamin D intake in CM-allergic children (Jensen et al. 2004). Hence, a dietitian is usually needed when the CMA therapy is started.

Having a dietitian is especially important when dietary intervention is needed for an infant with CMA. Hydrolysis of formulas reduces allergenicity in relation to the degree of hydrolysis. The first choices in the long-term management of CMA are extensively hydrolyzed formulas (de Silva et al. 2014, Muraro et al. 2014), which have been found to be safe and well-tolerated (Niggemann et al. 2008). A choice for infants over 6 months old used to be a soy-based formula, which is not on the market anymore. However, in some cases, amino-acid formulas may be more effective in reducing symptoms and also better tolerated than extensively hydrolyzed formulas (Hill et al. 2007, Niggemann et al. 2001).

2.2.3 Prognosis

Early studies suggest that food allergies to cow's milk, eggs, soy, and wheat are resolved rapidly over time, but that peanut and tree-nut allergies tend to be lifelong. However, prognosis of food allergies depends on the allergen and the allergy mechanism. Generally, the natural history of IgE- and non-IgE-mediated CMA offers a good prognosis since most CM-allergic children recover by 3 years old or at least by school age. Nevertheless, the resolution of CMA depends on the presence of IgE antibodies (Schoemaker et al. 2015, Saarinen et al. 2005, Skripak et al. 2007, Wood et al. 2013b). In fact, CM-allergic children with milk-specific IgE > 5 kU/l at the time of diagnosis seem to have the worst prognosis concerning tolerance development (Ahrens et al. 2012). The presence of IgE in more numerous epitopes of casein, one of the major CM proteins in sera, predicts persistent CMA (Järvinen et al. 2002).

A European study found that all children with non-IgE-associated CMA, but only 57% with IgE-associated CMA, became tolerant within one year of diagnosis (up to

age 30 months) or at least by 5 years old (Schoemaker et al. 2015). Instead, IgE-mediated CMA often persists to school age. In a Finnish study, all non-IgE-mediated milk-allergic children and 74% of IgE-mediated CM-allergic children were tolerant by age 5 years, and 15% by 8.6 years old still had CMA (Saarinen et al. 2005). A U.S. study consisting of only IgE-mediated CM-allergic children found that the recovery rates by age 8 years old were 42%; by 12 years old, 64%; and by 16 years old, 79% (Skripak et al. 2007). These studies indicate that after 5 years old, the rate of allergy recovery is slowing down, and CMA is commonly persisting into late childhood and even into adolescence. Thus, the burden of the disease is accumulating for IgE-mediated CMA with high sIgE to CM.

2.3 Oral immunotherapy

Specific immunotherapy (SIT) for an allergen already was discovered over a century ago, and SIT has been especially effective with allergic rhinitis and conjunctivitis (Dhami et al. 2017). For allergic rhinitis, SCIT is recommended for at least three years to ensure post-cessation effectiveness (Roberts et al. 2017). SIT is the only tolerance-inducing treatment and, thus, the only curative method against aeroallergens and insect sting allergies (Jutel, Akdis 2011). SIT modifies the immune response by treating the cause rather than the symptoms. The goal of effective immunotherapy is to change a patient's allergen-specific responsiveness from an allergic profile (Th2) to a non-allergic profile (Th1). According to one theory, this is accomplished through regulatory T (Treg) cells (Figure 1). With food allergies, the immune response is skewed toward a Th2-type cytokine-associated phenotype (Johnston, Chien & Bryce 2014).

Since the elimination diet only targets allergic symptoms, especially in severe CMA with potential risks of severe allergic reactions and even anaphylaxis, another option for treatment of ongoing food allergies is needed. The first report concerning allergen immunotherapy (AIT) for food allergies dates to the beginning of the 20th century. In 1908, *the Lancet* published a case report called "Egg Poisoning" (Schofield 1908). Other reports on oral immunotherapy (OIT) or specific oral tolerance induction (SOTI) from the 1970s involved limited numbers of patients.

Currently, the most studied immunotherapy route for food allergies is undoubtedly the oral route. Immunotherapy starts with a tiny amount of CM protein orally, with gradually increasing amounts over several weeks, then months, toward a

target dose of a small portion in a healthy child's daily diet. It also can lessen the fear of accidental ingestions of the offending food.

In a 2012 review, OIT was considered a promising approach in CMA management (Brozek et al. 2012). This review found that the probability of achieving tolerance with milk OIT treatment was 10 times higher compared with children taking a placebo or on an elimination diet. In 2014, the European Academy of Allergy and Clinical Immunology (EAACI) Food Allergy Guidelines still recommended that OIT be performed in specialized clinical settings under the supervision of an allergist with expertise in the field (Muraro et al. 2014). This means OIT was not considered a routine clinical treatment. However, the 2017 EAACI position paper on immunotherapy for IgE-mediated food allergies concluded that OIT may be an effective treatment for children (Nurmatov et al. 2017).

One problem is that published study designs and protocols differ considerably from each other. The age of patients has been between 2-17 years with or without a history of anaphylaxis. Starting doses have varied from 0.04 mg to 0.4 mg of CM protein. Some studies started by admitting children into the hospital for an ultra rush or a rush phase when more serious adverse events were involved (Longo et al. 2008, Skripak et al. 2008). Some began with a slow escalation phase with visits to the outpatient clinic (Meglio et al. 2004, Pajno et al. 2010), followed by a build-up phase, then a maintenance phase for months or years. The studies started with diluted milk and continued with fresh milk or milk powder, with doses incrementally raised every second day or every one to three weeks, mostly in the hospital. With most protocols, maintenance doses started at 0.5 g and ended at up to 7 g of CM protein. Study lengths varied from 4.5 months to 12 months. Most studies set the diagnosis of CMA with DBPCFC or open OFC before OIT trials and measured treatment success the same way, either with blind or open food challenges. But few have included a final challenge after a period off OIT therapy to distinguish desensitization from tolerance.

OIT consists of starting with an extremely low dose of the causative allergen swallowed, gradually increasing the amount over several months. This phase aims for a small daily dose for a healthy child, or at least an amount to protect the child from accidental exposure. In the case of milk, the dose is a 200-250 mL a day. The build-up period is called an escalation phase and is followed by a maintenance phase with an offending allergen. Desensitization is a state in which a patient tolerates a food challenge or a daily maintenance dose while on OIT. Instead, sustained unresponsiveness is tolerating a food challenge one to three months off OIT. This may be called remission as well. Since clinical reactivity often recurs, true tolerance

is rare. In a recent study of peanut OIT in children ages 9 to 36 months, the remission rate was 78 % (Vickery et al. 2017).

2.3.1 Oral tolerance

There is a dual-exposure hypothesis suggesting that cutaneous exposure causes sensitization, and that early oral exposure induces tolerance (Lack 2012). This theory is supported by early-introduction studies (Katz et al. 2010, Du Toit et al. 2015, Perkin et al. 2016). On the other hand, cow's milk is usually the first exposure to potential allergens early in life and also the culprit for the most common food allergy in children.

Primary oral tolerance is an active systemic inhibition of immune responses to non-harmful food antigens associated with prior exposure through the oral route (Chehade, Mayer 2005). If this default response fails, a food allergy develops. T-cells can cause tolerance or hypersensitivity when responding to an antigen in food. The key players in the induction and maintenance of oral tolerance are Treg cells (Su, Tang 2012). They suppress the production of IgE and further the development of allergies. In tolerance development, there are antigen-related factors such as dose and nature, and host-related factors such as genes, age, and intestinal microflora. The largest immunologic organ in the human body is the gastrointestinal tract. OIT studies starting with a rush phase fit well with the theory of high-dose tolerance development that causes lymphocyte anergy or deletion (Chehade, Mayer 2005, Vickery et al. 2011) (Figure 1.). According to this theory, another option for oral-tolerance induction is low-dose tolerance. This schema, consisting of repeated lower doses of antigen, like in other OIT studies, is mediated by Treg cells. Although immune mechanisms from food allergies are still obscure, it is known that food allergies develop when either oral tolerance to food antigens is breached or not acquired (Nowak-Wegrzyn, Szajewska & Lack 2016).

In the real world, the state of tolerance is defined as an ability to eat or drink an age-appropriate quantity of the offending food. Since tolerance in CMA is usually acquired spontaneously, it is important to re-evaluate children by performing an OFC at regular intervals, e.g., every 6-12 months (Muraro et al. 2014).

In many OIT trials, the ability to tolerate a CM challenge is referred to as a treatment success (Tordesillas, Berin & Sampson 2017). Desensitization is the state when the patient is still on the treatment and tolerates an OFC. Tolerating a challenge, e.g., for two weeks to three months after the treatment, is called sustained unresponsiveness, which is a proxy for clinical tolerance. It is proposed that this state

could be called remission since tolerance is permanently losing reactivity to an allergen (i.e. the patient can be considered cured).

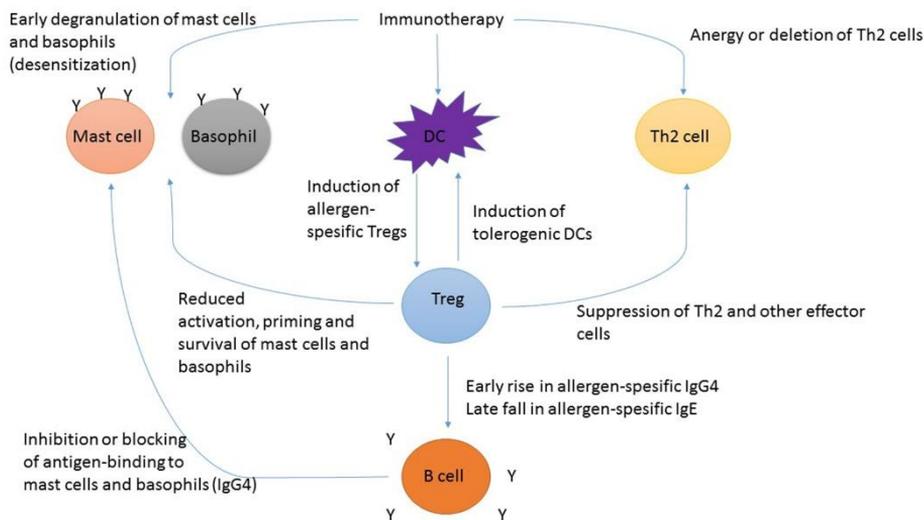


Figure 1. Potential mechanisms underlying OIT. Modified from Keet & Wood. (2014)

2.3.2 Efficacy

School-age children with severe CMA and high milk-specific IgE rarely outgrow their CMA (Saarinen et al. 2005, Skripak et al. 2007). In many studies, severe CM-allergic children are excluded, which means the results are difficult to generalize. Treatment success is the ability to tolerate a CM challenge off or on a maintenance dose involving daily consumption of milk or milk products.

Evidence from the RCT studies shows that milk OIT can desensitize most CM-allergic children. The short-term success rate of milk OIT has been 60-100% (Nowak-Wegrzyn, Albin 2015, Yeung et al. 2012). However, clinical studies to date have not yet proved that true tolerance as a permanent loss of clinical reactivity is possible.

Several studies have reported that if OIT is followed by a short period of allergen avoidance, the symptoms of CM or egg allergies will reappear (Rolinck-Werninghaus et al. 2005, Burks et al. 2012a, Keet, Corinne A., MD, MS et al. 2012, Staden et al. 2007). The available data demonstrated that this period may be as short as two days or as long as two months (Rolinck-Werninghaus et al. 2005). The symptoms can be

severe and systemic, such as anaphylaxis. Thus, OIT induces only transient tolerance, which, in most cases, is lost without ongoing exposure. Therefore, most investigators recommend milk consumption every day after successful OIT (Staden et al. 2007, Longo et al. 2008, Skripak et al. 2008, Vazquez-Ortiz et al. 2013).

2.3.3 Safety

Approximately 95% of patients experience side effects during OIT (Vazquez-Ortiz et al. 2013). The adverse events (AE), especially chronic intestinal symptoms with milk OIT, are common. Though AEs usually are mild and, transient, and need no medication, severe reactions also may occur. A review concerning milk, egg, and peanut OIT studies found that 10-20% of the patients withdraw because of side effects (Keet, Wood 2014). A meta-analysis of randomized, controlled milk OIT studies found almost a six-fold increase in intramuscular adrenalin use compared with the controls (Brozek et al. 2012). Particularly in studies that started with in-hospital treatment and a rush-up phase (Longo et al. 2008, Barbi et al. 2012), OIT was associated with serious side effects. To prevent AEs, some protocols have included daily oral antihistamine during OIT, reducing doses two to four weeks after reaching the maintenance dose of milk (Longo et al. 2008, Meglio et al. 2004).

Short-term safety has been assessed in many clinical trials (Barbi et al. 2012, Caminiti et al. 2009, García-Ara et al. 2013, Vazquez-Ortiz et al. 2013, Zapatero et al. 2008). However, data are scarce on long-term safety. In one report, three children developed eosinophilic esophagitis three to 14 months after reaching the maintenance dose in milk OIT (Sánchez-García et al. 2012). A systematic review suggested that up to 2.7% of either milk, peanut, or egg OIT patients may develop eosinophilic esophagitis due to regular consumption of allergens after OIT (Lucendo, Arias & Tenias 2014). This is a concern, especially with the data indicating that IgE-mediated food allergies themselves are a risk factor for developing eosinophilic esophagitis (Hill, Dudley & Spergel 2017).

Some recent milk OIT studies combined OIT with omalizumab, a monoclonal anti-IgE antibody, to increase safety (Nadeau et al. 2011, Wood et al. 2016). Although omalizumab may improve safety by reducing adverse effects during the dose-escalation phase of OIT, it does not seem to increase efficacy. Both studies used omalizumab as a pretreatment from nine weeks to four months before the OIT protocol started.

The use of CRD also has increased the safety of milk OIT. Children at risk for adverse effects have a higher number of IgE peptides in caseins before and during

OIT (Martnez-Botas et al. 2015). In addition, children who were more likely to attain desensitization had increased IgG4 binding and decreased IgE binding following OIT (Savilahti et al. 2014).

It is known that cooking reduces IgE-type allergenicity in milk, and as many as 75% of CM-allergic children tolerate heated milk (Nowak-Wegrzyn et al. 2008). Thus, there are milk OIT studies implemented with baked or heated milk (Amat et al. 2017, Goldberg et al. 2015, Takahashi et al. 2016). These studies produced even worse results in terms of safety compared with raw milk, milk dilution, or powdered-milk studies. Instead, in a recent Japanese study on heated milk, the authors concluded that the safety profile was good, and the desensitization rate increased from 45% to 80% in the one-year and four-year follow-ups, respectively (Takahashi et al. 2016).

2.3.4 Follow-up studies

There is only a small number of studies on the long-term outcome of milk OIT (Meglio et al. 2008, Keet et al. 2013, Luyt, Bravin & Luyt 2014). All thus-far-published, long-term, follow-up studies of milk OIT lacked control groups. In an Italian study, the long-term outcome after 4.5 years was that 14 (70%) of 20 patients were partially or completely desensitized (Meglio et al. 2008). In the follow-up study, combining two trials, the outcome three to five years later was that only 25% of 50 children consumed milk without symptoms (Keet et al. 2013). The other trial included there were also patients who started with milk SLIT and continued with OIT (Keet, Corinne A., MD, MS et al. 2012). The long-term success rate seems to be related to ongoing milk exposure, which decreases over time. In a British case-series study, 64% of 50 children achieved full or partial desensitization to milk after starting OIT, and in 42%, it persisted up to five years (Luyt, Bravin & Luyt 2014). A recently published paper on a four-year follow-up of peanut OIT and probiotics showed promising results (Hsiao et al. 2017). In the OIT group with probiotics (*Lactobacillus* CGMCC 1.3724) seven (58%) patients were unresponsive to peanuts eight weeks after OIT compared with one (7%) in the placebo group without probiotics.

2.3.5 Alternative immunotherapies

Several approaches for allergen-specific immunotherapy in food allergies have been investigated. Rectal immunotherapy with modified allergens has been studied in peanut allergies (Wood et al. 2013a). Modified peanut allergens were incorporated into *Escheria coli* EMP-123, which is a rectally administered vaccine. The phase 1 study was not promising because five of 10 peanut allergic patients presented with allergic reactions and two with an anaphylactic reaction. The risks for severe adverse effects outweighed the potential benefits.

Sublingual immunotherapy (SLIT) has been used mostly to treat food allergies triggered by hazelnuts and peanuts in many studies (Enrique et al. 2005, Fleischer et al. 2013, Kim et al. 2011). In this type of immunotherapy, allergen extracts are placed under the tongue for one to three minutes to induce desensitization, then are swallowed or spit out. One of the studies compared OIT with SLIT for treating CMA (Keet, Corinne A., MD, MS et al. 2012). All 30 patients began with milk SLIT for six weeks, then were randomized into either the OIT or SLIT arm. Treatment success was measured with a challenge test. The conclusion was that combining OIT with SLIT therapy was more effective than SLIT alone, but was associated with more severe side events. OIT was still the more efficient treatment when assessed at one and six weeks off therapy.

A novel, promising approach is epicutaneous immunotherapy (EPIT). This form of immunotherapy is taken through the skin by repeated applications of allergens in the form of a Viaskin patch (DBV Technologies SA, France). There is only one small extant pilot trial so far, published in CMA (Dupont et al. 2010). This study was done with a bi-center, double-blind, placebo-controlled design, using cumulative, tolerated dose in an open milk challenge, but the duration of the study was only three months. However, EPIT with the Viaskin Peanut patch for peanut allergies in 35 children ages 4 -11, than in 14 children and young adults ages 11-25, was more efficacious when defined by a treatment success in 5,044 mg peanut protein OFC, or achieving at least a 10-fold increase in successfully consumed doses from baseline to Week 52. This was a multicenter, double-blind, randomized, placebo-controlled study (Jones et al. 2017). It consisted of patients treated with EPIT for 52 weeks. These studies seem to confirm that EPIT is safer, but less effective, than OIT. With EPIT, the dose of the allergen is smaller, and the route of exposure through the skin is safer than through the mucosa in OIT.

Subcutaneous immunotherapy (SCIT) is effective with pollen and insect-sting allergies, but SCIT has not been safe enough to use with food allergies

(Oppenheimer et al. 1992). Thus far, there are no published studies on SCIT in CMA in children. (Tordesillas, Berin & Sampson 2017).

2.3.6 Immunoglobulins

The LEAP study on early introduction of peanuts to high-risk infants ages 4-11 months to prevent allergic reactions documented a significant induction of peanut-specific IgG4 after exposure to peanuts (Du Toit et al. 2015). In addition, infants with high levels of IgG4 antibodies to β -lactoglobulin and ovalbumin are more likely to consume these foods at the age of 4.5 years (Tomacic et al. 2009). This is in line with the findings of increased CM IgG4 levels in milk OIT studies (Pajno et al. 2010, Skripak et al. 2008). Also, in a Japanese egg OIT study, the rise in egg-specific IgG1 was associated with a good response for the treatment (Sugimoto et al. 2016). In a Finnish study, high IgE levels for the major CM allergens of α -lactalbumin, β -lactoglobulin, and casein predicted a lower success rate of OIT (Kuitunen et al. 2015).

In the development of allergies, IgE antibodies play a particularly central role (Kucuksezer et al. 2013). Soluble IgE is produced by B-cells and is either in circulation or bound to mast cells or basophils (Figure 2.) There are mast cells in the skin, gut, and respiratory tract. Despite the key role of IgE in food allergies, the regulation of IgE-producing B-cells is poorly understood (Tordesillas, Berin & Sampson 2017). Generally, the higher the level of milk-specific IgE, the more common severe adverse events in OIT and the longer takes to reach desensitization (García-Ara et al. 2013). In a follow-up study, which combined patients from a double-blind, placebo-controlled milk OIT trial (Skripak et al. 2008) and an open-label randomized trial comparing milk SLIT to milk OIT (Keet, Corinne A., MD, MS et al. 2012), there was no success in long-term clinical unresponsiveness if baseline CM-specific IgE was greater than 75kU/l (Keet et al. 2013). It has been shown in many studies that a significant decrease in CM-specific IgE occurs within 6-12 months after an initial rise (Longo et al. 2008, Martorell et al. 2011, Meglio et al. 2008). In a Spanish OIT study in 60 children ages 2 to 2.5 years, casein-specific IgE and skin reactivity to CM decreased at a one-year follow-up from the initial assessments (Martorell et al. 2011). The controls were CMA patients on a milk-free avoidance diet.

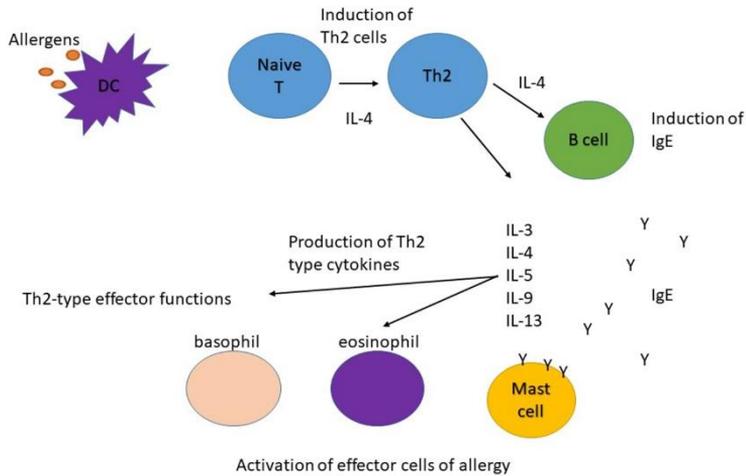


Figure 2. Initiation of allergy. Modified from Kucuksezer et al. (2013)

2.3.7 Interleukins

Most mechanisms underlying milk OIT have not been fully elucidated. There are many cytokines that are involved in either allergy or tolerance development. Interleukins (IL), such as IL-4, IL-5, and IL-13, are the key cytokines in the Th2-type allergic immune response (Akdis et al. 2011). Interestingly, mycobacteria, or their components, such as tuberculin, have reduced allergen-induced IL-4 and IL-13 responses (Savolainen et al. 2000).

IL-10 is a Treg-type anti-inflammatory cytokine. Also, in CMA, Treg cells are involved in desensitization and tolerance (Karlsson, Rugtveit & Brandtzaeg 2004). IL-10 is a key cytokine in the development of immune tolerance since it down-regulates both Th1 and Th2 responses (Akdis et al. 2011). In addition, IL-10 exert direct suppressive effects on mast cells, basophils, and T-cells in allergic reactions (Akdis, Akdis 2011). Since it increases the production of IgG4-blocking antibodies, it also may have a suppressive effect on both total and allergen-specific IgE production (Jutel, Akdis 2011). Already-food-allergic 14-month-old children seem to have lower levels of IL-10 and IL-6 compared with food-sensitized, but tolerant controls (Dang et al. 2013). In a double-blind, placebo-controlled peanut OIT trial, the allergen-specific Th2-type cytokine profile was suppressed (Varshney et al. 2011).

2.3.8 Adipokines

Macrophages and adipocytes secrete adipokines which are also mediators of immunity and inflammation (Fantuzzi 2005). Adiponectin is mainly an anti-inflammatory factor, whereas leptin, resistin, and adiponectin have pro-inflammatory properties. In autoimmune diseases, serum adiponectin levels increased, but with allergies, the role of adiponectin is unclear (Fantuzzi 2008). Adiponectin is considered a hormone involved mainly in lipid and glucose metabolism, but leptin controls Treg proliferation (De Rosa et al. 2007). Adipokines have not been studied in OIT, but during SLIT for pollen allergies, serum leptin levels rose in male patients (Ciprandi et al. 2009). Leptin is known to promote pro-inflammatory activity, but it also can switch cytokine profiles toward Th1 responses (Radon et al. 2008).

3 AIMS OF THE STUDY

The broad purpose of this thesis was to evaluate the management of cow's milk allergy (CMA) with oral immunotherapy (OIT) in school-age children.

The specific objectives were:

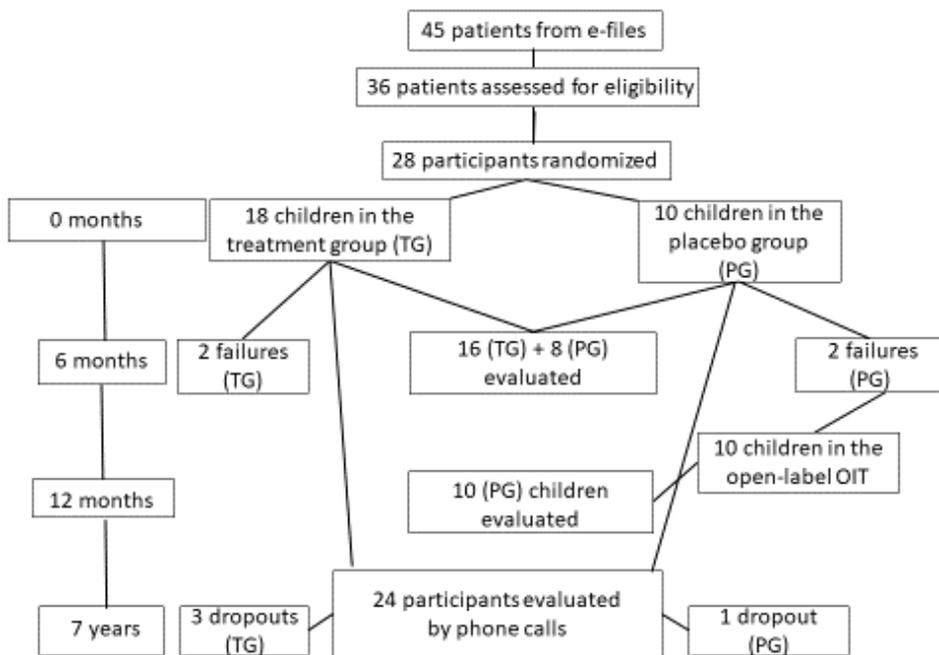
1. To study the effectiveness of OIT with a randomized, double-blind, placebo-controlled trial in school-age children with CMA from early childhood.
2. To evaluate immunological changes, such as allergy and inflammatory biomarkers, during the six-month OIT for CMA.
3. To determine the long-term outcome of OIT seven years later, measured through the consumption of milk or milk protein.
4. To assess measured immunological changes through allergy and inflammatory biomarkers to predict the long-term outcome of OIT in CMA.

4 MATERIAL AND METHODS

4.1 Study design

4.1.1 Course of the study

Flow chart of the study design:



4.1.2 Randomized controlled trial

Overall, 45 children from the electronic files of the hospital were assessed for eligibility for the study by calling and interviewing their parents. The study design was planned as a randomized, controlled trial (RCT), done as a placebo-controlled

trial. Some parents declined because they considered the double-blind trial too laborious, while other children were either too old for the study, passed the preceding milk challenge showing recovery from CMA, or there was no evidence that the children's CMA was IgE-mediated.

Thirty-six patients treated for CMA when they were less than 2 years age were chosen for this study. After detailed oral and written information was provided, 28 were willing to participate, with 18 randomized to the active-treatment arm and 10 to the control arm. We assumed that the success rate would be 70% in the active cases and 10% in the controls, or a 60% case-control difference. If the probability of the type-I error is settled to 0.05, and the case-control allocation rate is 2:1, the power would be 0.9 to reject the null-hypothesis that the difference in the success rates is less than 60 %, when uncorrected chi-square statistics are used. The power calculations were made, and the conclusion was that a sample size of 36 cases is needed.

Twenty-eight school-age children with CMA from early childhood were recruited for the study. The patients were randomized by a 2:1 ratio in the blocks of six (4+2) into the two OIT arms, with 18 in the active group and 10 in the placebo group. The random allocation by block randomization was carried out by the chief pharmacist at the Tampere University Hospital Pharmacy. The study doctors opened the sealed envelopes during the six-month control visits.

4.1.3 Open trial

In the control group, the six-month OIT was followed by an open OIT with an identical protocol, including an escalation phase of six months. All 10 controls, including the two who were considered failures in the double-blind phase, participated.

4.1.4 Patients

A total of 28 children ages 6-14 years were enrolled in the study in May 2008. The patients had been treated for CMA from early childhood in the Department of Pediatrics at Tampere University Hospital in Finland. The median age of the 18 children in the active group was 10.3 years (range: 6-14), and 56% were female. The respective figures in the placebo group were 9.8 years (7-13) and 60%. Most of the patients (79%) had doctor-diagnosed asthma. Nine children (32%) were considered

to have severe CMA since they had presented with anaphylaxis to milk prior to the OIT study. All patients had a history of either pollen allergies or another food allergy besides milk, reported by parents. Other food allergies included eggs, cereals, nuts, or cross-reacting foods with pollen, such as fruits, vegetables, and spices.

Interestingly, the study included three pairs of brothers from three different families. The eligibility criteria of this OIT study, in addition to being between 6 and 14 years old, included the presence of IgE-mediated CMA, which means the diagnosis had to be proved by an immediate (< 4h) reaction in an open milk challenge test during the previous three months. In the event of recent milk anaphylaxis during the previous six-month period a challenge test was abandoned. Additionally, either a positive ≥ 3 mm SPT reaction to CM or serum CM-specific IgE > 3.5kU/l was required.

At the first study visit, hospital records were checked, and the children and parents were interviewed to confirm that the study's enrollment criteria were fulfilled. In addition, the threshold doses of CM that have produced induced symptoms earlier were recorded.

The children's backgrounds included the following: 21 (75%) were asthmatics, 26 (93%) had allergic rhinitis, 26 (93%) had atopic eczema, and 26 (93%) had other food allergies: 20 to eggs, three to soy, 15 to cereal, and 20 to pollen cross-reacting foods.

4.1.4.1 Basic and outcome data of treatment group (Appendix 1)

More-specific data on the active-treatment group can be found in Appendix 1.

4.1.4.2 Basic and outcome data of placebo group (Appendix 2)

More-specific data on the placebo group can be found in Appendix 2.

4.2 Oral immunotherapy

4.2.1 Daily milk-dosing schedule

The schedule of daily milk doses was modified by the first large open study, by Meglio et al., published in 2004, and the maximum dose was the same 6,400mg of

CM protein, equal to 200 mL of milk (Meglio et al. 2004). First, milk (pasteurized 2.5% fresh milk) and placebo (oat drink, rice drink, or soy drink) products were prepared by the Mother's Milk unit of Tampere University Hospital. The placebo was chosen based on the allergy status of each child. All milk products were flavored with sugar.

The schedule started with one drop of solution containing 1 part CM and 25 parts water. For the first five weeks, patients used a milk dilution, and the rest of the time, pure milk. The CM protein amount gradually was increased from 0.006 mg to 6400 mg (equal to 200 mL of milk). The first dose and eight more doses were given at the outpatient clinic: 0.006 mg (Day 1), 0.013 mg (Day 8), 0.026 mg (Day 15), 2.0 mg (Day 36), 4.0 mg (Day 38), 8.0 mg (Day 42), 30 mg (Day 56), 60 mg (Day 64) and 130 mg (Day 78). The dosage then was increased by 0.3 mL daily for two weeks (from day 78 till day 91). Then 0.5 mL daily (day 92 to 105), 1.0 mL (day 106 to 119), 2.0 mL (day 120 to 133), 4.0 mL (day 134 to 147), and finally by 5.0 mL daily until Day 162. Most of the dose escalations occurred at home, and the amount of CM protein was doubled once a week. The OIT protocol lasted 23 weeks, consisting of 10 outpatient visits to the Department of Pediatrics at Tampere University Hospital. These visits were supervised at the outpatient clinic for two hours by two study nurses and two study doctors. The maintenance dose of 6,400 mg after the escalation phase was given at home on Day 162. The control visit at the outpatient clinic was held within two weeks. The patients were on a maintenance dose of antihistamine during the OIT protocol, and during the control visit, antihistamine use was discontinued.

4.2.2 Symptoms during OIT

In case of any symptoms, the parents were given a phone number for the study doctors or study nurses, one of whom answered 24 hours a day. Families were required to have antihistamines, self-injectable adrenaline, and corticosteroid tablets at home if any severe symptoms occurred. Parents were asked to report in a diary the 1-3 most significant symptoms from their perspectives (Thesis article I). The symptoms were reported as dermal, intestinal, oral, nasal, angioedema, or no symptoms during both blind and open phases. All 16 patients in the active group who completed the study had symptoms at some point in the study. These symptoms were mostly oral or intestinal, self-limiting, and needed no medication. There were no cases of anaphylaxis due to OIT treatment in the blind or open phases (Thesis article I).

4.3 Laboratory tests

Blood samples were collected from 28 children during the baseline visit and at the end of the study. In addition, serum was collected from the original placebo group of 10 children at the 12-month mark, when the open OIT was completed. Two serum samples were not analyzed for technical reasons (i.e., not enough serum), and one control patient did not participate in the six-month study visit. The total numbers of eosinophils were counted by an automated cell counter, then expressed as cells $\times 10^9/L$. Nephelometry (Siemens N Latex IgE mono assay) was used for serum total IgE (Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany). Serum CM-specific IgE was measured using the CAP-FEIA technique (Phadia Diagnostics, Uppsala, Sweden) and expressed as kiloUnits per liter (kU/L).

The rest of the samples were stored at -70°C until later analysis (Thesis article II). An enzyme immunoassay (EIA) using commercial reagents determined serum concentrations of EDN, IL-4, IL-5, IL-6, IL-10, IL-12p70, adipsin, adiponectin, leptin, and resistin. The DuoSet ELISA (R&D Systems Europe Ltd; Abingdon, Oxfordshire, UK) measured IL-4, IL-5, IL-12p70, adipsin, adiponectin, leptin, and resistin. IL-6 and IL-10 were measured by PeliPair ELISA (Sanquin; Amsterdam, Netherlands), ECP with the ECP ELISA kit (MBL International; Woburn, MA, U.S.) and EDN with the EDN ELISA kit (MBL International; Woburn, MA, U.S.). Serum CM-specific IgG and IgG4 levels were measured by the Immuno CAP 100 assay (Phadia AB; Uppsala, Sweden), then expressed as milligrams of allergen per liter (mgA/L).

4.4 Follow-up

4.4.1 Phone questionnaire (Appendix 4)

Twelve months after the blind study started (six months after the open OIT), the study doctor (SS) contacted and interviewed the parents of 27 children by phone, 10 from the original placebo group and 17 from the original active group. The parents of one family were interviewed at the outpatient visit. The milk or CM-product consumption and CM-related symptoms during the last six months were recorded through a questionnaire. The parents of all 28 children were contacted by phone with an identical phone interview 3.0-3.5 years later.

Seven years after enrollment, in June 2015, the other study doctor (MP) contacted the subjects by phone and administered a questionnaire for 20 subjects who continued to use milk. They were asked to report daily milk consumption during past 14 days, including the dose, and adverse reactions, classified as intestinal, dermal, nasal, or respiratory symptoms, and any medication needed.

4.4.2 Postal questionnaire (Appendix 4)

Questionnaires for the outcome data were sent to the study subjects three, four, and five years after enrollment. The consumption of CM products was recorded during the previous 12 months. The study subjects reported daily doses, any adverse reactions, any medication needed and, whether discontinuation of daily milk consumption had occurred. The reactions were classified as dermal symptoms, such as itching skin, flaring eczema, or urticarial; nasal symptoms such as runny or stuffy nose; intestinal symptoms including stomach cramps, diarrhea or vomiting; and respiratory symptoms such as a cough, stridor or wheezing. Any episodes of mouth itching, stinging, and angioedema, defined as facial or lip swelling, were recorded. If symptoms from two or more organ systems treated by intramuscular adrenalin were reported, they were labelled anaphylactic reactions and recorded.

4.5 Statistical analyses

The statistical analyses were performed with SPSS statistical software Version 19.0-22.0, for Windows (SPSS Inc; Chicago, IL, U.S.).

We used Fisher's exact test when the success rate and short-term outcome were analyzed in relation to categorized total IgE and milk-specific IgE, as well as for comparison of symptoms during the study between the two groups.

We used the Mann-Whitney U-test to compare continuous variables such as immunological, allergy, and inflammatory parameters between the groups, as well as the Wilcoxon test to compare continuous variables within the groups. The results were provided for comparisons of active and placebo groups, and for comparisons of successful and non-successful cases combining the actively treated groups during the blind and open OIT phases. The baseline point for both groups was May 2008, when the study was launched. The short-term outcome was evaluated for the original active-treatment group six months later, at the end of the escalation phase of OIT,

and for the original placebo group 12 months later, when the open OIT for them ended. Two-tailed p values of <0.05 were considered statistically significant.

We used multivariable Cox regression to find risk factors for the long-term OIT failure during the seven-year follow-up session. We considered failure as discontinuation of OIT, daily milk consumption of less than 200 mL, or CM protein consumption of less than 6,400 mg at the latest follow-up date. Kappa statistics were used to assess the co-existence between wheat and egg allergies, and since it was significant, we combined wheat and/or egg allergies into one variable. The independent variables at enrollment were age, gender, baseline milk-specific IgE, asthma, and combined wheat and/or egg allergies.

When assessing immunological, allergy, and inflammatory parameters all data were analyzed as continuous variables. Since the data were not normally distributed, we used the nonparametric Mann-Whitney U Test.

4.6 Ethics

The study protocol was approved by the Ethics Committee of Tampere University and University Hospital. Before the study started, the children and their parents provided written consent. The study was registered in ClinicalTrials, Gov. ID No. NCT01361347. The study consisted of the blind, randomized, controlled OIT, and open OIT for original controls, plus the seven-year follow-up for both groups.

The study was assumed to be laborious for participants and their parents due to placebo-controlling. Therefore, one member of the Ethics Committee suggested that all placebo patients receive open OIT after the blind phase. Thus, this was implemented. Safety precautions were taken, i.e., the aforementioned emergency medication and study nurses or doctors that were available 24 hours a day. The study required a high level of commitment from parents, patients, and study staff.

5 RESULTS

5.1 Efficacy of oral immunotherapy for cow's milk allergy (I)

There were no significant differences between the children in the active and placebo groups in either age or sex distributions or in clinical characteristics such as asthma, atopic eczema, allergic rhinitis, and other food allergies in the exploratory statistical analysis.

The study consisted of 28 children, 6-14 years old, who previously had been treated for IgE-mediated CMA in Tampere University Hospital's Department of Pediatrics in Tampere, Finland. Sixteen (88.9%) patients from the active-treatment group and eight (80.0%) from the placebo group completed the six-month OIT protocol. Two children in the active group discontinued the protocol due to abdominal symptoms and were considered failures. Another was admitted to the hospital because of abdominal cramps on Day 11 after a dose of two drops of milk dilution. One child in the placebo group discontinued because of a lack of motivation, and the other suffered accidental CM-induced anaphylaxis.

Altogether, 26 (92.9%) children completed the 6-month OIT protocol: 16 cases under the blind OIT and all 10 controls under the subsequent open OIT. Before the study began, 10 of the 28 children had suffered anaphylactic reactions associated with food. One case was attributed to a hen's egg and the others to CM. We also could demonstrate that the success rate of OIT was 80.0% (8 in 10) for children with CMA presented earlier with an anaphylactic reaction due to accidental exposure.

Before OIT, the amount of CM protein tolerated was 6 mg (range 0.05 – 162 mg). After the blind OIT, 14 children tolerated the full amount of 6,400 mg and another two tolerated 1,920 and 960 mg.

At the 12-month mark, after the blind-OIT study had ended, 13 children from the active-treatment group and all 10 children from the original placebo group used milk products daily, corresponding to 6,400 mg of CM protein. Two failures from the active-treatment group were advised to follow strict CM avoidance. Three children who completed the protocol did not use the target amount of milk products: two tolerated 6,400 mg of CM protein during OIT, and one with very high total IgE and CM-specific IgE tolerated 1,920 mg. Furthermore, the child who had tolerated

only 960 mg during OIT now tolerated the full 6,400 mg. Thus, 23 (82%) of 28 children were able to use significantly more CM 6-12 months after the initial assessment than at the beginning of the study.

5.2 Safety of oral immunotherapy for cow's milk allergy (I, III)

Parents were asked to report the most significant symptoms related to milk intake, but no more than three such symptoms during the escalation phase (Table 1 in Article I). All 16 patients in the active group who completed the six-month study had symptoms at some point. These were mostly mild symptoms, such as itchy mouth or abdominal pain, that did not require treatment. On the other hand, antihistamines regularly were administered during both blind and open OIT until two weeks after reaching the target dose of 200 mL of milk. There were no systemic reactions reported in the active group, nor among controls, when they were given CM. In the placebo group, one patient withdrew because of accidental CM-induced anaphylaxis.

5.3 Outcome at 3.5 years (I)

One child discontinued using milk products by the time of the follow-up 3-3.5 years later because of difficult asthma and atopic-eczema symptoms. After the patient returned to the strict CM-avoidance diet, however, the boy experienced accidental milk-induced anaphylaxis. He had discontinued the blind OIT trial in the placebo group because of the aforementioned accidental CM anaphylaxis, but successfully completed the open OIT. Thus, the 3.5-year success rate, when the original active-treatment and placebo groups were analyzed and combined, was 22 out of 28 (79%).

5.4 Outcome at seven years (III)

The long-term efficacy seven years after the start of the study was 50% (14 of 28), including 58% of the 24 who participated in the follow-up study. Most of the patients used 200-500 mL of milk or a corresponding amount of CM products daily. When desensitization was reached, it was sustained for at least seven years in 14 of 24 (58.3%) cases. Still, 18.3% of the participants reported milk-related symptoms at the seven-year follow-up mark, compared with 50.0% at the three-year mark (Table 1 in

Article III). The high baseline milk-specific IgE correlated with poor outcomes at the seven-year mark (Table 2 in Article III).

5.5 Changes in biomarkers during oral immunotherapy (II)

There were no significant differences in immunological status, i.e., allergy or inflammatory parameters at the baseline between the active OIT group and the placebo control group (Article II). The only exception was serum milk-specific IgG4, which was higher in the active group (Table 1 in Article II).

Serum IL-6 and IL-10 were significantly higher in the active group than in the placebo group at the six-month mark (Table 2 in Article II). When the results of the blind OIT at six months and open OIT at 12 months were combined, serum total IgE and blood eosinophils decreased (Table 2 in Article II), and milk-specific IgG4 and IgG, serum IL-4 and IL-6, and adipocytokines resistin and leptin were significantly higher compared with baseline levels (Table 4 in Article II).

5.6 Biomarkers as prognostic factors (I, III, IV)

Serum milk-specific IgE was high, at 70 IU/L and 313 IU/L, in both children who were unable to complete the six-month OIT protocol, and only three of 16 children who completed the protocol had milk-specific IgE >70 IU/L. Thus, milk-specific IgE > 70 IU/L seemed to predict failure in completing the OIT protocol.

The high baseline serum milk-specific IgE could predict the long-term seven-year failure after the OIT trial-in-maintenance phase ($p=0.021$) (Table 2 in Article III). In addition, serum adipon, an inflammatory marker, at the end of the six-month OIT protocol for CMA, was significantly higher in the unsuccessful group than in the successful OIT group of milk-users at the seven-year follow-up point (Table 2 in Article IV).

6 DISCUSSION

6.1 Oral immunotherapy in cow's milk allergy

The main result in our blind OIT study was achieved desensitization measured by daily milk usage with minimum symptoms in 89% of active-treatment group participants. The effect was sustained for at least three years in 79% of the participants. In addition, we included school-children with severe CMA in our study, and their success rate was 80%.

The latest Cochrane review on OIT for milk allergies concludes that OIT is an effective method in inducing desensitization for CMA patients (Yeung et al. 2012). The pooled success rate assessed after OIT was 62%, compared with the respective figure of 89 % in the present study. Our study protocol, modified from that of Meglio et al. (Meglio et al. 2004), differed from most extant OIT studies which have used a three-step procedure consisting of rush, build-up, and maintenance phases (Longo et al. 2008, Skripak et al. 2008). Some studies consisted only of two steps: rush and maintenance phases (Staden et al. 2008, Martorell et al. 2011). The rush phase, consisting of frequent dosing in a rapid build-up process, particularly is associated with a risk for severe adverse events (Barbi et al. 2012, Longo et al. 2008). Our study consisted of a six-month protocol of incrementally increasing doses, then daily milk consumption that should continue throughout one's life. Thus, our protocol may be considered less risky for severe CM-allergic patients. Although all children in the active group suffered from milk-related allergic symptoms during the study, only one severe reaction occurred, which was due to accidental milk exposure in the control group. Our placebo-controlled, double-blind study confirmed that milk OIT was effective in desensitizing CM-allergic schoolchildren. The short-term success rate was 89% when assessed immediately after the six-month OIT and 79% when assessed three years later. The long-term success rate at the seven-year follow-up mark was 50%, which means the children with CMA from early childhood were able to consume milk or milk products daily.

The Cochrane review (Yeung et al. 2012) included only five RCTs on milk OIT (Longo et al. 2008, Martorell et al. 2011, Pajno et al. 2010, Skripak et al. 2008), including the present study (Article I). No more RCT studies were done from 2012

to the present, but a large number of clinical trials have been published (Meglio et al. 2004, Zapatero et al. 2008, Caminiti et al. 2009, Barbi et al. 2012, García-Ara et al. 2013, Vazquez-Ortiz et al. 2013). Furthermore, many trials are ongoing (Tordesillas, Berin & Sampson 2017). In addition to the present study (Article I), only one RCT included in the Cochrane review was double-blind and placebo-controlled (Skripak et al. 2008). One was single-blind and placebo-controlled (Pajno et al. 2010), and two were open trials, controlled by an avoidance diet (Longo et al. 2008, Martorell et al. 2011). In all studies, the controls were on milk-free avoidance diets. These five extant studies are summarized in Table 1. There were 196 child patients in all, consisting of 106 actively treated patients and 90 controls. In one study, patients were younger with a better prognosis of tolerance development, at 60 out of 196 (31%) (Martorell et al. 2011) compared with 110 out of 196 (56%) in other studies (Longo et al. 2008, Skripak et al. 2008, Pajno et al. 2010). However, only seven participants (8%) in the control groups, compared with 66 (62%) in the OIT groups, achieved desensitization to full servings of CM, which was only 15mL in one study (Skripak et al. 2008) and 150-200 mL in other studies, including the present study. The development of tolerance could not be ruled out in the present study since post-OIT elimination-challenge tests were not done. Most of the studies assessed the primary outcome as treatment success confirmed with a blind food challenge on a maintenance dose. However, no reliable biomarkers are yet available for measuring true permanent tolerance instead of desensitization, demanding continuous daily exposure to maintain the positive effect.

Table 1. Five RCT studies in the Cochrane review

Author	Study design	Patients	Age range (years)	Withdrawal (n)	OIT (weeks)	Maintenance dose protein (g)	Maintenance dose reached (%)	Desensitization (%)
Longo et al. 2008 Italy	open	30 Active 30 CM-free diet	5 - 17	3	52	5*	36	(23% unrestricted diet) 0%
Skripak et al. 2008 U.S.	double-blind	13 Active 7 Placebo	6 - 17	1	23	0.5*	92	15% 0%
Pajino et al. 2010 Italy	single-blind	15 Active 15 Soymilk	4 - 13	3	18	6.6*	67	67% 0%
Martorell et al. 2011 Spain	open	30 Active 30 CM-free diet	2 - 2.5	6	16	6.6*	90	(90% unrestricted diet) 23%
Thesis article I Finland	double-blind	18 Active 10 Placebo	6 - 14	4	23	6.4*	89	(72% unrestricted diet) 0%

*5g CM protein=150ml cow's milk

6.2 Biomarkers in oral immunotherapy

Several studies have shown how Th2 responses are downregulated, Th1 responses are increased, and Treg cells are activated when food OIT is successful. Peanut OIT studies have documented a decrease in allergen-specific IgE and SPTs, as well as in IL-4, IL-5, and IL-13 cytokine productions, and an increase in allergen-specific IgG and IgG4, as well as in IL-10 and TGF- β productions (Blumchen et al. 2010, Jones et al. 2009, Varshney et al. 2011). In the short-term follow-up of 3-17 months of milk OIT patients, milk-specific IgE decreased, IgG4 increased, and skin-test reactivity to milk disappeared (Narisety et al. 2009).

We did not determine casein-, α -lactalbumin-, or β -lactoglobulin-specific IgG or IgG4 antibodies, even though there are interfering IgG antibodies for bovine albumin in most sera. Two blind studies determined the sum of casein-, α -lactalbumin-, and β -lactoglobulin-specific IgG4 as a surrogate measure, and their results, showing a CM-specific IgG4 level increase in the active group, were in line with our study when we combined the treated groups from the blind and the open-label studies (Skripak et al. 2008, Pajno et al. 2010). In fact, the induction of allergen-specific IgG4 antibodies is considered to be one of the key OIT mechanisms (Ponce et al. 2016). In line, the sum of the casein-, α -lactalbumin-, and β -lactoglobulin-specific IgG4 antibody levels was used as a surrogate in the single-blind RCT study (Pajno et al. 2010). In all, the increases of CM-specific IgG and IgG4 that we found when the active and control groups were analyzed as combined were in line with these two other RCTs.

Our study was powered for the double-blind OIT, but it was small and probably not strong enough to find significant associations in the biomarkers. In addition, the biomarkers were studied only at the baseline, at six-months for the active and control groups after double-blind OIT and at 12 months for the control group after open OIT. In the Italian follow-up study that continued for 4.5 years, there was a significant reduction in milk-specific IgE (Meglio et al. 2008), which we could not document in the short term.

Thus far, our study is the only one showing that OIT affects serum adipokines. The preliminary finding of leptin and resistin increasing significantly during OIT warranted confirmation. Both adipokines may possess anti-allergic activity, like many cytokines do (Fantuzzi 2005). Leptin is known to switch Th2 profiles toward a Th1 response. Resistin has been reported to enhance Th1-type response (Tarkowski et al. 2010).

6.3 Safety of oral immunotherapy

Our study showed that milk OIT can be a safe treatment for school-age children with CMA, including those with severe symptoms from previous exposure. Only one child during the three-year follow-up presented with a severe allergic reaction, treated as anaphylaxis with self-injectable adrenalin. This episode was tied to asthmatic symptoms during birch pollen season, as well as an accidentally large dose of milk products. These associations refer to the view that other factors, e.g., physical exercise, menses, fever, and acute infection, may affect immune systems, elicit symptoms, and increase the severity of allergic reactions to CM, even after successful desensitization (Caminiti et al. 2007, Varshney et al. 2009). We advised our OIT patients to avoid physical exercise 1.5 hours before and after CM intake, and in case of fever, or respiratory or gastrointestinal infection, the CM dose was not to be increased until a patient fully recovered.

In our study, children continued an age-appropriate dose of antihistamine daily during OIT until the check-up at an outpatient clinic two weeks after reaching the maintenance dose. Antihistamines also were used in other studies (Longo et al. 2008, Meglio et al. 2004). They may prevent or treat mild allergic symptoms, such as oral or pharyngeal symptoms, during OIT, but in the event of severe symptoms, intramuscular adrenalin is needed (Muraro et al. 2007, Sampson et al. 2006).

The safety aspect and long-term commitment must be discussed with families and children taking part in OIT studies. For safety reasons – not to regain clinical reactivity – many experts recommend milk consumption every day (Longo et al. 2008, Skripak et al. 2008, Staden et al. 2007, Vazquez-Ortiz et al. 2013). Case reports have been published on life-threatening reactions associated with milk during OIT protocols (Nieto et al. 2010, Vazquez-Ortiz et al. 2014). All children were teens with asthma in these studies. Teen years are known to be challenging for asthma patients (Couriel 2003). Thus, it is not surprising that poor compliance in relation to asthma and food-allergy treatment may even lead to fatal consequences (Bock, Muñoz-Furlong & Sampson 2001). The presence of asthma also has been linked to severe allergic reactions in accidental exposures (Boyano-Martínez et al. 2009). Thus, asthma may be considered a risk factor for severe side effects during OIT, even during the maintenance phase. In our study, the only patient who needed intramuscular adrenalin had asthma treated with inhaled corticosteroids. However, as many as 75% of the children in our study had asthma, but adrenalin was required only this one time.

6.4 Long-term follow-up

We assessed long-term efficacy based only on phone interviews, when structured questionnaires were administered. The patients were not studied clinically, nor were they challenged with milk during the follow-up. The long-term outcome was settled in a corresponding way in all other milk OIT follow-up studies (Keet et al. 2013, Luyt, Bravin & Luyt 2014, Meglio et al. 2008). All three studies lacked control groups and one included both OIT- and SLIT-treated children (Keet et al. 2013).

Our long-term success rate is in line with other prospective follow-up studies. However, it was lower – 50% of all 28 children who enrolled in the study and 58% of those 24 who attended the seven-year follow-up – than immediately after the six-month OIT (86%). In the U.S. study, only 31% tolerated a full maintenance amount of milk with minimal symptoms or no symptoms at a median follow-up of 4.5 years (Keet et al. 2013). In the Italian study, the desensitization rate was 65% at the 4.5-year follow-up mark (Meglio et al. 2008).

High milk-specific IgE is a potential risk factor for discontinuing milk consumption, as was seen in the present study. In two other studies, participants with baseline milk-specific IgE > 75kU/L did not reach the target CM dose and were symptomatic (Keet et al. 2013).

6.5 Methodological aspects

6.5.1 Strengths of the study

The key strength of the study was its randomized, placebo-controlled, and double-blind design. There is only one previous milk OIT study that was conducted in this fashion (Skripak et al. 2008). On one hand, this type of study design may have reduced the number of participants, but on the other hand, the participants and their parents had to be motivated to take part in such a laborious study. Overall, only a few randomized controlled trials in milk OIT have been published so far (Longo et al. 2008, Skripak et al. 2008, Pajno et al. 2010, Martorell et al. 2011). Some have been conducted with no control group (Meglio et al. 2004, Zapatero et al. 2008, Caminiti et al. 2009, Barbi et al. 2012, Viquez-Ortiz et al. 2013) or they are merely case series (Luyt, Bravin & Luyt 2014).

The milk product used as the placebo was either a soy drink, oat drink, or rice drink, depending on which was suitable for each child. The active CM product was

pasteurized 2.5% fresh milk. The Mother's Milk unit at the hospital prepared all products for identical packages. Both active-treatment and placebo products were flavored with sugar. In the other placebo-controlled study, blinding of the participants and investigators was done, but not described in the text (Skripak et al. 2008). It may be assumed that the same products used for the blind challenge test at the baseline were used for the protocol as well. The other single-blind study originally was designed as a double-blind, controlled study, but the authors did not consider soy milk as a true placebo, as its taste could be distinguished from the taste of CM (Pajno et al. 2010).

Our study protocol was for outpatient visits, and several dose escalations were performed at home, which may be considered a strength, compared with starting with an in-hospital phase for 10 days (Longo et al. 2008).

The long follow-up period for the patients involved is a clear strength, even though a control group was lacking. The original control group treated with a placebo later was treated with open OIT for CM. This motivated the patients to participate in the study, and we considered it ethically mandatory.

6.5.2 Shortcomings of the study

The main limitation of the present study was the size of the study population for the biomarker and follow-up studies. The study was powered only for the double-blind OIT intervention. This allows for possible Type II errors, especially because there could have been significant changes in biomarkers that we could demonstrate. On the other hand, we tested as many as 16 biomarkers, which presents the problem of multiplicity. No multi-testing corrections were done for the few positive findings. Our study is the largest done with a placebo-controlled, double-blind design for OIT in CMA, including a long-term prospective follow-up.

At the beginning of the OIT, study a diagnosis of CMA was not confirmed by DBPCFC, which is currently considered the gold standard for diagnosing food allergies. We relied on a robust history of CMA, or, in some cases, a recent documented anaphylaxis episode. Likewise, sustained unresponsiveness was not confirmed by post-treatment milk-elimination challenge after OIT. This means that desensitization was confirmed, but not tolerance.

We are aware of the lack of systematic, prospective symptom monitoring during the double-blind and open OIT. Maybe we should have used a diary to register all symptoms every day. However, when parents reported from one to three of the most

significant symptoms, this might have given us even more reliable information. The most serious adverse reactions were recorded.

We did not study follow-up serology at the seven-year follow-up mark, which could have been more informative concerning the risk factors for OIT failure.

Also, we did not examine both groups through a challenge test after a double-blind phase to assess desensitization. Our treatment success was measured by reaching the maintenance dose instead of passing the challenge, as we viewed desensitization as the ability to tolerate more milk after the trial than before, a more realistic goal. Even though a state of desensitization requires ongoing allergen exposure, it is protective against accidental milk exposures. In addition, we assumed that there were hardly any tolerant children in the placebo group, since the recovery of CMA is rare in school-age children (Saarinen et al. 2005, Skripak et al. 2007). Furthermore, in other RCT studies, no tolerant controls were found when the trials assessed clinical tolerance with a milk-challenge test (Longo et al. 2008, Pajno et al. 2010, Skripak et al. 2008).

6.6 Future research needed

Though new treatment trials are underway, there are no ongoing registered studies of cost-effectiveness of laborious OIT for CMA. Even though OIT is beneficial for a single patient a health-economic analysis needs to be done.

Patient selection also is crucial for choosing which CMA children are most amenable to the therapy. Therefore, biomarkers should be studied further for predicting the outcome of OIT for CMA.

The OIT protocols differ from each other tremendously, so a unified protocol would be useful when comparing studies with each other. Also more comparisons of OIT, SLIT and EPIT studies are needed.

Some data show how milk OIT improves quality of life for patients and parents during and at the end of OIT (Carraro et al. 2012, Rigbi et al. 2017). More data, especially concerning the effect on quality of life for the OIT patients in the long run, are needed.

Finally, more research on the mechanisms and safety, as well as long-term outcomes and safety of immunotherapy for food allergies, is crucial. The safety of OIT has been shown to increase through co-treatment with omalizumab but is this worth the cost (Wood et al. 2016)? Interestingly, probiotics showed promising results of unsustained responsiveness in peanut OIT (Hsiao et al. 2017), but this has not

been studied in milk OIT. The safety of OIT vs. continued elimination needs to be assessed because if we can demonstrate that OIT is safer than continued avoidance, it will be easier to mandate its use in further studies.

7 CONCLUSION

We found that OIT is effective in raising the threshold of reactivity in school-age children with IgE-mediated CMA. In the double-blind study, the effect was 16 of 18 study participants (89%), and including the open study, 26 of 28 (93%). Our study revealed the benefits of OIT and also showed how common milk-related reactions are during OIT -- fortunately mild. The treatment was accomplished totally at an outpatient clinic, so it was less time-consuming and resource-intensive than past studies with a rush episode in a hospital.

We confirmed that interleukins IL-10 and IL-6 increased in the active group during the blind OIT study. When the immunological changes were combined from both treated groups, active in the blind phase and placebo in the open phase, allergy markers such as blood eosinophils and total IgE decreased during milk OIT. Also, milk-specific IgG4 and IgG increased, as did IL-6 and IL-4. A novel finding was the rise of leptin and resistin -- adipokines linked to Th1-type response -- during OIT. The role of adipokines in food OIT remains to be examined.

The effect of OIT remained at least for seven years in 58% of participants when assessed in milk-protein consumption. The rate of symptoms due to milk consumption also decreased over the years.

We did not find any reliable biomarkers other than high milk-specific IgE to predict poor outcomes in milk OIT treatment in the long run. Adipocytokine adiponin was the only marker that was elevated during the OIT protocol, which is associated with non-use of milk at the seven-year mark.

Before milk OIT can be considered a clinical practice outside of allergy clinics, universally proved and standardized protocols are needed. In addition, safety must be ensured through well-conducted, placebo-controlled studies.

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10 APPENDIX

Table 1. Protocol for cow's milk dosing schedule used in oral immunotherapy

Day	Diluted milk (drops)	Raw milk (drops)	Raw milk (mL)	CM protein (g)
1	1			0.00006
8	2			0.00013
15	4			0.00026
22	8			0.0005
29	16			0.001
36		1		0.002
43		5		0.008
50		10		0.016
56		20		0.03
64			2	0.06
78			4	0.13
92			8	0.26
106			16	0.5
120			32	1
134			64	2
148			128	4.1
162			200	6.4

Table 2. Basic and outcome data for the 18 children in the active treatment group for oral immunotherapy

No	Gender, Age (years)	Asthma	Other allergies	Serum IgE (IU/L) Total/ specific	Prior CM challenge/ accidental exposure Dose, reaction	The CM dose at the end of oral immunotherapy
1.	Boy, 12y	No	Cereals, Eggs, Nuts, Other foods, Birch	901 / 1,8	Drop, Itching	200 mL
2.	Girl, 7y	Yes	Cereals, Eggs, Dogs	444 / 6,2	10 mL, Anaphylaxis	200 mL
3.	Boy, 11y	Yes	Cereals, Other foods, Birch, Dogs	3690 / 15	2 mL, Angioedema	200 mL
4.	Girl, 13y	Yes	Cereals, Eggs, Other foods, Birch, Timothy, Mugwort	8100 / 33	162 mL, Wide Urticaria	200 mL
5.	Boy, 10y	Yes	Birch, Timothy	621 / 39	50 mL, Urticaria	195 mL
6.	Boy, 10y	Yes	Cereals, Eggs, Nuts, Fish	228 / 4,4	2 mL, Angioedema, Intestinal ^A	200 mL
7.	Girl, 9y	Yes	Birch, Timothy, Cats, Alder, Mugwort	7410 / 229	Drop, Anaphylaxis ^A	200 mL
8.	Girl, 11y	Yes	Cereals, Soy, Eggs, Nuts, Other foods, Birch, Timothy, Alder, Mugwort	10100 / >400	Drop, Dermal	60 mL
9.	Boy, 7y	Yes	Nuts, Other foods, Birch, Alder, Cats, Dogs	495 / 0,7	10 mL, Nasal, Itching ^A	200 mL
10.	Girl, 9y	Yes	Cereals, Eggs, Nuts, Birch, Timothy	778 / 3,8	2 mL, Itching	30 mL
11.	Boy, 7y	Yes	Cereals, Nuts, Birch, Timothy, Mugwort, Cats	473 / 22	Drop, Dermal	195 mL
12.	Girl, 9y	No	Eggs, Nuts	865 / 21	2 mL, Dermal, Itching	200 mL
13.	Boy, 6y	Yes	Soy, Eggs, Fish, Other foods, Birch, Cats, Dogs, Horses, Cows	2150 / 133	Drop, Anaphylaxis ^A	200 mL
14.	Girl, 13y	No	Birch	638 / 2,6	160 mL, Wide Urticaria	200 mL
15.	Girl, 10y	No	Eggs, Nuts, Other foods, Birch, Timothy, Mugwort	409 / 5,1	62 mL, Urticaria	200 mL
16.	Girl, 9y	Yes	Nuts, Other foods, Birch, Cats, Dogs, Horses, Cows	240 / 3,4	62 mL, Itching, Intestinal	200 mL
17.	Boy, 14y	Yes	Cereals, Eggs, Fish, Alder, Dogs	4790 / 70	Drop, Itching	Interruption: four drops of milk ¹
18.	Girl, 6y	Yes	Eggs, Nuts, Other foods, Timothy, Cats	5230 / 313	Milk powder in candy, Anaphylaxis ^A	Interruption: two drops of dilution ²

200 mL of milk corresponds to 6,400 mg of milk protein.

Allergies other than CMA allergies were parent-reported. Other food allergens were mainly from fruits, vegetables, and spices cross-reacting with seasonal pollens, based on the medical history reported by parents.

Nasal symptoms refer to runny or stuffy nose, dermal symptoms entail itching skin or flaring eczema, urticaria was recorded separately, and intestinal symptoms meant vomiting, diarrhea, or stomach cramps. Itching means mouth itching or stinging. Anaphylactic reaction was defined as symptoms from two or more organ systems that needed intramuscular epinephrine as first aid. Angioedema means facial swelling, usually in the lips.

^ Accidental exposure in five cases

¹The study was discontinued on Day 42 (dose four drops of CM) due to stomach pain. ²The study was discontinued on Day 11 (dose two drops of milk dilution) due to stomach cramps.

Table 3. Basic and outcome data for the 10 children in the placebo group for oral immunotherapy

No	Gender, Age (years)	Asthma	Other allergies	Serum IgE (IU/L) Total/ specific	Prior milk challenge / accidental exposure Dose, reactions
1.	Girl, 8y	Yes	Cereals, Soy, Eggs, Nuts, Other foods, Birch, Dogs	5040 / 14	2 mL, Urticaria
2.	Girl, 10y	No	Cereals, Eggs, Nuts, Other foods, Birch, Alder, Cats, Dogs	2840 / 7,8	2 mL, Nasal, Itching, Intestinal
3.	Boy, 8y	No	Eggs, Nuts, Other foods, Birch	623 / 5,7	2 mL, Angioedema
4.	Boy, 9y	Yes	Cereals, Eggs, Nuts, Other foods, Birch, Timothy, Mugwort	656 / 2,8	2 mL, Itching [^]
5.	Girl, 10y	Yes	Other, Birch, Cats, Dogs, Horses	Not available	2 mL, Angioedema
6.	Girl, 13y	Yes	Cereals, Eggs, Fish, Birch, Timothy, Mugwort	731 / 3,8	2 mL, Itching
7.	Girl, 9y	Yes	Birch, Mugwort	662 / 13	2 mL, Itching, Abdominal
8.	Girl, 11y	Yes	Cereals, Eggs, Nuts, Other foods, Birch, Timothy, Mugwort, Cats, Dogs, Horses	1060 / 11	50 mL, Dermal, Intestinal
9.	Boy, 7y ¹	Yes	Eggs, Nuts, Birch, Cats, Dogs, Horses	354 / 27	Milk powder in candy, Anaphylaxis [^]
10.	Boy, 13y ²	Yes	Eggs, Nuts, Birch, Alder, Timothy, Cats, Horses	941 / 15	Drop, Itching

Questionnaire

Milk OIT for school-aged children

Milk-related symptoms

(≤1 year after treatment ended)

**Patient's name and birth
date:**

Date:

Milk or milk products used daily (circle the correct answer): yes no

The amount of milk or milk products (when used last time):

Milk-related symptoms (circle the correct answer)

0 = no symptoms, 1 = mild symptoms, 2 = moderate symptoms, 3 = severe symptoms

1. Mouth itching or stinging	0	1	2	3
2. Nasal symptoms, runny nose, sneezing	0	1	2	3
3. Eyes itching, red, lacrimation	0	1	2	3
4. Dermal symptoms (urticaria, erythema)	0	1	2	3
5. Dermal symptoms (atopic eczema flare-up)	0	1	2	3
6. Vomiting, stomach pain, diarrhea	0	1	2	3
7. Respiratory symptoms	0	1	2	3
8. Other symptoms (describe the symptoms)				
_____	0	1	2	3

Treatment for symptoms (circle the correct answer):

Oral antihistamine yes no

Corticosteroid yes no (iv / im / po)

Adrenalin yes no (iv / im)

If you wish you may use the other side of the paper for any comments.

Thank you!

11 ORIGINAL PUBLICATIONS

- I Salmivesi, S., Korppi, M., Mäkelä, M.J., Paasilta, M. (2012). Milk oral immunotherapy is effective in school-aged children. *Acta Paediatrica*, 102, 172 – 176.
- II Salmivesi, S., Paasilta, M., Huhtala, H., Nieminen, R., Moilanen, E., Korppi, M. (2016). Changes in biomarkers during a six-month oral immunotherapy intervention for cow's milk allergy. *Acta Paediatrica*, 105, 1349 – 1354.
- III Paasilta, M., Salmivesi, S., Mäki, T., Helminen, M., Korppi, M. (2015). Children who were treated with oral immunotherapy for cows' milk allergy showed long-term desensitization seven years later. *Acta Paediatrica*, 105, 215 – 219.
- IV Salmivesi, S., Paasilta, M., Huhtala, H., Nieminen, R., Moilanen, E., Korppi, M. (2017). Elevated serum adipsin may predict unsuccessful treatment for cows' milk allergy but other biomarkers do not. *Acta Paediatrica*, 107, 328 – 332.

REGULAR ARTICLE

Milk oral immunotherapy is effective in school-aged children

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Clinical trial registering: ClinicalTrials, Gov Id
NCT01361347**ABSTRACT****Aims:** To study the efficacy of oral immunotherapy (OIT) in schoolchildren with cow's milk (CM) allergy (CMA).**Methods:** Twenty-eight children aged 6–14 years with CMA documented by oral challenge were enrolled into a randomized, double-blind, placebo-controlled OIT study. In the active treatment, CM protein amount was increased during 23 weeks from 0.06 mg to a maximum of 6400 mg (200 mL of milk).**Results:** Twenty-four (86%) patients completed the protocol: 16/18 in active and 8/10 in placebo groups. All children in the active and 2/3 in the placebo group suffered from symptoms considered by the parents as induced by milk. The children were contacted by phone 12 months later, and 13 (81%) used daily CM or milk products corresponding 6400 mg of CM protein. After double-blind OIT, all 10 children in the placebo group completed successfully an open-label OIT by an identical protocol, and all used daily CM or milk products 6 months later. Three to 3.5 years later, one child more had discontinued daily milk use. Thus, the long-term success rate was 22/28 (79%).**Conclusions:** This placebo-controlled, double-blind study confirmed that OIT was effective in desensitizing schoolchildren with CMA. With occasional exceptions, the reached desensitization sustained for more than 3 years.**INTRODUCTION**

Cow's milk (CM) allergy (CMA) is common in young children and improves usually spontaneously before school age, but in 10–15% of cases, continues until adolescence and may continue even until adulthood. Currently, the standard care for food allergy is strict allergen avoidance, restricting the everyday life of allergic persons. However, many recent studies have reported encouraging results concerning oral immunotherapy (OIT) in the treatment CMA or hen's egg allergy (1–7). The results have ranged from protection against small accidental exposures to tolerance of substantial amounts of the allergen. However, the majority of these studies have been small, with limited characterization of patient populations and dosing protocols. The follow-ups published thus far have continued from 3 months to 4.8 years (8,9).

Only one study, published by Skripak et al. (10), has evaluated the efficacy and safety of OIT in children with CMA using a randomized, placebo-controlled, double-blind design. Nineteen patients attended the trial, and only 13 of them were re-examined during open-label CM dosing after the trial (9). In that study, OIT was effective in desensitizing children with CMA (10), and the threshold dose to elicit allergic symptoms further increased over time when CM was in daily use (9). This necessity of daily CM consumption to maintain desensitization has been observed also in

previous studies (1,4,11). Whether OIT can lead to tolerance, that is, a permanent loss of reactivity to an allergen has been questioned (4).

When reached, desensitization sustained at least for 3 years

PATIENTS AND METHODS

Schoolchildren who were treated for CMA in the Department of Pediatrics, Tampere University Hospital (Finland), were invited to participate in the study. The criteria for the recruitment were the presence of immunoglobulin E (IgE)-mediated CMA, that is, an immediate (<4 h) reaction in an open CM challenge test performed within 6 months and the

Key notes

- Allergen avoidance is the standard care of food allergy.
- This randomized, placebo-controlled, double-blind study in 28 children aged 6–14 years showed that oral immunotherapy is effective in desensitizing children with milk allergy.
- When reached, desensitization sustained at least for 3 years.

age of 6–14 years. The challenge test was not repeated, if an immediate reaction had associated with an accidental CM exposure within 6 months, or if the child had been hospitalized because of an anaphylactic reaction to CM. A positive, >3 mm skin prick test (SPT) response to CM extract, or the CM protein-specific IgE concentration >3.5 kU/L, was demanded to confirm the IgE-mediated mechanism. Thirty-six patients treated for CMA from <2 years of age were invited to attend the study, and after detailed oral and written information, 28 were willing to participate. The CMA diagnosis was based on a challenge test in 21 cases and on an accidental exposure with a severe systemic reaction in seven cases.

The design of the OIT study was a randomized, placebo-controlled, double-blind trial. The study participants were randomized by a 2:1 ratio in the blocks of six (4 + 2) into the two OIT arms, 18 patients into the active treatment and 10 into the placebo group. At the first study visit, the hospital records were checked and the children and parents were interviewed to confirm that the enrolment criteria of the study were fulfilled and to record the threshold doses of CM to induce symptoms. The reactions were classified into dermal (itching skin and/or flaring eczema, or redness and/or urticaria, as recorded separately), nasal (runny or stuffy nose), intestinal (vomiting, diarrhoea or stomach cramps) or respiratory (cough, stridor or wheezing). In addition, mouth itching and/or stinging, and angioedema, defined as facial or lip swelling, were recorded if present. Anaphylactic reaction was considered if there were symptoms from two or more organ systems treated by intramuscular adrenaline. Serum total IgE and CM protein-specific IgE were measured at the first study visit by electrochemiluminescence.

The mother's milk unit of the hospital prepared the milk (pasteurized 2.5% fresh milk) and placebo (oat milk, rice milk or soy milk, depending on the allergy status of the child) products and packed them in identical bottles. Both active treatment and placebo products were flavoured with sugar. In the active treatment group, the amount of milk protein increased daily, being doubled every week, from 0.06 to 6400 mg (Table S1). The target dose was 6400 mg in the study of Meglio et al. (1) and 8240 mg in the study of Skripak et al. (10). The first dose (0.06 mg) and eight later doses were given in the outpatient clinic as follows: 0.12 mg on day 8; 0.24 mg on day 15; 2.0 mg on day 36; 4.0 mg on day 38; 8.0 mg on day 42; 40 mg on day 57; 80 mg on day 64; and 160 mg on day 78. The children were monitored at the outpatient clinic for 2 h. The final dose of 6400 mg was given at home on day 162, and the control visit was held within 2 weeks. All other dose increases were performed at home according to a prospective, daily schedule (Table S1). If the parents noted any symptoms, they were advised to phone to the study doctors who were attainable 24 h a day by telephone. For emergency situations, study subjects were required to have antihistamines, self-injectable adrenaline and prednisolon tablets at home. In case of symptoms either at home or at the outpatient clinic, the study doctors modified the protocol as needed. Four subjects interrupted the study, and 26 completed the protocol within 162 (+14) days.

At the end of the double-blind OIT programme, one of the authors (SS or MP) examined the patients. The symptoms during OIT were summarized by interviewing the parents. The presence of the symptoms presumably associated with food allergy, as well as emergency room visits if present, was asked. If the parents reported symptoms, they were asked to assess which symptoms were the three most common in the order of importance. Thereafter, the code was opened. The children who belonged to the active treatment group, and who had completed the OIT protocol totally or partially, were advised to continue daily CM use, either 200 mL (6400 mg milk protein) or a corresponding amount of CM products, or a lower amount reached during OIT. The children who belonged to the placebo group were invited to an open-label OIT. The dosing schedule and the registration of allergic symptoms were exactly the same as in the treatment group of the placebo-controlled phase of the study, and one of the authors (MP) examined the patients and interviewed the parents at the end of the programme.

Twelve months after the placebo-controlled OIT (6 months after the open OIT), one of the authors (SS) contacted by phone the parents of 27 children, 17 from the original active treatment and 10 from the original placebo group. In addition, one family was contacted at their regular outpatient visit in the allergy centre. The use of CM or CM products and the symptoms during the last 6 months of open-label milk use were charted by using a questionnaire. An identical phone interview was performed to the parents of all attending 28 children 3.0–3.5 years later.

Exploratory statistical analysis revealed that there were no significant differences between the children in the two treatment arms in either age or sex distribution or in clinical characteristics, including asthma, allergic rhinitis, atopic eczema and other food allergies (data not shown). The median age of the 18 children in the active treatment arm was 10.1 years (range, 7–14), and 44% were boys. The respective figures in the placebo arm were 9.8 years (7–13) and 40%. Fisher's exact test was used when the outcome was analysed in relation to categorized serum total IgE and CM protein-specific IgE and in the comparison of the symptoms during the study between the two treatment arms.

The Ethics Committee of Tampere University and University Hospital approved the study protocol. A written consent was obtained from the children and their parents. The study has been registered in ClinicalTrials, Gov Id number NCT01361347.

RESULTS

Sixteen (89%) children in the active treatment group and 8 (80%) in the placebo group successfully completed the OIT protocol. Two children in the active treatment group discontinued the study because of abdominal complaints. In the placebo group, one child discontinued because of lacking motivation and one because of accidental CM-induced anaphylaxis. In addition to CMA, 21 (75%) children had asthma, 26 (93%) allergic rhinitis and 26 (93%) atopic

dermatitis. Other food allergies were present in 26 (93%) children: 20 to egg, 15 to cereal, three to soya and 20 to other foods like fruits, vegetables and spices cross-reacting with seasonal pollens.

Serum total IgE was high, 4790 and 5230 IU/L, in all two children who were not able and in 3/16 who were able to complete the OIT protocol ($p = 0.065$). Likewise, serum CM protein-specific IgE was high, 70 and 313 IU/L, in these two children, and the value was >70 IU/L in only two children who completed the protocol ($p = 0.039$).

The median amount of CM protein tolerated before OIT was 6 mg (range, 0.05–162 mg). After OIT, 14 children tolerated 6400 mg, and other two 960 and 1920 mg (Table S2). All 16 children who completed the protocol in the active treatment group and two-thirds of those 8 in the placebo group suffered, based on the interview of the parents, from possibly study milk-induced symptoms during the double-blind OIT (Table 1). Abdominal and oral symptoms, difficult to confirm objectively, were the most common ones. Wheezing was reported in 5 (19.2%) of cases, but no emergency rooms visits were needed. Anaphylactic reactions were reported in no cases.

All 10 children in the placebo group attended the open-label OIT and tolerated milk 200 mL (6400 mg) daily (Table S3). Like the double-blind OIT, abdominal and oral symptoms were the most common ones (Table 1). In this group, no children had serum total IgE over 4790 IU/L or CM protein-specific IgE over 70 IU/L.

Twelve months after the double-blind OIT, 13 children from the active treatment group and 6 months after the open-label OIT, all 10 children from the original placebo group used daily CM or CM products corresponding 6400 mg of CM protein. Two children in the active treatment group who were not able to complete the protocol followed still strict CM elimination. Three children who

completed the protocol did not use CM or CM products: two of them tolerated 6400 mg CM protein during OIT, and one with very high total IgE and CM-specific IgE tolerated 1920 mg. Instead, the child who tolerated only 960 mg during OIT tolerated now the full amount of 6400 mg. Thus, among all 28 children, 23 (82%) were able to use significant amounts of CM 6–12 months after desensitization.

During the 6–12 months open-label milk consumption, parents reported immediate symptoms probably induced by CM in 16/26 (62%) children. None of the children needed treatment in emergency room or in hospital, and no asthma exacerbation was associated with milk consumption. Oral itching and/or stinging were the most common symptoms. Only one child suffered from frequent symptoms (eczema flares). Thus, CM-induced symptoms were present in 13/23 (57%) of those who continued CM consumption.

In the follow-up 3.0–3.5 years later, only one child more had stopped using CM products because of severe atopic eczema and severe asthma. In addition, an accidental CM-induced anaphylactic reaction took place when milk avoidance was restored. Thus, the combined, long-term success rate of the OIT was 22/28 (78.5%). Only 4/22 (18%) reported CM-induced symptoms during the preceding 12 months.

Before the study, 10/28 (36%) children had suffered from an anaphylactic reaction associated with ingested food; the causative allergen was hen's egg in one case and CM definitely in four cases and probably in five cases. Six children belonged to the active treatment group and four completed the OIT protocol. Four children belonged to the placebo group, and all completed the open-label OIT protocol. Thus, the success rate of OIT was 80% in children with CMA presenting with an anaphylactic reaction.

DISCUSSION

There are three main results in the present study on OIT in schoolchildren with CMA. First, OIT by gradually increasing CM amounts led to a significant desensitization in 89% of the patients, although all children suffered from presumably milk-induced symptoms. Second, the reached desensitization sustained at least 3 years in 79% of the children with CMA, if CM or CM products were daily used. However, CM-induced symptoms were still reported by over half of the children 6–12 months after the daily open-label CM use. Third, preliminary evidence was found that OIT may be unsuccessful, if total and CM protein-specific IgE values are very high. The present OIT study is the second one performed by a randomized, placebo-controlled, double-blind design in children with CMA (13). Children with severe CMA presenting with anaphylaxis were also included, and OIT succeeded in 80% of them.

In the first randomized, placebo-controlled, double-blind OIT study, Skripak et al. (10) allocated 12 schoolchildren with CMA into the active treatment and seven into the placebo arms. Before OIT, the median CM threshold dose was 40 mg of CM protein in both groups. After OIT, the threshold dose was 5140 mg (range, 2540–8240 mg) in the active

Table 1 Parent-reported symptoms during the 24 weeks of OIT in the 24 children who completed the double-blind study protocol and in the 10 children who completed the open-label study protocol

Symptoms	Double-blind OIT		Open-label OIT
	Active group (N = 16) ^A	Placebo group (N = 8) ^B	Placebo group (N = 10) ^C
Intestinal	8 (50.5%)	3 (37.5%)	8 (80%)
Oral	8 (44.4%)	1 (12.5%)	3 (30%)
Nasal	2 (11.1%)	2 (25%)	1 (10%)
Dermal	4 (22.2%)	3 (37.5%)	1 (10%)
Angioedema	2 (12.5%)	0	1 (10%)
No symptoms	0	3 (37.5%)*	2 (20%) [†]

Parents were allowed to report only 1–3 symptoms that they considered most significant.

Among 28 children, 2^A in the active group and 2^B in the placebo group did not complete the protocol; all 10^C in the placebo group completed the open-label protocol

* $p = 0.056$ between the placebo and active treatment groups ($p = 0.013$ when the four interrupted patients are included).

[†]One child reported no symptoms, and one child reported nausea after every milk administration, but completed the protocol.

treatment group, but remained as 40 mg in the placebo group. CM protein-specific IgE did not change significantly in either group, but CM-specific IgG increased in the active treatment group (10). CM protein challenges were repeated in 13 participants 3–17 months after open-label dosing (9), and six tolerated 16,000 mg with no reaction, and seven reacted at 3000–16,000 mg.

In the present study including 28 schoolchildren with CMA, the median baseline CM threshold inducing allergic symptoms was even lower, 6 mg, but after OIT, as many as 72% of the patients tolerated a dose of 6400 mg. During the 6–12 months open-label dosing, this CM protein amount was tolerated by 88% of those who completed the OIT protocol and 3.0–3.5 years later still by 85%. Thus, our results confirm in a larger study including also children with anaphylactic reactions to CM the results of Skripak et al. (10) that OIT is effective in desensitizing children with CMA, and that the threshold dose to induce symptoms may even increase over time, if CM is daily consumed (9).

The efficacy rates have been 35–86% in previous controlled open-label OIT studies in children with CMA (1,4,5). The result of the present double-blind OIT study was even better, 89%, and as good as 82% at 12 months and 79% at 3–3.5 years when the results of the open-label OIT were also included. In an optimal case, OIT may result in tolerance, defined as a permanent loss of reactivity to a previously allergenic substance. The 3.5-year results that over 80% were free of symptoms suggest that some of the patients have reached tolerance. In an Italian study, 70% of the children were able to use milk products 4–5 years after an open-label OIT (8). However, a more realistic goal set is desensitization, defined as the ability to tolerate more allergenic substance after than before treatment, which is likely to be protective against severe reactions by accidental exposures.

Staden et al. (4) compared OIT and dietary elimination in a randomized, controlled, open-label study in 47 children with CMA or hen's egg allergy. They found the same efficacy rates for tolerance in the OIT (36%) and elimination (35%) groups. When tolerance and desensitization were combined, the efficacy rate of OIT was 64%. In the present study, 36% of the children (43% of those who used CM products daily) had no CM-induced symptoms 6–12 months after open-label CM consumption.

Clinical symptoms have been reported in 50–60% of the cases in open-label OIT studies (1,3,4). Skripak et al. (10) also reported frequent symptoms in their placebo-controlled, double-blind study; the figure was 45% in association with milk doses and 11% in association with placebo doses. In the present study, all children in the active treatment group and two-thirds in the placebo group reported harmful symptoms during OIT, but the symptoms were not severe and never led to emergency room visits or hospitalizations. However, the high frequency of symptoms implicates that a successful completion of the protocol requires commitment.

The main strength of the present study was that the trial was randomized, placebo-controlled and double-blind. The researchers, parents and children were all unaware of to which treatment arms the patient belonged. Although the

number of study participants was rather small, the number was larger than in the earlier placebo-controlled, double-blind or open-label studies in children with CMA (4,9,10). Because there were only two treatment failures, no evaluation of the factors associated with successful or unsuccessful outcome was possible. Preliminary evidence was found that those children who could not complete the OIT protocol, or who could not continue milk consumption after OIT, had very high total and CM protein-specific IgE values.

The lack of systematic, prospective symptom monitoring during the double-blind and open-label OIT is a clear shortcoming of the study. However, the symptoms registered by the parents were summarized at the end of the double-blind OIT, which when focusing on significant symptoms, may even give more reliable information. Thus, we know the symptoms per study participants but not per exact study milk doses. The design of the study did not allow the identification of the cases with spontaneous recovery during the follow-up. A widely accepted view has been that milk allergy which continues until school age, only rarely recurs spontaneously (14,15), but in the study of Skripak et al. (12), spontaneous recovery took place until adolescence. The recovery rates were, depending on definitions, 9–12% between 6 and 8 years, and 8–12% between 8 and 10 years, thus being much less than the desensitization rates of 79–88% reached in the present study.

In conclusion, this randomized, placebo-controlled, double-blind study in 28 children aged 6–14 years showed that oral immunotherapy is effective in desensitizing children with milk allergy. When reached, desensitization sustained at least for 3 years.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 The daily cow's milk dosing schedule used in the oral immunotherapy.

Table S2 Basic and outcome data in the 18 children in the treatment group of oral immunotherapy.

Table S3 Basic and outcome data of the 10 children in the placebo group of oral immunotherapy.

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REGULAR ARTICLE

Changes in biomarkers during a six-month oral immunotherapy intervention for cow's milk allergy

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ABSTRACT

Aim: Oral immunotherapy (OIT) is a promising but still experimental method to treat children with cow's milk (CM) allergy (CMA). We evaluated changes in allergic, immunological and inflammatory parameters, which happened during the six-month OIT for CMA.

Methods: We treated 28 school-aged children with CMA using OIT with a double-blind placebo-controlled design. After the controlled study finished, the placebo group was treated with the same but open-label OIT protocol. Sixteen immune variables were tested before and after the six-month OIT.

Results: Before OIT, the median serum CM-specific immunoglobulin (Ig) E was 18.0kIU/L in the intervention group and 9.4kIU/L in the placebo group ($p = 0.46$). At six months, interleukin (IL)-6 and IL-10 were significantly higher in the intervention group. When the changes during the blinded and open OIT were analysed together for both groups, blood eosinophils and serum total IgE decreased and milk-specific IgG and IgG4, serum IL-4 and IL-6, and serum leptin and resistin increased significantly.

Conclusion: Preliminary evidence was found that markers of allergy such as blood eosinophils and serum IgE decreased and milk-specific IgG and IgG4 increased during OIT. Adipokines, leptin and resistin, which functionally are cytokines linked to Th1-type response, increased during OIT.

INTRODUCTION

Cow's milk (CM) allergy (CMA) is the most common childhood food allergy. High serum concentration of CM-specific immunoglobulin (Ig) E, or the presence of IgE to distinct epitopes of CM proteins, predicts the persistence of CMA (1,2). About 75% of children with CMA can tolerate heated forms of CM, and these patients are more likely to become tolerant over time (3). Consuming heated milk, oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) are promising therapeutic approaches. Several studies have confirmed that OIT can also be used for children with severe and persistent food allergies (4–8). Mild and self-limiting adverse reactions are frequent during OIT, but severe and life-threatening reactions may also occur (5,9).

The mechanisms leading to desensitisation or even tolerance to food allergies are mainly unknown, but they may be similar to those seen in standard subcutaneous immunotherapy (SCIT). SCIT is an established treatment for pollen allergies, which releases IL-10 and transforming growth factor (TGF)- β from the T cells. In children with peanut allergy, OIT has been shown to lead to weakened responses in skin prick tests (SPT), less antigen-specific

basophil activation and decreased allergen-specific IgE concentrations in serum, and, correspondingly, to increased allergen-specific IgG1 and IgG4 and higher regulatory T-cell (Treg) counts and higher Treg-associated cytokine levels (10,11). In egg allergy, OIT has been shown to lead to increased egg-specific IgG4 in serum and

Key notes

- Oral immunotherapy (OIT) is an experimental treatment for food allergy. The mechanism of OIT is not known.
- Blood eosinophils and serum IgE decreased and milk-specific IgG and IgG4 increased during OIT. Th2-type IL-4, regulatory IL-10 and pro-inflammatory IL-6 cytokines, and inflammatory adipocytokines leptin and resistin increased.
- We found that OIT induced some beneficial immunological changes. A novel finding was that leptin and resistin, linked to Th1-type response, increased during OIT.

weakened responses to SPTs, but no changes have been observed in egg-specific IgE (12). One study showed that when CMA was treated with a 16-week OIT programme, the SPT responses to CM and serum CM-specific and casein-specific IgE decreased when they were compared to the initial assessments (13). In an 80-week follow-up after milk OIT, another study showed that serum CM-specific IgE decreased significantly, but this was not seen in milk SLIT (14). Meanwhile, another study showed that short-term changes in milk-specific IgE were not seen, but milk-specific IgG1 and IgG4 increased (15).

T-helper (Th) lymphocytes are the major source of cytokines and can be further divided into a number of subtypes, such as Th1 and Th2 (16). IL-12 is considered to be a key player in the development and maintenance of Th1 cells. Th2-type cytokines include IL-4 and IL-5, which are associated with the promotion of IgE-mediated and eosinophilic responses. IL-6 is a multifunctional pro-inflammatory cytokine produced in systemic inflammation. IL-10 seems to be more an anti-inflammatory than a pro-inflammatory cytokine, and it enhances eosinophil cell death. Eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) are secreted by activated eosinophils (17).

Adipokines, such as leptin, adiponectin, adiponectin and resistin, are mediators secreted by adipocytes and macrophages (18). Leptin, adiponectin and resistin have pro-inflammatory properties, whereas adiponectin mainly has anti-inflammatory properties. Adiponectin is a hormone that regulates lipid and glucose metabolism, and leptin plays a key role in the control of Treg proliferation (19).

Recently, we used OIT for 28 cow's milk allergic schoolchildren for six months in a placebo-controlled intervention (6). Sixteen (89%) of the 18 children in the active group completed successfully the protocol. Subsequently, ten children of the placebo group were treated with an identical but open-label protocol, and all of them completed the protocol.

Our hypothesis was that during OIT, atopy and eosinophil activity markers, and the Th2-type cytokines that regulate these markers, would decrease, and the markers of Th1-type immunology and the respective cytokines would increase.

The aim of this study was to evaluate the role of allergy and immunology markers in CMA in schoolchildren and the changes in markers during the six-month prospective, placebo-controlled OIT. The markers we monitored were the following: total IgE, eosinophils, ECP, EDN, CM-specific IgE, milk-specific IgG and IgG4, Th2-type cytokines IL-4 and IL-5, Th1-type cytokine IL-12, regulatory cytokine IL-10, pro-inflammatory cytokine IL-6 and the adipokines such as adiponectin, leptin, resistin and adiponectin.

SUBJECTS AND METHODS

Subjects

As previously described (6), 28 children between six and 14 years of age were recruited to this randomised, double-

blind, placebo-controlled OIT study. All of them had been treated with a complete avoidance diet for CMA from early childhood in the Department of Pediatrics, Tampere University Hospital. There were 18 children in the intervention and 10 in the placebo group. The diagnosis of CMA was based on either a positive challenge test or an anaphylactic reaction after accidental exposure during the preceding six months. In all cases, IgE-mediated mechanism had been confirmed with either positive >3 mm responses in SPTs or with CM-specific IgE >5kU/L in serum (6). Two children in each group failed to complete the six-month study, leaving 16 in the active treatment group and eight in the placebo group.

Laboratory methods

Blood samples were obtained from all patients before the start of the study and at the end of the study. In addition, blood samples were obtained from the original placebo group after they completed the open-label OIT at 12 months, which was performed immediately after the blinded phase of the study. One of the dropouts in the active group did not participate in the six-month study visit, and occasional blood samples were not analysed for technical reasons (i.e. not enough serum). Total eosinophils were counted in the blood samples by an automated cell counter and expressed as cells $\times 10^9/L$. Serum total IgE was determined nephelometrically using a Siemens N Latex IgE mono assay (Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany). Serum CM-specific IgE was measured using the CAP-FEIA technique (Phadia, Uppsala, Sweden) and expressed as milligrams of allergen per litre (mgA/L). The rest of the samples were stored at -70°C until they were analysed. Serum concentrations of IL-4, IL-5, IL-6, IL-10, IL-12p70, EDN, adiponectin, adiponectin, leptin and resistin were determined by enzyme immunoassay (EIA) using commercial reagents. IL-4, IL-5, IL-12p70, adiponectin, adiponectin, leptin and resistin were measured by the DuoSet ELISA (R&D Systems Europe Ltd, Abingdon, Oxfordshire, UK), IL-6 and IL-10 (PeliPair ELISA, Sanquin, Amsterdam, the Netherlands), EDN with the EDN ELISA kit (MBL International, Woburn, MA, USA) and ECP with the ECP ELISA kit (MBL International, Woburn, MA, USA). Serum allergen-specific IgG and IgG4 levels (Phadia AB, Uppsala, Sweden) were measured by the Immuno CAP 100 assay (Phadia AB, Uppsala, Sweden).

Statistics

Statistical analyses were performed with spss statistical software for Windows version 20.0 (SPSS Inc, Chicago, IL, USA). The Mann-Whitney *U*-test was used to compare continuous variables between the two groups, and the Wilcoxon test was used to compare continuous variables within the groups. The results for the two groups are given both separately and combined. The baseline was the same for both groups, but the outcome for the original placebo group was at 12 months, rather than six, when their phrase of the open OIT ended. The protocol was the same in the

double-blinded and open OITs. We considered a two-tailed *p* value of <0.05 as statistically significant.

Ethics

Ethical approval for the study was obtained from the Ethics Committee of Tampere University and University Hospital. The children and their parents provided written informed consent. The study was registered at clinicaltrials.gov NCT01361347.

RESULTS

At the baseline, there were no significant differences between the active OIT group and the placebo control group in the immunity and inflammatory markers (Tables 1 and 2). The only exception was the average milk-specific IgG4 level, which was higher in the intervention group. This may have been due to by chance because of large variation in that variable (Table 1).

At six months, serum IL-6 and IL-10 levels were significantly higher in the group that had been treated with OIT than in the control group (Table 2). There were no other significant differences in the immunity and inflammatory markers between the two groups (Tables 1 and 2).

When the markers in the active group were compared at baseline and at six months after the OIT intervention, there were no significant differences in any of the markers (Tables 1 and 2).

The control group was placed on the open-label OIT intervention after six months. This started immediately after the double-blind OIT study and used exactly the same

protocol. Serum resistin was significantly higher at 12 months in this group than at the start of the six-month intervention (Table 3).

Finally, we combined the active and control groups and measured the values before either group took part in the OIT intervention, together with the six-month values for the first intervention group and 12-month values for the second intervention group, who acted as the control placebo group for the first six months (Table 4). This showed that blood eosinophils and serum total IgE had decreased significantly after the intervention and allergen-specific IgG and IgG4, serum IL-4 and IL-6, and serum leptin and resistin had increased significantly.

DISCUSSION

We were able to confirm that serum total IgE, and blood eosinophils decreased significantly during OIT, when the data from the double-blind and open-label OITs were combined. Milk-specific IgG and IgG4 increased in line with this. Also in other studies, OIT has been associated with a rise in allergen-specific IgG4 (8,10,12). One indicator for successful milk OIT seems to be an increase in serum IgG4 to milk components such as α -lactalbumin, β -lactoglobulin, casein and lactoferrin (20).

When immunotherapy with an allergen is effective, it changes the patient's allergen-specific response from an allergic (Th2) to a nonallergic (Th1) profile. This is promoted by Treg cells that release IL-10, which further induce IgG4, TGF- β and IgA production (16). As previously reported, the OIT intervention provided during the present

Table 1 Immunity markers: comparisons between the intervention and placebo groups at the start of the study and at six months after the double-blind milk oral immunotherapy study ended

	Placebo group		Intervention group		<i>p</i> *	<i>p</i> **	<i>p</i> ***
	Median (range)	<i>n</i>	Median (range)	<i>n</i>			
Baseline (0 months)							
Eos 10E9/L	0.86 (0.28–1.09)	9	0.58 (0.20–1.25)	17	0.287	0.031	0.302
IgE kU/L	731.00 (354.00–5040.00)	9	822.00 (228.00–10100.00)	18	1.000	0.078	0.804
CM IgE kU/L	9.40 (2.80–27.00)	8	18.00 (0.66–400.00)	18	0.461	0.094	0.793
ECP μ g/L	30.10 (7.90–76.60)	9	19.50 (7.10–143.00)	17	0.560	0.109	0.229
EDN pg/mL	60.12 (38.41–186.00)	10	58.57 (21.83–272.20)	18	0.796	0.214	0.408
IgG mgA/L	75.70 (57.20–90.50)	10	75.05 (61.3–111.0)	16	0.816	0.086	0.638
IgG4 mgA/L	19.85 (16.10–24.00)	10	21.80 (13.70–112.40)	16	0.027	0.075	0.754
At 6 months							
Eos 10E9/L	0.43 (0.14–1.75)	8	0.43 (0.14–0.79)	16	0.742		
IgE kU/L	853.00 (308.00–3850.00)	8	831.50 (119.00–7280.00)	16	0.928		
CM IgE kU/L	6.65 (2.50–73.00)	8	11.00 (0.41–400.00)	16	0.528		
ECP μ g/L	15.45 (6.90–50.40)	8	11.75 (6.40–31.90)	16	0.291		
EDN pg/mL	45.79 (25.49–115.36)	9	62.05 (24.20–120.02)	16	0.187		
IgG mgA/L	79.10 (62.50–141.00)	9	78.25 (65.50–123.0)	14	0.643		
IgG4 mgA/L	23.40 (18.50–216.00)	9	21.20 (15.00–238.00)	14	0.688		

**p* value for intervention group vs. placebo group 0 and 6 months.

***p* value for placebo group 0 vs. 6 months.

****p* value for intervention group 0 vs. 6 months.

Table 2 Inflammatory markers: comparisons between the intervention and placebo groups at the start of the study and at six months after the double-blind milk oral immunotherapy had ended

	Placebo group		Intervention group		p*	p**	p***
	Median (range)	n	Median (range)	n			
Baseline (0 months)							
IL-4 pg/mL	1.95 (1.95–1.95)	10	1.95 (1.95–7.30)	18	0.160	0.068	0.553
IL-5 pg/mL	2.05 (1.03–15.90)	10	1.90 (1.03–52.40)	18	0.555	0.889	0.955
IL-6 pg/mL	0.50 (0.30–3.40)	10	0.60 (0.30–9.20)	18	0.245	0.214	0.729
IL-10 pg/mL	1.85 (1.20–7.30)	10	2.65 (1.00–6.20)	18	0.191	0.401	0.134
IL-12p70 pg/mL	3.90 (3.90–4.80)	10	3.90 (3.90–3.90)	18	0.689	0.317	0.317
Adiponectin µg/mL	3.69 (1.76–7.57)	10	3.93 (2.09–6.84)	18	0.759	0.374	0.569
Adipsin ng/mL	833.56 (619.36–1109.94)	10	829.08 (597.35–1200.25)	18	0.869	0.859	0.877
Leptin ng/mL	3.64 (0.65–20.11)	10	3.83 (0.32–22.19)	18	1.000	0.859	0.215
Resistin ng/mL	3.94 (1.51–5.90)	10	2.98 (1.73–6.01)	18	0.382	0.260	0.501
At 6 months							
IL-4 pg/mL	1.95 (1.95–4.00)	9	1.95 (1.95–23.30)	16	0.329		
IL-5 pg/mL	1.30 (1.03–9.40)	9	2.00 (1.03–35.40)	16	0.251		
IL-6 pg/mL	0.50 (0.30–3.70)	9	0.95 (0.30–7.70)	16	0.032		
IL-10 pg/mL	1.50 (0.40–4.70)	9	2.25 (1.20–10.00)	16	0.023		
IL-12p70 pg/mL	3.90 (3.90–3.90)	9	3.90 (3.90–139.70)	16	0.637		
Adiponectin µg/mL	3.43 (1.34–5.31)	9	4.22 (2.05–7.43)	16	0.095		
Adipsin ng/mL	878.57 (703.31–1140.27)	9	879.21 (510.55–1139.32)	16	1.000		
Leptin ng/mL	5.14 (0.70–28.04)	9	4.32 (1.30–23.45)	16	0.846		
Resistin ng/mL	4.33 (1.30–7.56)	9	3.17 (1.61–5.70)	16	0.207		

*p value for intervention group vs. placebo group 0 vs. 6 months.

**p value for placebo group 0 vs. 6 months.

***p value for intervention group 0 vs. 6 months.

Table 3 Immunity and inflammatory markers in the initial control group: comparison of the results at the start of the study and after their subsequent open-label milk oral immunotherapy at 12 months

	Median (range)	n	Median (range)	n	p
Eos 10E9/L	0.86 (0.28–1.09)	9	0.47 (0.24–1.20)	10	0.044
IgE kU/L	731.00 (354.00–5040.00)	9	657.50 (340.00–3960.00)	10	0.173
CM IgE kU/L	9.4 (2.80–27.00)	8	9.55 (2.50–57.00)	10	0.176
ECP µg/L	30.10 (7.90–76.60)	9	36.85 (4.60–139.00)	10	0.214
EDN pg/mL	60.12 (38.41–186.00)	10	67.40 (37.00–159.70)	10	0.721
IgG mgA/L	75.70 (57.20–90.50)	10	96.25 (71.60–124.00)	8	0.012
IgG4 mgA/L	19.85 (16.10–24.00)	10	89.40 (19.00–114.40)	8	0.017
IL-4 pg/mL	1.95 (1.95–1.95)	10	1.95 (1.95–25.40)	10	0.068
IL-5 pg/mL	2.05 (1.03–15.90)	10	1.35 (1.03–6.80)	10	0.959
IL-6 pg/mL	0.50 (0.30–3.40)	10	1.45 (0.3–8.3)	10	0.014
IL-10 pg/mL	1.85 (1.20–7.30)	10	1.65 (0.7–5.8)	10	0.240
IL-12p70 pg/mL	3.90 (3.90–4.80)	10	3.9 (3.9–85.3)	10	0.317
Adiponectin µg/mL	3.69 (1.76–7.57)	10	2.75 (1.2–5.3)	10	0.059
Adipsin ng/mL	833.56 (619.36–1109.94)	10	898.80 (560.0–1290.60)	10	0.241
Leptin ng/mL	3.64 (0.65–20.11)	10	5.15 (1.00–19.70)	10	0.139
Resistin ng/mL	3.94 (1.51–5.90)	10	4.80 (2.10–8.60)	10	0.007

p value for placebo group 0 vs. 12 months.

study was clinically effective (6). OIT led to a desensitisation, defined as the ability to tolerate more milk or milk products after than before treatment, in 89% of patients in the active group. In line with this, serum IL-10 was higher in the active group than in the control group at the end of the OIT study. However, the rise of IL-10 between the start

and the end of OIT was not significant in either of the two groups or when the results for both groups were combined.

Infants with food allergies have been reported to present with lower plasma IL-10 and IL-6 than sensitised but tolerant nor nonsensitised infants (21). Defects in Treg cell activity, due to suppressed immune responses by

Table 4 Immunity and inflammatory markers in the combined intervention and control groups

	Median (range)	n	Median (range)	n	p
Eos 10E9/L	0.60 (0.20–1.25)	26	0.41 (0.14–1.20)	26	0.003
IgE kU/L	778.00 (228.00–10100.00)	27	732.50 (119.00–7280.00)	26	0.048
CM IgE kU/L	12.00 (0.66–400.00)	26	10.40 (0.41–400.00)	26	0.088
ECP µg/L	20.55 (7.10–143.00)	26	16.30 (4.60–139.00)	26	0.853
EDN pg/mL	58.57 (21.83–272.20)	28	62.83 (24.20–159.70)	26	0.849
IgG mgA/L	75.05 (57.20–111.00)	26	83.15 (65.50–124.00)	22	0.039
IgG4 mgA/L	21.00 (13.70–112.40)	26	25.35 (15.00–238.00)	22	0.039
IL-4 pg/mL	1.95 (1.95–7.30)	28	1.95 (1.95–25.40)	26	0.011
IL-5 pg/mL	1.90 (1.03–52.40)	28	1.85 (1.03–35.40)	26	0.780
IL-6 pg/mL	0.60 (0.30–9.20)	28	1.00 (0.30–8.30)	26	0.008
IL-10 pg/mL	2.35 (1.00–7.30)	28	2.20 (0.70–10.00)	26	0.459
IL-12p70 pg/mL	3.90 (3.90–4.80)	28	3.90 (3.90–139.70)	26	0.109
Adiponectin µg/mL	3.87 (1.76–7.57)	28	4.05 (1.20–7.43)	26	0.970
Adipsin ng/mL	833.56 (597.35–1200.25)	28	879.21 (510.55–1290.60)	26	0.304
Leptin ng/mL	3.83 (0.32–22.19)	28	5.02 (1.00–23.45)	26	0.022
Resistin ng/mL	3.45 (1.51–6.01)	28	3.66 (1.61–8.60)	26	0.008

Comparison between pre-OIT values (at the start of the study) and post-OIT values (at six months in the intervention group and at 12 months in the former control group).

p value for placebo and intervention group combined 0 vs. 6 or 12 months.

regulatory cytokines such as IL-10 and TGF- β , may contribute to the development of food allergy (22). However, our patients were school-aged children, even though they had suffered from milk allergy since infancy. In a previous OIT study for peanut allergy, an upregulation of IL-10 was not seen, even though the children ranged from three to 14 years of age. This suggests that cytokine production may have been measured too late (8). In another peanut OIT study, IL-10 was measured immediately after OIT, like in our present study, and the increase in IL-10 was clearly seen (10). Treg cells secreting IL-10 and IL-6 are involved in the induction of desensitisation and tolerance in CMA (23), but we were only able to demonstrate a significant rise in IL-6.

To our knowledge, this is the first study that has documented that OIT affects serum adipokines. It has been reported that preseasonal SLIT courses significantly increased serum leptin in allergic rhinitis, but only in males (24). Our finding was that OIT resulted in significant increases in serum leptin and resistin levels and one explanation could be that leptin and resistin, as well as many cytokines, possess anti-allergic activity (18).

Leptin, at least, is known to influence cytokine production by switching the phenotype towards a Th1 response. Resistin has also been reported to act as an endogenous ligand of toll-like receptor 4 (TLR4) that enhances classical macrophage activation and Th1-type response (25). Hence, increased resistin and leptin levels in our OIT intervention can be regarded as an effector and also a biomarker for the shift of Th2-type immune responses towards Th1-type dominance. Future studies are warranted to confirm the role of leptin and resistin in food allergy and in monitoring the effect of OIT.

The results of this study should be interpreted with caution. The study population was small, which means that the study did not have enough power to find all the significant associations. The short follow-up period is another limitation, and blood samples were only obtained at the start and end of OIT, not later as in many other OIT studies (5,8,10,12–14), where markers were monitored for one to 86 weeks after the active OIT.

In one egg OIT study, children were assessed 4–6 weeks after they finished immunotherapy (12), and in a milk OIT versus milk SLIT study, patients were tested for sustained unresponsiveness at one and six weeks after therapy (14). Peanut OIT was also discontinued for two weeks before the final double-blind placebo-controlled food challenge to assess long-term tolerance (8). Monitoring hen's egg and cow's milk-specific IgE has been particularly useful for predicting prognoses after OIT (26). The rise especially in allergen-specific IgG1 correlated with good response in a previous Japanese egg OIT study (27).

CONCLUSION

This study provides preliminary evidence that markers of allergic inflammation, such as blood eosinophils and serum IgE decreased during milk OIT, while allergen-specific IgG and IgG4 and the pro-inflammatory cytokine IL-6 increased. Notable and novel findings were that leptin and resistin, which are two adipocytokines linked to Th1-type response, increased during OIT.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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REGULAR ARTICLE

Children who were treated with oral immunotherapy for cows' milk allergy showed long-term desensitisation seven years later

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ABSTRACT

Aim: This was a follow-up of 28 schoolchildren with cows' milk allergy (CMA) who attended a randomised double-blind placebo-controlled oral immunotherapy (OIT) study. In the original study, 26 (92.9%) completed the six-month escalation phase, and 25 (89.3%) used milk daily at 12 months and 24 (85.7%) at 36 months. This study evaluated the outcome seven years later, with special attention paid to milk consumption and symptoms.

Methods: Outcome data were collected through a postal questionnaire completed three, four and five years after enrolment and by a phone questionnaire after seven years. We asked about the daily dose of milk products, any adverse reactions, any medication needed and possible discontinuation of daily milk consumption.

Results: Data were available at the seven-year point for 24 children and 14 (58.3%) of these continued to use milk (≥ 200 mL) or milk products (protein ≥ 6400 mg) daily for seven years. However, three (21.4%) of these still reported symptoms associated with milk consumption. Of the 10 remaining children, two children used milk products daily but consumed less due to symptoms and eight (33.3%) had discontinued milk consumption.

Conclusion: Oral immunotherapy was an effective and safe way of desensitising schoolchildren with persistent CMA.

INTRODUCTION

Cows' milk allergy (CMA) and hens' egg allergy are the most common food allergies in children, affecting 2–3% of the child population (1). Many food allergies improve before school age but, in some cases, CMA persists even into adulthood (1,2).

The current standard treatment for food allergies is limited to strict nutritional counselling, dietary avoidance and emergency treatment of severe adverse reactions (3). However, many studies have reported achieving encouraging results using oral immunotherapy (OIT) to treat CMA or hens' egg allergies (4–22). Meta-analyses have shown that studies to date have included small numbers of patients (13–15) and some of them have also included toddlers, which is a stage when milk allergies often resolve themselves naturally (6,11). Dosing protocols have varied from small allergen amounts that aim to protect against accidental exposures to large allergen amounts that aim to induce tolerance (14–16). There are a few published

studies, which had a limited number of subjects, on the long-term outcomes of milk OIT. These ranged from follow-up at three to 12 months of age (17,18) to four to five years (19–21). However, longer follow-ups are not available.

Daily cows' milk consumption is considered necessary to maintain desensitisation (6,22). Around 10–20% of patients are unable to tolerate the initial escalation period of OIT and discontinue the protocol due to adverse effects. An additional 10–20% fail to achieve the full planned maintenance dose, which means they have not achieved complete

Abbreviations

CMA, Cows' milk allergy; IgE, Immunoglobulin E; IU/L, International units per litre; kU/L, Kilounits per litre; mg, Milligram; mL, Millilitre; OIT, Oral immunotherapy; SPT, Skin prick test.

Key notes

- This was a seven-year follow-up of 28 schoolchildren with cows' milk allergy (CMA) who received oral immunotherapy, with outcome data collected through postal and phone questionnaires.
- Data were available for 24 children seven years after they received oral immunotherapy and 14 (58.3%) of these continued to use milk or milk products daily.
- Oral immunotherapy was an effective and safe way of desensitising schoolchildren with persistent cows' milk allergy.

desensitisation (23). This means that about 75% of children with CMA who have received OIT have tolerated the maintenance dose (22,25), but at least 20% have reported temporarily limiting milk consumption because of symptoms, for example, during exercise or illness (17).

The original study that this research was based on comprised 28 schoolchildren with CMA who attended a randomised double-blind placebo-controlled OIT study. Of these, 26 (92.9%) completed the six-month escalation phase of the milk OIT protocol, during either the double-blind phase or the subsequent open-label phase of the study, which included treating the double-blind phase controls, and 25 (89.3%) used milk daily at 12 months and 24 (85.7%) at 36 months after the start of the study (10). The aim of this seven-year follow-up study was to evaluate the long-term efficacy and safety of OIT in schoolchildren with persistent CMA. Special attention was paid to milk consumption and symptoms.

PATIENTS AND METHODS

Details of the study involving the 28 schoolchildren, aged six to 15 years old, who were previously treated for CMA in the Department of Pediatrics, Tampere University Hospital, Finland, and participated in the double-blind placebo-controlled OIT study have already been published (10). The criterion for participation was the presence of persistent immunoglobulin E (IgE)-mediated CMA. In 21 cases, an immediate reaction was documented in an open cows' milk challenge test in the six-month period before the start of OIT (10). In seven cases, the parents reported an immediate reaction associated with an accidental cows' milk exposure within six months and the challenge test was not repeated (10). A positive ≥ 3 mm skin prick test (SPT) response to cows' milk extract, or the cows' milk-specific IgE concentration of >3.5 kU/L, was necessary to confirm the IgE-mediated mechanism. Data on asthma and other allergies were collected at the start of the study.

At enrolment, the median age of the 28 children was 10.3 years, with a range of six to 15 years, and 57% were female. We randomised 18 of these children to the active treatment arm of the six-month, double-blind escalation period of the OIT study and 10 to the placebo control arm (10). After that, the 10 controls received the six-month open-label OIT using an identical protocol. In the first three years of the study, four children stopped consuming cows' milk. Thus, 24 children took part in this study, which focused on their milk consumption and symptoms three to seven years after the start of the milk OIT study.

Questionnaire data

Outcome data were collected through questionnaires sent to the study subjects three, four and five years after enrolment. The questions concerned the use of milk products during the previous 12 months, daily doses, any adverse reactions, any medication that was needed and possible discontinuation of daily milk consumption. The reactions were classified as separately recorded dermal

symptoms, such as itching skin and, or, flaring eczema or urticaria, nasal symptoms such as a runny or stuffy nose, intestinal symptoms including vomiting, diarrhoea or stomach cramps and respiratory symptoms such as a cough, stridor or wheezing. In addition, any episodes of mouth itching and, or, stinging and angioedema, defined as facial or lip swelling, were recorded. An anaphylactic reaction was recorded if there were symptoms from two or more organ systems treated by intramuscular adrenalin (24).

In June 2015, seven years after enrolment, one of the authors (MP) called all of the 20 subjects who had not discontinued milk OIT and completed a phone questionnaire for each subject. They were asked questions about milk consumption during the preceding 14 days, including the dose per day, adverse reactions, classified as any dermal, nasal, intestinal or respiratory symptoms, and any medication that they required.

Ethics

The Ethics Committee of Tampere University and the University Hospital approved the study protocol. Written consent was obtained from the children and their parents. The original OIT study was registered at ClinicalTrials.gov (number NCT01361347).

Statistics

Multivariable Cox regression was used to find possible risk factors for OIT failure. Either the discontinuation of OIT or not using the full daily milk (>200 mL) or milk products (protein >6400 mg) at the latest follow-up date were considered as failures. Kappa statistics was used to assess the correlation between wheat and egg allergies. These variables were combined in further analyses, indicating one or both of these allergies. The independent variables we used were age at enrolment, gender, baseline serum milk-specific IgE, combined wheat and, or, egg allergies and the presence of asthma. SPSS Statistics version 19.0 (IBM Corporation, Armonk, NY, USA) was used for the analyses, and *p* values less than 0.05 were considered as statistically significant.

RESULTS

During the six-month escalation period of OIT, two children stopped participating in the study, and one more child discontinued cows' milk consumption by the end of the first year and one more child discontinued cows' milk consumption by the end of the third year of the OIT maintenance period. This meant that 24 children participated in this study that covered the follow-up period from three to seven years.

In this study, two more children discontinued cows' milk consumption due to milk-related symptoms. One child, who used 100–200 mL of milk or milk products per day on an irregular basis, was advised to discontinue at 4.5 years during a scheduled follow-up visit due to significant symptoms of urticaria and feeling that their throat was swollen after exercise. Another child, who used of less than

50 mL of milk or milk products per day on an irregular basis, discontinued at 5.5 years due to mild itching in the mouth and delayed worsening of atopic eczema. Two children used milk or milk products regularly but, due to their symptoms, they consumed less than the planned 200 mL per day. There were four subjects, and their parents, who could not be reached by telephone.

Seven years after recruitment, 14 (58.3%) of the 24 children reported daily use of milk (200 mL) or milk products (6400 mg). As seen in Table 1, milk-related symptoms reduced over time and were reported by 50% of subjects after three years and by only 19% after seven years. The symptoms were mainly mild to moderate in severity and were mostly resolved with antihistamines (Table 1). One child with birch allergy required intramuscular adrenalin for severe symptoms, including asthma attacks and oral angioedema induced by an accidentally large dose of milk consumed during the birch pollen season. The patient and the family were very highly motivated to continue, and the patient had no previous milk-related concerns. Therefore, after we instigated a significant dose reduction, carried out an asthma check-up and controlled the other atopic diseases, the patient continued milk OIT and finally reached the daily dose of 200 mL, under careful monitoring and without any symptoms.

During the seven-year follow-up period, the high baseline serum milk-specific IgE predicted the nonuse of milk products in Cox regression analysis ($p = 0.021$) (Table 2). As allergy to either wheat or hens' eggs were moderately correlated ($Kappa = 0.429$, $p = 0.012$), the combined variable was used in the regression model along with other independent variables. The figures were similar in the successful (71.4%) and unsuccessful (70.0%) groups.

The study also included seven children with severe CMA, who presented with anaphylaxis prior to the milk OIT and two of them consumed 3–5 dL milk per day without any symptoms seven years later. We were unable to contact two

subjects at seven years, but they had used 2 dL milk per day at five years with mild or no symptoms. The remaining three subjects had discontinued the OIT.

DISCUSSION

There were four main results in the seven-year follow-up study on daily milk use and milk-related symptoms during free milk consumption, following desensitisation by OIT. Firstly, the desensitisation when achieved sustained for at least seven years in 58% of the children with CMA. Secondly, around 20% of those who continued milk consumption still suffered from mild milk-related symptoms. Thirdly, high cows' milk-specific IgE values were related to the long-term risk of discontinuing milk consumption. However, seven children with severe CMA presenting with prior milk anaphylaxis were included in this study and two of them consumed 3–5 dL milk per day

Table 2 Clinical data in relation to full tolerance (successful) and partial or failed tolerance (unsuccessful) achieved with milk oral immunotherapy, based on the seven-year phone interview

Clinical data	Successful [†] (n = 14)	Unsuccessful [‡] (n = 10)
Prior milk anaphylaxis, n = 7 (%)	2 (14.3)	3 (30.0)
Pre-OIT milk IgE Median (range) IU/L	9.4 (0.7–133.0)	24.5 (2.6–700.0)*
Wheat or egg allergy (%)	10 (71.4)	7 (80.0)
Asthma (%)	12 (85.7)	7 (70.0)

*Cox regression hazard ratio estimate, $p = 0.021$; no other significant differences between the groups.

[†]Successful = 200 mL milk or 6400 mg of milk protein daily.

[‡]Unsuccessful = 50–100 mL/day (n = 2) or discontinuation (n = 8). We were unable to reach three subjects over 18 years of age and the parents of one subject under 18.

Table 1 Type and prevalence of symptoms reported in questionnaires at three to seven years after initiation of milk OIT

Symptoms and rescue treatments	Questionnaire at three years (n = 20)	Questionnaire at four years (n = 17)	Questionnaire at five years (n = 13)	Questionnaire at seven years (n = 16)
Symptoms				
Intestinal	4	3	1	2
Oral	4	4	4	0
Dermal	8	5	3	1
Angioedema	1	0	2	0
Asthma	3	0	2	0
Conjunctival	1	0	0	0
Nasal	0	0	1	0
No symptoms	10 (50.0%)	10 (58.8%)	8 (61.5%)	13 (81.3%)
Treatment for symptoms				
Oral antihistamine	5	5	5	3
Oral corticosteroid	2	1	2	1
Adrenalin injection	1	0	0	0
No treatment for symptoms	15 (75.0%)	12 (70.6%)	8 (61.5%)	13 (81.3%)

without any symptoms seven years later. Fourthly, discontinuation of milk use mainly occurred during the first year of the OIT and rarely occurred three to seven years later during the maintenance period.

The success rate for desensitisation to milk has ranged from 25% to 70% in previous studies (17,22,25). Just three studies have been published so far with a follow-up period of more than three years. The first two studies reported a full tolerance of 250 mL/day in 46% (19) and 65% (21) of cases, respectively. In the third long-term follow-up study, 31% of the 16 subjects tolerated full servings of cows' milk with minimal or no symptoms (20) after 4.5 years of milk OIT. The figures achieved in the present study were 50.0% of all the children who enrolled in the study ($n = 28$) and 58.3% of those who completed the first three years of the study ($n = 24$). These figures were higher than those in the two long-term studies published to date (19,20).

In the Cochrane review that comprised 106 subjects on milk OIT, adverse reactions were seen in 92% of subjects, although most were local and mild (13). The vast majority (82%) of the adverse reactions took place during the escalation period and 40% took place during the long-term follow-up period (19). We recorded symptoms less frequently in 50% of the patients at three years, in 31.5% at five years and in 21.5% at seven years. In line with a previous study (18), the overall rate of symptoms decreased over time.

In our study, a high pre-OIT cows' milk-specific IgE was a risk factor for discontinuing OIT. In other studies, cows' milk-specific IgE >50 kU/L, a wheal of larger than 9 mm in the skin prick test (22) and symptom severity at the baseline milk challenge have been associated with OIT failure (7,13,18–22). Interestingly, a long-term British study (19) showed that subjects with asthma or other allergies were more likely to achieve full tolerance. Neither of the two children who discontinued in our study at three to seven years after enrolment were asthmatic, had milk-specific IgE >20 kU/L, had reacted to 2 mL of milk in the milk challenge test prior to OIT or had egg and nut allergies. In line with other studies (13,19–21), most adverse reactions affected the skin and gastrointestinal tract.

It is important to note that the systemic reactions that required adrenalin administration in previous studies occurred during home administration as well as during the escalation period (13,19–21). In the Cochrane review on milk OIT, for every 11 patients who received milk OIT, one required intramuscular adrenalin (13). In a study of 15 severely milk allergic children with a median baseline milk-specific IgE of 29.9 kU/L, adrenalin was required in four (27%) subjects (18). In our study, one patient (4%) reported adrenalin administration during the follow-up period from three to seven years at home and had received emergency medical assistance on one occasion, but did not discontinue OIT.

Whether milk OIT will promote full tolerance, defined as the adaptive immune system not responding to antigens, or merely desensitisation, which is reduced immunological reactivity, remains to be determined. The number of fully tolerant patients in milk OIT studies has varied from 25% to

50% (13,18–20,26,27). In this study, more than 80% of the 16 children with complete seven-year data available reported no symptoms at that time. Whether this represents sustained unresponsiveness, that is tolerance induced by milk OIT, or only transient desensitisation remains unknown.

The main strengths of the present study were the long seven-year follow-up period, the systematic collection of data on milk consumption and milk-related symptoms and the good subject retention rate. Only children who had persistent CMA at more than six years of age were included. As a result, our patients were beyond the age at which natural tolerance was most likely to develop and this meant that they had a confirmed persistent milk allergy. As children with anaphylactic reactions were included, the study covered the whole spectrum of severe persistent CMA. The main shortcoming of the study was the small number of patients. For example, this did not allow us to use adjusted analyses and evaluate all the factors that were possibly related to the treatment failures. In addition, the data were only collected through postal questionnaires, supplemented with a telephone interview at seven years, so both the use of milk or milk products and the presence of symptoms were self-reported. Another shortcoming was that re-challenge tests or re-evaluations of SPT or milk-specific IgE status were not conducted at the end of the follow-up period of seven years.

CONCLUSION

In conclusion, the long-term success rate of milk OIT was 50% of all of the 28 children who enrolled in the study and 58% of those 24 who completed the first three years of this study. When desensitisation was reached it was sustained for at least seven years in nearly all cases. The rate of symptoms decreased over time, and at seven years, about 20% of the subjects reported milk-related symptoms. Large prospective studies with long follow-up periods are necessary to evaluate patient selection to OIT, the outcomes of different groups and the safety of different OIT protocols. So far, OIT has proved itself to be a promising therapy, but it is far from being adopted into routine clinical practice.

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REGULAR ARTICLE

Elevated serum adipsin may predict unsuccessful treatment for cows' milk allergy but other biomarkers do not

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INTRODUCTION

Oral immunotherapy (OIT) has been used as immunomodulatory treatment for children with cows' milk allergy for over a decade (1–7). Currently, OIT can be considered an effective treatment for immunoglobulin E (IgE) mediated food allergy, but it is associated with a risk of local and systemic adverse events (8). The short-term success rate of OIT for cows' milk allergy has been good, at around 60%–100% (2,3), but less is known about the long-term success rates. The 2017 position paper on biomarkers in allergen immunotherapy for allergic asthma and rhinoconjunctivitis, published by the European Academy of Allergy and Clinical Immunology, recommended using allergen-specific IgG4 as a biomarker for compliance (9). High milk-specific IgE has been shown to predict a risk of failure for OIT interventions (10,11), but the usefulness of other pre-OIT markers is poorly known. In particular, the predictors of the long-term outcome after OIT are not known. Better knowledge would help to select the patients who would benefit most from this OIT protocol, which is laborious to carry out, means that

ABSTRACT

Aim: This study evaluated whether 15 allergy, immunology or inflammatory markers predicted the long-term use of cows' milk or milk products seven years after the start of oral immunotherapy (OIT) for cows' milk allergy in children.

Methods: The following laboratory parameters were measured before the OIT at Tampere University Hospital, Finland, and after the six-month escalation phase: serum total immunoglobulin (Ig) E, milk-specific IgG and IgG4, eosinophil cationic protein, eosinophil-derived neurotoxin, interleukins 4, 5, 6, 10 and 12p70 and serum adipokines adiponectin, adipsin, leptin and resistin. Follow-up data from a seven-year phone questionnaire in 2015 were available for 24 children: 14 successful and 10 unsuccessful milk users.

Results: There were no significant differences in any of the 15 markers measured at the start of the study between the subjects who later formed the successful and unsuccessful groups. At the end of the six-month escalation phase of OIT, serum adipsin was higher in the group who were unsuccessful milk users at the seven-year follow-up study.

Conclusion: None of the 15 allergy, immunology or inflammatory markers were useful in predicting the outcome of OIT. Preliminary evidence was found that high serum adipsin after the six-month escalation phase of OIT might predict unsuccessful outcome.

patients need to tolerate daily symptoms for months and requires lifelong commitment.

We treated 28 school-aged children with cows' milk allergy using OIT with a double-blind placebo-controlled design, and the immediate success rate was 89% (12). Allergy markers, such as blood eosinophils and serum milk-specific immunoglobulin (Ig) E, decreased and milk-specific IgG and IgG4 and adipokines such as leptin and resistin increased during the escalation phase of OIT (13). Seven years after the start of the OIT treatment, we had data on 24

Key notes

- This study evaluated how 15 allergy, immunology or inflammatory markers predicted the long-term use of cows' milk or milk products after oral immunotherapy (OIT).
- We measured the markers before and after the escalation phase of OIT and evaluated the outcome using a questionnaire seven years later in 14 successful and 10 unsuccessful subjects.
- None of the 15 allergy, immunology or inflammatory markers proved useful in predicting the outcome of OIT.

Abbreviations

Ig, Immunoglobulin; IL, Interleukin; OIT, Oral immunotherapy.

children and this showed that 14 (58%) were still able to consume daily milk or milk products and 10 were not. The baseline serum milk-specific IgE level before starting OIT was higher in those individuals who had discontinued using milk or milk products during the seven-year follow-up period (11). We had already published the short-term outcomes, based on the baseline and six-month data of the blood eosinophils, serum total IgE, milk-specific IgG and IgG4, eosinophil cationic protein, eosinophil-derived neurotoxin, interleukins (IL) 4, 5, 6, 10 and IL-12p70 and the serum adipokines adiponectin, adipisin, leptin and resistin (13).

The aim of this study was to determine the value of these 15 allergy, immunology or inflammatory parameters, measured before OIT and at the end of the six-month escalation phase, in predicting the long-term use of cows' milk or milk products seven years after the start of OIT for cows' milk allergy in children.

MATERIALS AND METHODS

Design

As published previously (12), we treated 28 school-aged children with a cows' milk allergy using OIT with a double-blind placebo-controlled design. After the controlled escalation phase of the study, the placebo group was treated with the same protocol, but on an open-label basis (11). The combined immediate success rate in the two groups was 92.9%.

We carried out telephone interviews with 24 of the original 28 children in June 2015, seven years after the start of OIT (11) and they formed the subjects of this study. The mean age of the 24 children was 17.0 years and the range was 13.1–22.0 years, with 10 males and 14 females. All 24 had previously taken part in the questionnaire studies at three, four and five years after OIT. The 14 (58.3%) children who still continued to use 200 mL of daily milk or milk products corresponding to 6,400 mg of cows' milk protein formed the successful OIT group and the 10 (41.7%) others formed the unsuccessful OIT group. As published previously (11), serum milk-specific IgE before OIT was higher in those individuals who had discontinued using milk or milk products during the seven-year follow-up.

Markers

We measured blood eosinophils, and serum total IgE, milk-specific IgG and IgG4, eosinophil cationic protein, eosinophil-derived neurotoxin, interleukins (IL) IL-4, IL-5, IL-6, IL-10 and IL-12p70 and serum adipokines – namely adiponectin, adipisin, leptin and resistin – before and after the six-month escalation phase of OIT. The detailed laboratory methods, and the changes in the markers during the six-month escalation phase of OIT, have previously been published (13).

Ethics

The study was approved by the Ethics Committee of Tampere University and University Hospital, Finland, and

registered at ClinicalTrials.com (NCT01361347). Written, informed consent was obtained from the children and their parents.

Statistics

SPSS statistical software version 22 for Windows (IBM Corp, New York, USA) was used for the statistical analyses. All data were analysed as continuous variables. As the parameters were non-normally distributed in the exploratory data analyses, the nonparametric Mann–Whitney *U* Test was used. A two-tailed *p* value of <0.05 was considered statistically significant.

RESULTS

Of the 24 children who were included in the phone interviews at seven years, 14 were continuing to consume cows' milk or milk products on a daily basis and the other 10 were not. These findings enabled us to split them into two groups – successful OIT and unsuccessful OIT – and then compare the 15 markers measured at baseline and at six months after the OIT treatment started to see if they could predict whether the treatment would be successful in the long term.

As seen in Table 1, there were no significant differences in any of the 15 allergy, immunology or inflammatory markers measured at the start of the study between the 14 subjects in the successful OIT group and the 10 subjects in the unsuccessful OIT group.

When the markers were studied at the end of the six-month escalation phase of OIT and compared between the same groups, the only significant difference we found between the successful and unsuccessful OIT groups was in the serum adipisin concentrations. The median level of serum adipisin was 814.35 ng/mL (range 510.55–1,170.00) in the successful OIT group and 989.65 ng/mL (range 814.07–1,290.60) in the unsuccessful OIT group (*p* = 0.016) (Table 2). These represented nonsignificant increases from baseline to six months of 1.85% and 9.82%, respectively. However, having higher levels of serum adipisin six months after treatment commenced predicted treatment failure at the seven-year follow-up point. It also suggests that adipokines, which are cell signalling proteins secreted by adipose tissue, may play a role in cows' milk allergies. This result was unexpected because serum adipisin did not increase significantly during the escalation phase.

There were no other significant differences between the successful and the unsuccessful OIT groups in the other 14 parameters measured after the six-month escalation phase of the OIT (Table 2).

DISCUSSION

We studied 15 allergy, immunology and inflammatory markers before the start of OIT and at the end of the six-month escalation phase and only serum adipisin showed any association with the long-term outcome of OIT at the seven-year follow-up. Higher serum adipisin after the

Table 1 Results of the 15 biomarkers measured at the start of oral immunotherapy for cows' milk allergy in 24 children, based on which children had or had not conquered their allergy seven years after the intervention

	Successful group* (n = 14)		Unsuccessful group (n = 10)		p
	Median (range)	Tested (n)	Median (range)	Tested (n)	
Eos 10E9/l	0.60 (0.24–1.25)	12	0.60 (0.20–1.09)	10	0.771
IgE kU/L	662.00 (228.00–5040.00)	13	751.50 (240.00–10100.00)	10	0.605
IgG g/L	9.55 (7.51–12.12)	12	11.02 (8.33–12.78)	9	0.193
ECP µg/L	19.00 (9.40–143.00)	12	27.55 (7.10–76.60)	10	0.771
EDN pg/mL	51.64 (38.41–74.79)	14	75.39 (21.83–186.00)	10	0.138
IgG4 mgA/L	20.75 (16.10–112.40)	14	22.25 (13.70–24.90)	8	0.616
IL-4 pg/mL	1.95 (1.95–2.90)	14	1.95 (1.95–2.80)	10	0.625
IL-5 pg/mL	2.60 (1.03–15.90)	14	1.75 (1.03–52.40)	10	0.341
IL-6 pg/mL	0.45 (0.30–3.60)	14	0.65 (0.30–9.20)	10	0.212
IL-10 pg/mL	2.05 (1.00–7.30)	14	2.45 (1.00–5.10)	10	0.709
IL-12p70 pg/mL	3.90 (3.90–3.90)	14	3.90 (3.90–4.80)	10	0.709
Adiponectin µg/mL	3.49 (2.26–7.57)	14	4.29 (2.09–5.40)	10	0.666
Adipsin ng/mL	799.48 (619.36–987.61)	14	901.15 (597.35–1083.09)	10	0.285
Leptin ng/mL	3.83 (0.32–22.19)	14	3.48 (0.65–10.29)	10	0.931
Resistin ng/mL	2.85 (1.51–5.90)	14	4.05 (2.43–6.01)	10	0.122

*Able to consume milk (≥ 200 mL) or milk products (protein $\geq 6,400$ mg) daily.

Table 2 Results of the 15 biomarkers measured at the end of the six-month escalation phase[#] of the oral immunotherapy for cows' milk allergy in 24 children, based on which children had or had not conquered their allergy seven years after the intervention

	Successful group* (n = 14)		Unsuccessful group (n = 10)		p
	Median (Range)	Tested (n)	Median (Range)	Tested (n)	
Eos 10E9/l	0.54 (0.14–0.88)	14	0.36 (0.20–1.20)	8	0.525
IgE kU/L	732.50 (269.00–3960.00)	14	524.50 (119.00–7280.00)	8	0.616
IgG g/L	8.96 (6.80–10.90)	14	9.97 (7.78–12.72)	8	0.110
ECP µg/L	23.25 (4.60–139.00)	14	9.75 (6.40–59.20)	8	0.330
EDN pg/mL	66.73 (31.17–159.70)	14	53.61 (24.20–140.20)	8	0.441
IgG4 mgA/L	52.35 (15.00–238.00)	12	21.20 (16.30–226.00)	10	0.456
IL-4 pg/mL	1.95 (1.95–7.80)	14	1.98 (1.95–25.40)	8	0.482
IL-5 pg/mL	1.80 (1.03–6.80)	14	3.75 (1.03–35.40)	8	0.238
IL-6 pg/mL	0.80 (0.30–2.40)	14	0.85 (0.30–8.30)	8	0.330
IL-10 pg/mL	2.05 (0.70–10.00)	14	2.25 (1.70–3.50)	8	0.365
IL-12p70 pg/mL	3.90 (3.90–12.30)	14	3.90 (3.90–139.70)	8	0.482
Adiponectin µg/mL	3.53 (1.70–7.43)	14	5.10 (2.05–5.30)	8	0.127
Adipsin ng/mL	814.35 (510.55–1170.00)	14	989.65 (814.07–1290.60)	8	0.016
Leptin ng/mL	3.63 (1.00–23.45)	14	5.58 (2.40–10.72)	8	0.441
Resistin ng/mL	3.66 (1.61–7.00)	14	3.42 (1.78–7.60)	8	1.000

[#]Data from the placebo-controlled and open-label escalation phases have been combined.

*Milk (≥ 200 mL) or milk products (protein $\geq 6,400$ mg) daily.

six-month escalation phase of OIT predicted poorer outcome at this stage, namely discontinuing the use of milk or milk products. Serum adipsin measured before the escalation phase, or any other marker measured before or after the escalation phase of OIT, did not predict the long-term outcome.

There are no other published data, apart from our present study series, on the role of adipokines in OIT for cows' milk allergy or other food allergies. The earlier phases of this study documented that the levels of proinflammatory adipokines, leptin and resistin, increased during the escalation phase of OIT, but the rise for adipsin was not

significant (13). Adipsin is a nonspecific adipokine secreted by adipocytes that may be increased in different diseases and inflammatory conditions (14). Thus, our result is preliminary and should be confirmed or ruled out in further studies.

The result of our present OIT study that serum adipsin did not increase significantly during the escalation phase, but that having a higher serum adipsin at the end of the escalation phase predicted poorer long-term outcome, was against our expectations. In a previous study, serum adipsin was higher in adults with seasonal allergic rhinitis than in controls and the highest levels were in those treated with

sublingual immunotherapy (15). As a result, adipsin might be considered to have both anti-inflammatory and proinflammatory properties. Our finding of higher adipsin levels after the escalation phase of OIT in those who did not succeed with OIT fits well with the theories that adipsin is a Th2-type proinflammatory cytokine that promotes allergic activity, or, *vice versa*, that it is also an anti-inflammatory cytokine reducing Th1-type activity. However, serum adipsin did not increase or decrease significantly during the escalation phase of OIT (13), which makes it difficult to draw any strong conclusions about its impact on food allergies or immunotherapy. Two other proinflammatory adipokines, namely leptin and resistin, increased during the escalation phase of OIT (13), but neither of these levels had any association with the later seven-year outcome.

Data on milk-specific IgE as a predictor of the seven-year outcome in this study have previously been published (11). On average, high serum milk-specific IgE concentrations before OIT predicted poor outcome, as the concentration was higher in those who discontinued using milk or milk products during the seven-year follow-up (11). However, any cut-off limits could not be assessed, possibly due to the small number of patients in the study. In the other long-term follow-up studies on OIT for cows' milk allergy, milk-specific IgE was either not measured at all (16), a significant reduction was found that was considered to be a prognostic indicator of cows' milk tolerance (17) or a baseline level greater than 75 kU/L predicted poor long-term outcome (10).

Blood eosinophils and serum total IgE were used as the markers of allergy in the present study and when they were measured before OIT and at the end of the six-month escalation phase, they played no role in predicting the seven-year outcome. Likewise, milk-specific IgG and IgG4, which increased during the escalation phase (13), indicating a Th1-type nonallergic reaction pattern, had no association with later outcome. The 2017 position paper recommended using allergen-specific IgG4 to predict compliance in allergen immunotherapy for allergic asthma and rhinitis (9).

There were some limitations in the present study. We did not measure blood or serum markers during, or at the end of, the seven-year follow-up. On the other hand, the primary purpose of the study was to evaluate whether there were some markers before OIT that had a predictive value in assessing long-term outcomes of OIT in children with cows' milk allergies. We also wanted to know which markers could help us to select the most appropriate patients for OIT. The number of study subjects was rather small and did not allow us to carry out any stratified analyses or the use of multivariate models. The power calculation was made for the original intervention (12), but not for the present seven-year follow-up study (11). There was a risk for type-2 errors, which mean that all the differences that would be present in real life could not be found in the study. On the other hand, as many as 15 biomarkers were tested, which means that there was a risk of a type-1 errors due to multitestting. The clear strengths were the ongoing participation rate of 85% and the long prospective follow-up of seven years.

CONCLUSION

When the serum level of the inflammatory marker adipsin was measured at the end of the six-month escalation phase of OIT for cows' milk allergy, it was later shown to be significantly higher in the unsuccessful OIT group than the successful OIT group at the seven-year follow-up point. None of the other 14 biomarkers were capable of predicting the long-term outcome of OIT. In addition, the difference in serum adipsin between those with and without successful long-term outcome was rather small and the changes during the six-month escalation phase did not differ between the groups. This means that high milk-specific IgE, as reported previously (11), was the only biomarker that predicted poor outcome in this cohort.

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CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

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