

TITLE OF STUDY

Milk Desensitization and Induction of Tolerance in Children

Milk Allergy is a very common problem in children. Milk-induced symptoms effect up-to 20% of children, with detrimental effects on nutrition during critical periods of growth. Moreover, approximately 2-5% of children make IgE antibodies against cow's milk, which are responsible for severe allergic reactions and anaphylaxis. Milk is the second most common food causing life-threatening anaphylaxis in North America and Europe, and the most common food to cause life-threatening symptoms world-wide. Due to the ubiquitous nature of dairy products in our diet, milk is extremely difficult to avoid. Treatment of milk allergy is currently based on strict avoidance, and patients must carry injectable adrenalin (e.g. Epipen). Our study will assess a novel and potentially life-changing therapy, by actively treating milk allergy with Oral Immunotherapy. This may allow patients to **safely** consume dairy products. Treatment with oral-immunotherapy has been piloted in the USA and Europe, but there is no current research into milk-immunotherapy in Canada, depriving our population of a potential cure for this very common problem. We propose to perform a well-defined clinical study, using proper control groups and immunological measures to properly understand the ideal patients and the safest and most efficacious methodologies. If successful, this study will clearly increase the margin of safety for children and young adults who suffer from life-threatening milk allergy. It can also increase the consumption of dairy products within this population, providing nutritional benefits including improved bone mineralization and growth. We will also disseminate optimal milk- specific oral-immunotherapy protocols to practitioners for general use.

Background: Cow's Milk Allergy (CMA) is a very common problem in children with important nutritional impact. Moreover, IgE mediated cow's milk allergy is among the most common causes of severe allergic reactions and anaphylaxis. Due to the ubiquitous nature of dairy products in our diets, milk is extremely difficult to avoid. In spite of this, treatment of CMA is currently based on strict avoidance, as well as instructions for patients to carry injectable adrenalin (such as an Epipen). This study will assess a novel and potentially life-changing therapy, by actively treating CMA using Oral Immunotherapy, which may allow patients to safely consume milk and other dairy products. Treatment with oral immunotherapy (OIT) has been performed in the USA and Europe, but there are few rigorous, blinded clinical trials, with adequate controls and outcomes. In addition, there is no current research into OIT for CMA in Canada, depriving our population of a potential cure for this very common problem. A well defined clinical trial with adequate controls and immunological outcomes is essential for understanding the ideal patients and the safest and most efficacious methodologies, and for dissemination of successful milk specific OIT protocols to practitioners for more general use.

2.0 Hypothesis: Children who are allergic to milk can be treated via a supervised program of oral immunotherapy (OIT). Successful treatment will be indicated by the ability to ingest dairy products daily with no adverse symptoms. This will be accompanied by a decrease in specific IgE to milk, an increase in IgA and IgG4 blocking antibodies and by increases in milk-specific Regulatory T-cells (Treg) and Regulatory B-cells (Breg).

2.1 Specific Objectives:

- 1) To determine the efficacy of Oral immunotherapy (OIT) treatment for children with IgE mediated cow's milk allergy(CMA)
- 2) To determine the safety of OIT for treatment of children with IgE-mediated CMA
- 3) To determine if successful OIT for children with CMA is associated with increases in blocking antibodies, including milk specific IgA and IgG4
- 4) To determine if successful OIT for children with CMA is associated with increases in Treg and/or Breg.

Experimental Approach: This is a randomized control study with a cross-over design. Eighty four boys and girls with between 6 to 20 years of age, diagnosed with IgE-mediated cow's milk allergy using strict skin testing and

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serological criteria, will be recruited for this study. 42 will undergo oral immunotherapy, while 42 will be followed as natural history controls but will be offered similar therapy, should it be successful, at the completion of one year. OIT subjects will initiate therapy with a 2-day rush desensitization treatment using oral doses of milk, in our Clinical Investigation Unit. They will then continue the highest tolerated dose of milk at home for two weeks. Subsequently, they will return for weekly increases in doses until a maximum of 200 ml of milk is ingested daily. The primary clinical outcome will be a comparison of the amount of milk consumed safely on oral challenge performed prior to OIT, when the OIT dose has reached its maximum (200 ml or highest tolerated dose) and after one year of therapy. Patients will also be followed with clinical symptom scores and adverse event diaries and we will monitor immunological parameters such as changes in milk-specific IgE, blocking antibodies (IgG4 and IgA) and regulatory T and B cells. We expect that we will be able to see important improvement in the ability to safely ingest milk and other dairy products, and this will be accompanied by significant decreases in IgE and increases in Regulatory T and B cells.

BACKGROUND INFORMATION:

1.0 Food allergy, a common, significant problem: Foods are reported to be extremely common triggers of anaphylaxis, accounting for 33.2% to 56% of all anaphylaxis cases (1, 2). Food-induced anaphylaxis is an increasing reason for hospital admission, primarily in the first 2 decades of life. At present, the only treatment for food allergy is to avoid the causative food, while the principle treatment for an allergic reaction is prompt administration of intramuscular epinephrine (3, 4). Cow milk allergy (CMA) is the most common food allergy in children. CMA is associated with severe and frequent allergic reactions, life-threatening anaphylaxis, and multiple nutritional deficiencies. Due to the ubiquitous use of cows' milk in Western diets, it is almost impossible to avoid (5). Studies suggest that quality-of-life of patients and families with milk allergy is highly impaired (6). The large burden of this disease requires development of new treatment strategies apart from avoidance to alleviate this problem. This proposal will examine oral immunotherapy to cow's milk as a treatment for CMA. We will perform the first Canadian placebo-controlled trial of milk oral immunotherapy (OIT), and evaluate the clinical and immunological parameters that will help determine the ideal protocols, and predict ideal candidates to have maximal impact of this treatment strategy on the large population of children and adolescents with CMA in Canada.

12 : Adverse reactions to cow's milk protein: Adverse reactions to cow's milk are frequently reported, although only a small number can be properly substantiated. For example, in a European telephone survey of more than 40,000 contacts, the most common identified food sensitivity was milk; up to 38.5 % of children with food allergy (n=438) were reported sensitive to milk, and 26% of food-sensitive adults in this survey identified problems with milk (7, 8). This included both perceived symptoms and physician diagnoses. To make the issue more complex, when assessing reports of milk allergy, several immunological and biochemical mechanisms are implicated. For example, deficiency of the enzyme lactase leads to lactose intolerance, a common cause of milk-induced gastrointestinal symptoms (abdominal bloating, pain, diarrhea) in many individuals. This rarely, if ever, causes life-threatening symptoms, and lactase enzyme-supplemented products are common, thus obviating nutritional problems. Other disorders (such as FPIES, the Food induced Enterocolitis and Proctocolitis Syndrome) can cause intestinal symptoms such as vomiting, diarrhea and/or GI bleeding in infants and young children. This may involve immune mechanisms, such as T-cell mediated reactions to milk, and while FPIES can cause severe symptoms, the problem almost always resolves in early childhood (9, 10).

In contrast, **IgE-mediated allergy** is the cause of adverse reactions to milk associated with the most serious potential consequences. IgE antibodies are the common denominator in all allergic diseases, including food allergy, hay fever and asthma. IgE-mediated CMA can present with rapid onset of symptoms, starting within seconds following ingestion of dairy products. Symptoms can involve a single organ, such as skin (rash, eczema, and hives), or the intestinal tract (vomiting and abdominal pain), but milk ingestion can also cause life-threatening anaphylaxis, which involves the skin (swelling, hives, edema) mucous membranes (angioedema), respiratory tract (congestion, cough, wheezing, bronchospasm), intestinal tract (severe cramping, vomiting, diarrhea) and cardiovascular system (dizziness, hypotension, shock). Studies on food allergy-induced fatalities have consistently identified cow's milk among the most common causes of food induced mortality, along with tree nuts and peanuts (11).

13 : Diagnosis of IgE mediated CMA: IgE-antibody mediated CMA is caused by the presence of specific IgE antibodies against milk. It can affect all ages, from early in the first year of life through adulthood, and is the primary immunological mechanism that can cause anaphylaxis. When strict diagnostic criteria are applied, combining medical history and diagnostic testing, the rate of true IgE-mediated milk allergy in infants is estimated to range between 2 to 5% (2). The diagnosis of IgE- mediated CMA is predicated upon a history of initiation of the classical symptoms within seconds to less than 2 hours after ingestion of dairy products. This is further confirmed by the presence of a positive reaction on **allergy skin testing** using validated techniques, and/or by the presence of **specific IgE** to milk proteins on serological testing. A confirmatory **oral challenge** to milk in the setting of an allergy clinic can also be performed; this is employed in the research setting, and is also clinically useful to confirm unusual symptoms or to determine if the child has outgrown the allergy. It is important to note that skin testing or blood testing alone, without a clinical history of a reaction, is a poor measure of milk allergy, as children with no personal history of reaction can successfully pass a challenge in spite of the positive testing (5).

14 Pathophysiology of anaphylaxis and allergy: If a child has confirmed allergy, the symptoms that ensue are due to the presence of IgE antibodies on **mast cells** in the dermis, on mucosal surfaces, in the airways and gastrointestinal tract and in the vasculature (11). Mucosal Mast Cells (and circulating **basophils**) harbor IgE molecules on specialized receptors, Fc epsilon R1. Once an antigen such as milk is trapped by bound IgE, it triggers the mast cell to release a complex cascade of protein and lipid-based mediators that may induce swelling, redness, urticaria, bronchospasm, dizziness and shock that can accompany anaphylaxis. The mediators of anaphylaxis include histamine, leukotrienes, platelet-activating factor as well as other cytokines and chemokines (3). At present, the primary treatment for these reactions is injectable adrenalin (11). Strict avoidance of the food is currently the single effective strategy to preventing allergic reactions (3).

15 The impact of CMA: IgE mediated milk allergy is associated with a high frequency of unwanted reactions (12). In US surveys, it was ranked as second or third most common food requiring adrenalin treatment in children with food allergy (13, 14). This was due to the high frequency of dairy ingredients in common foods as well as medication and supplements (15, 16). Complete avoidance of dairy is thus difficult for families, and for the affected children and young adults.

The natural history of IgE mediated milk allergy is variable. It was reported that symptoms of CMA resolve for approximately 80% - 90% of children before the age of 5 (17). However, more recent studies indicate a later resolution of milk allergy and fewer children outgrowing CMA . In a study by Skripak et al, the median age for tolerance to cow's milk was 7 years \pm 11 months and at least 15% - 20% of their population retained their sensitivity into the second decade of life (18). This represents resolution at **older ages** than earlier studies (5). In addition, at least 20% of children who are diagnosed with CMA will have **life-long allergy** (5). The consequences of later resolution and prolonged allergy include important nutritional deficiency especially at critical times of bone growth and mineralization (19-21), in addition to the high risk of life-threatening events that accompany the increased exposures experienced by teens and young adults.

As there is a global increase in food allergy in general, and particularly in milk allergy, the issues reviewed above are not insignificant. To date, the primary treatment of any food allergy, including CMA, is strict avoidance in order to minimize the risk of allergic reactions. Although the symptoms of CMA resolve spontaneously for many patients, a significant number remain. The ubiquitous presence of milk products makes strict avoidance of milk more difficult than most other foods. In addition, the nutritional consequences of avoiding all dairy products, such as poor bone growth and osteoporosis, are much more protean than if one needs to avoid other common food allergens such as peanuts. We thus need to determine a better strategy for treating those with milk allergy. In developing treatments, we need to determine what are the optimal protocols, and what are the changes in the immune response to CMA that predict successful treatment. Defining these immunological differences may help develop novel treatments to help individuals to resolve their symptoms of CMA.

1.6 Treatment of CMA: Immunotherapy has been utilized for inhalant allergies, such as for grass, tree or ragweed hay fever, for over a century (22). Allergen immunotherapy is based on injection of incrementally increasing amounts a specific allergen over a prolonged period. However, attempts at food based immunotherapy led to severe adverse effects including fatalities (23). Treatment of food allergy and prevention of anaphylaxis based on allergen immunotherapy was not re-attempted until recently (22). Instead of injected immunotherapy, recent studies have attempted oral administration of

graded amounts of the food over a period of weeks to desensitize patients.

To date several studies have examined oral immunotherapy (OIT) for milk, eggs, and peanut allergies. Several uncontrolled studies have been published, although few controlled trials of oral immunotherapy to milk have been performed. A recent meta-analysis found only 4 studies of oral immunotherapy for CMA that included control groups (24). The authors found that the protocols were overall successful, with up to 10 fold improvements in milk tolerance in treated patients compared to untreated or placebo treated controls. These protocols reveal encouraging results with significantly increased thresholds to ingesting milk after oral immunotherapy (25-28). However, all studies had relatively small sample sizes. The authors of the meta-analysis also observed that for each study, treatment was accompanied by a significant number of reactions to milk (24). Most reactions were mild to moderate, and none were life-threatening. The authors concluded that more controlled studies should be performed to determine the safest and most efficacious protocols, whereby patients have the maximum benefit with risks minimized.

1.7 Questions regarding OIT treatment for CMA: There are no known guidelines describing the optimal patient candidates for desensitization nor are there criteria for the safest and most effective dosing schedules. To date, published milk desensitization protocols start treatment with very small doses, and the doses are increased weekly or bi-weekly. Alternatively, “rush” protocols which perform the initial increases rapidly in hospital over a 1-3 day period are employed, in order to speed up treatment. Rush protocols appear to be effective, although may be associated with more side effects during the in-hospital period. Conclusions as to the optimal protocols are limited by the low number of studies performed with each one. Therefore, major questions exist regarding the efficacy of the immunotherapy, the quantity/ frequency of allergen consumption required to cause desensitization and the optimal protocol that can balance efficacy and minimize side effects.

In addition, there are major gaps in the understanding of the immunological changes that accompany successful OIT. Individuals who outgrow food allergy have decreased size of allergy skin testing, and exhibit decreases in specific IgE antibodies to milk. Perhaps counter-intuitively, serum IgE against milk does not have to be undetectable, nor the skin test completely negative before a patient can tolerate milk (29). No firm cut-offs for skin test or IgE that will predict tolerance exist, although guidelines have been established that help predict who may undergo oral challenges successfully(30). In order for a child who has CMA to safely ingest milk, other changes in the immune system clearly are required to counter-balance the IgE antibodies that cause the disease. We will review several of the proposed immune system changes that are associated with tolerance to CMA.

1.8 Pathogenic and blocking antibodies: Antibodies are produced by B-lymphocytes (B-cells). To produce antibodies, B-cells must interact with T-lymphocytes, and receive specific signals from cell-cell contact and from intracellular communication molecules known as cytokines. For example, to synthesize IgE, B-cells must receive Interleukin (IL) 4 and/or IL-13 from T-cells. Importantly, patients who become tolerant to an allergen naturally or via immunotherapy exhibit an increase in antibodies of the **IgG4 class** (31). IgG4 may be a sign of tolerance, or may actually act as a “blocking antibody” to counteract IgE. Recently, in subjects being desensitized for peanut allergy, peanut specific **IgA** was also found to increase with treatment. IgA and IgG4 are produced when B-cells are exposed to IL-10 and Transforming Growth Factor β (TGF β), and both IgG4 and IgA may act as blocking antibodies, especially in the intestinal tract (32). It is not yet known if milk specific IgA increases with milk OIT. In this proposal we will follow the progression of milk specific IgE, IgA and IgG4 and correlate these with clinical outcomes.

1.9 Regulatory T-cells: T-cells are crucial coordinators of immune responses. They play an essential role in host defense and inflammation, via Effector T-cells (Teff) designated by surface molecules CD4+CD25-. T-cells are also key players in immune regulation via Regulatory T-cells (Treg) designated as CD4+CD25+. Human studies report that food allergens induce specific Teff cells, and studies have shown that Teff cells can proliferate in response to milk proteins in allergic individuals. In developing tolerance to milk, it is possible that Teff are diminished. Alternatively, tolerance to milk may be associated with increases in Treg that are specific for milk, which inhibit immune-mediated allergic reactions to milk

proteins. A potential suppressive role of Treg in food allergies was exemplified both in animal models (33, 34) and in human studies. At least two studies demonstrated increased Treg cells in peripheral blood in children who spontaneously outgrew milk allergy (35, 36). Our laboratory has shown that antigen-specific Treg attenuate airway inflammation in a mouse model of asthma (37, 38). This is due in part to production of regulatory cytokines, including IL-10 and TGF β . It is not known if increases in Treg during the natural outgrowth of milk allergy are mirrored by similar increased in Treg following active oral immunotherapy. In a limited study of 10 children, Mori et al reported no changes in total Treg expression in subjects undergoing milk OIT. However, this study was limited by small sample size, relatively short period of follow up (6 months), and they did not examine **antigen specific** Treg. Thus, we will investigate the effect of immunotherapy to milk on the levels of milk-specific Teff and Treg cells. We will carefully monitor the numbers of Treg, by analyzing blood cells that have the characteristics of CD4+CD25+ and the key regulatory molecule **Foxp3**. We will also determine if children who have undergone OIT have decreased T-eff responses to milk than children who have not been treated, and determine the cytokines that are produced by the Treg.

1.10 Regulatory B-cells in food allergy: B-lymphocytes are cells specialized for producing antibodies, and play a crucial role in the pathogenesis of milk allergy by producing IgE, the primary trigger for CMA associated with anaphylaxis. In addition to this already important role, B-cells likely play other crucial roles in the ability tolerate milk proteins. B-cells can produce cytokines such as IL-13, which may contribute to allergic inflammation in lungs and sinuses (39) and increase IgE production. Additionally, as with T-cells, B-cells can play an active role as regulatory cells (B-reg) in various infectious and inflammatory processes. B-reg produce regulatory cytokines, such as IL-10 and TGF β , similarly to T-reg, which can influence the production of IgG4 and IgA. An imbalance in B-reg has been shown to be important to the pathogenesis of Multiple Sclerosis (40). Recently, Noh et al showed that children with eczema and sensitivity to milk had fewer milk specific B-reg than children who could drink milk without worsening of their skin symptoms (32, 41). No studies have examined the correlation between B-reg and IgE-mediated milk allergy and anaphylaxis. Since B-cells can make both pathogenic IgE (which requires IL-13) and regulatory antibodies IgG4 and IgA, (which require IL-10) understanding the contribution of B-reg to milk desensitization is an important facet of clarifying the immunological network that is in play in this disease. Thus we will examine the total numbers of B-reg (designated by the surface markers CD19 and CD5 as well as the cytokines IL-10 and TGF β) and determine changes in these cells in children undergoing oral immunotherapy to milk compared to an observation group of untreated children with milk allergy.

1.11 Vitamin D in desensitization: Several environmental factors have been implicated in the pathogenesis of food allergies. One of the factors that can be measured and manipulated is vitamin D level. There is growing interest in the potential role of sunlight/vitamin D status on allergic conditions. Some ecological studies suggest a protective effect of vitamin D on the development of food allergies, (42, 43) others fail to substantiate this association; some even suggest an inverse effect, i.e. high levels of vitamin D are associated with increased food allergy risk(44). Because of our northern climate, fluctuations in vitamin D may play a role as a confounding variable in our study. As such, vitamin D levels will be measured with and correlated with symptoms, milk-specific IgE and IgG4 levels as well as T and B reg levels.

1.12 Both immunoglobulin (Ig) isotype and conserved Fc glycosylation sites often dictate the downstream activity of an Ab where complexity and degree of glycosylation contribute to its ability to bind Fc receptors (FcRs) and activate complement. Most information on the effects of glycosylation center on IgG in cancer therapy and autoimmunity. In cancer therapy, glycosylation modifications that enhance affinity for activating FcRs are utilized to facilitate immune-mediated tumor cell killing. In autoimmunity, disease severity has been linked to alterations in the presence, location, and composition of Fc glycans. Significantly less is understood about the role of glycosylation in the setting of allergy and asthma. However, recent data demonstrate that glycosylation of IgE at the asparagine-394 site of C ϵ 3 is necessary for IgE interaction with the high affinity IgE receptor but, surprisingly, glycosylation has no effect on IgE interaction with its low-affinity lectin receptor, CD23. Variations in the specific glycoform may modulate the interaction of an Ig with its receptors. Significantly more is known about the functional effects of glycosylation of IgG than for other Ig isotypes. Thus, the role of glycosylation is much better understood in the areas of autoimmunity and cancer therapy, where IgG is the dominant isotype, than in the field of allergy, where IgE predominates. Participant serum and/or plasma will be assessed for IgE glycosylation by Dr Galit Alter's laboratory at Harvard University

1.13 Does oral immunotherapy (OIT) give patients the ability to normally ingest dairy products? In treating allergic conditions, we may achieve either temporary **desensitization**, or complete **tolerance**. Desensitization refers to a change in the amount of food antigen needed to cause allergic symptoms; it is a temporary state and is dependent on regular exposure to the substance in question, as is done for penicillin allergic patients who require drug desensitization. When the treatment with penicillin is ended, the patient loses the ability to safely ingest it within days to weeks, unless the therapy continues. In contrast, **tolerance** refers to long-term immunologic changes associated with the ability to ingest a food without symptoms and without ongoing therapy. Both scenarios are an improvement over the status quo; desensitization as therapeutic goal allows individuals more freedom from the risk of accidental ingestion in everyday settings; however, frequent if not daily exposure to the food would be required to maintain the desensitized state. Limited reports of three subjects suggested a loss of protection within 48h to weeks after elimination of the food from the diet (45-47). A more recent trial suggested that as long as participants ingested the maintenance dose of milk at least twice a week no allergic reactions were likely to occur(48). Achieving long-term clinical tolerance would allow safe food ingestion without ongoing therapy by inducing lasting immunologic changes; given the abundance of dairy products in our diet, it is crucial, to determine if we can achieve tolerance using our clinical protocol. We will utilize oral food challenges at the end of oral immunotherapy and one month following cessation of therapy to evaluate this question.

1.14 Project Feasibility: Our team at the McGill University Health Center is well positioned to carry out this project. We have a very active program studying food allergy, with projects that encompass peanut allergy, sesame allergy, patients with multiple severe food allergies and we have extensive clinical and research data bases that catalogue food allergy patients. The division of pediatric allergy and immunology at MUHC is the largest academic allergy faculty in Canada and has extensive experience in performing food challenges and management of patients with milk allergy. Dr. Ben Shoshan has coordinated several large clinical studies and has hands on experience in investigating and managing severe food allergies (49-56). Dr. Mazer is a clinician scientist with expertise in food allergy and his laboratory is recognized as a leading Canadian laboratory in understanding B-cells and Treg in allergic inflammation (37-39, 57-60). The Research Institute of the MUHC includes laboratories studying T and B-reg in a variety of chronic conditions, including Diabetes, Multiple Sclerosis and Rheumatoid arthritis. Thus we have an expertise that is unique in Canada, and the experience that will ensure that the operating procedures for sample preparation and processing are in place.

1.15 Summary:

Ingestion of dairy products has tremendous health benefits for children and is a crucial source of nutrients for optimal growth and bone health. Children with persistent milk allergy not only cannot benefit from these important health effects but may suffer severe, untoward reactions and even fatalities with ingestion of small amounts of milk. At present no treatment is available in Canada, and avoidance is almost impossible. Successful oral immunotherapy will provide improved patient safety and undoubtedly contribute to improved quality of life through optimal nutrition. The uniqueness of our study includes, for the first time in Canada assessment of oral immunotherapy to milk, use of a control group and the thorough molecular and immunologic investigation of our subjects to provide data that will allow others to adapt these protocols safely. The integrated multidisciplinary team at the MUHC covers all facets of epidemiology, allergy, basic immunology and nutrition and has a proven track record in food allergy research.

2.0 Hypothesis: Children who are allergic to milk can be treated via a supervised program of oral immunotherapy (OIT). Successful treatment will be indicated by the ability to ingest dairy products daily with no adverse symptoms. This will be accompanied by a decrease in specific IgE to milk, an increase in IgA and IgG4 blocking antibodies and by increases in milk-specific Regulatory T-cells (Treg) and Regulatory B-cells (Breg).

2.1 Specific Objectives: 1) To determine the efficacy of Oral immunotherapy (OIT) treatment for children with IgE mediated cow's milk allergy (CMA)

2) To determine the safety of OIT for treatment of children with IgE-mediated CMA

3) To determine if successful OIT for children with CMA is associated with increases in blocking antibodies, including milk specific IgA and IgG4

4) To determine if successful OIT for children with CMA is associated with increases in Treg and/or Breg.

2.2 Rationale: CMA is a very common problem in children with important nutritional impact. Moreover, milk is among the most common foods associated with severe allergic reactions and anaphylaxis, and is extremely difficult to avoid. Treatment of CMA is currently based on strict avoidance, which is difficult to adhere to due to the ubiquitous nature of dietary dairy products. Treatment with oral immunotherapy (OIT) has been performed in the USA and Europe, but there are few rigorous, blinded clinical trials, with adequate controls and outcomes. However, there is no current research into OIT for CMA in Canada, depriving our population of a potential cure for this very common problem. A well defined clinical trial with adequate controls and immunological outcomes is essential for understanding the ideal patients and the safest and most efficacious methodologies, and for dissemination of successful milk specific OIT protocols to practitioners for more general use.

3.0 Research Methods

3.1 Subject Selection: Eighty four boys and girls, between 6 to 20 years of age, diagnosed with IgE-mediated milk allergy, will be recruited for the study. Participants will be recruited from the allergy clinic at the Montreal Children's Hospital, Hopital Ste Justine, and the Centre de santé et de services sociaux de Chicoutimi.

Inclusion criteria: Children 6 years and older who satisfy **all** the following criteria will be included:

a. A **history** suggestive of IgE-mediated allergy to milk. An IgE-mediated reaction to a specific food is defined as a minimum of 2 mild symptoms and/or 1 moderate and/or 1 severe symptom that began within 1-20 minutes after ingestion or contact. Mild IgE-mediated symptoms include: pruritus, urticaria, flushing, or rhinoconjunctivitis. Moderate symptoms include angioedema (of face or lips), throat tightness, gastrointestinal complaints (vomiting, cramping, pain and/or diarrhea), or airway involvement (cough, nasal blockage, mucous); severe symptoms include bronchospasm, wheezing, hypoxia, cyanosis, low blood pressure, or circulatory collapse (shock) (appendix A ,table 1) (61).

b. The presence of **at least one** of the following confirmatory tests:

- (a) Positive skin prick test to milk (weal diameter 3 mm larger than that of the normal saline control). The allergen used will be commercially available milk extracts (Omega Labs, Montreal, QC). Skin tests will traced in ink, tape transferred to paper and wheal diameter measured by computer assisted planometry.
- (b) Detection of serum specific IgE (>0.35 kU/L) to milk or any of its proteins, measured by fluorescence enzyme immunoassay (Immunocap, Phadia, Uppsala, Sweden). The range for Immuncap is 0.35 – 100 and changes over time can be monitored effectively.

Following explanation of all facets of the protocol, informed consent and assent forms will be signed by the parents or legal guardian and subject, if applicable (Appendix B).

Exclusion criteria.

1. Patients with uncontrolled asthma or other uncontrolled respiratory diseases .
2. Malignancies, autoimmune diseases and/or severe primary or secondary immune deficiencies.
3. Patients receiving immunosuppressive therapy.
4. Patients receiving β -blockers (including topical formulations).
5. Associated diseases contraindicating the use of epinephrine: cardiovascular disease or severe hypertension.

Through a process of stratified randomization (according to sex), participants will be randomly assigned to one of 2 study groups. The first group will receive active desensitization, and the second group will not receive any treatment but will be followed up regularly (Appendix A Table 2). Desensitization will be offered to the observation groups following the trial, should the outcome be successful in the treatment group.

3.2 Oral challenge test to milk. Prior to initiating desensitization and following treatment, the subjects will undergo a placebo controlled oral challenge to milk. These challenges will determine the level at which the children react and will be the baseline to follow their improvement in oral tolerance following therapy.

Oral challenges will be single blinded and will be performed with fresh cow's milk, with increasing doses taken orally every 10 to 15 minutes up to a total of 150ml of milk (doses of 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, 45ml, 60 mL) ^{(49,82) (83)}. Patients will receive 2 challenges per day, 1 placebo and 1 with the active substance (milk). Nurses in the Medical Day Unit/Center for Investigative Medicine will prepare the food and randomize the placebo challenges. Unless contraindicated, the placebo will be soy or rice-based milk. If the challenge is negative after 150 ml of milk, results will be confirmed by means of open challenge, using 250ml of yogurt or ice cream 1 hour after completion of the blinded portion(29) according to the recommendations of the European Academy of Allergology and Clinical Immunology (5). After completing the challenge, patients will be kept under observation for at least 2 hours. Only objective symptoms will be considered to be indicative of a positive allergic reaction (29). Food challenges will be scored as positive if a single symptom or any of the following objective clinical reactions will be observed: urticaria, angioedema, wheezing, rhinitis, vomiting, diarrhea, protracted abdominal pain, exacerbation of atopic dermatitis, or shock. A single episode of vomiting will not be considered as a positive reaction, while severe and repetitive vomiting will. If the challenge is **negative**, ie, the subject is asymptomatic, he/she will be excluded from the study. Based on our experience, approximately 80% of those subjects ages 6 or greater, with positive history and positive skin tests, approached will fail the oral challenge and thus be eligible.

3.3 Oral Immunotherapy to Cow's Milk Protein: The desensitization protocol will be performed at the Medical Day Unit / Center for Investigative Medicine, under the direct supervision of the medical and nursing staff, and with full cardiopulmonary resuscitation measures available for the treatment of possible allergic reactions that could occur during the procedure. Oral and intravenous doses of diphenhydramine and intramuscular epinephrine and IV corticosteroid will be at the bedside at all times(3).

The desensitization process is adapted from Martorelli 2011 al (26) (Appendix B). We have based our protocol on this study, as it was used for infant desensitization and was associated with high resolution of allergy and low incidence of side effects.

3.3.1 2 Day Initial Rush Desensitization: After placement of an intravenous catheter, the desensitization will begin with a first dose of 1ml of diluted milk (1/100). If tolerated, subjects will then receive approximately doubling doses every 60 minutes until they reach 8 ml of 1/100 dilution, or the highest tolerated dose without adverse reaction. Those who reach the 8 ml dose will be administered 1.6 ml of a 1/10 dilution of whole cow's milk. On the second day the dose of 1.6 ml of the 1/10 dilution is repeated, and then doubling doses every 60 minutes are administered till a dose of 12 ml 1/10 dilution of milk is reached. If 12 ml (1/10) is tolerated, the final dose on day 2 will be 2.5 ml undiluted milk.

The patients will not be admitted, but will remain under observation for 2 hours after the final dose each day, with the possibility for increasing this period if required. A full list of doses can be found in Appendix B.

3.3.2 Escalation phase

Once subjects reached the dose of 2.5 ml undiluted milk, they will be instructed to repeat this dose at home daily for 2 weeks. They will then come to the research unit, and will be given an increased dose of 4 ml. This will be continued for one week, with incremental increases given weekly for 16 weeks, until a dose of 200 ml is reached. If a patient is unable to increase past a particular dose they will continue on this dose until the end of the 16 week acceleration phase.

3.3.3 Maintenance phase

Patients who reach the maintenance dose of 200 ml will be instructed to continue and consume 200 ml of milk daily for 1 month. They will then undergo an oral challenge (see above), with the maximum dose of 300ml.

If this 300 ml dose is tolerated, they will be permitted to consume dairy products without restrictions and attend regular follow up visits for 12 months. It will be recommended to consume dairy at least twice weekly, if not more, to maintain the desensitization.

Subjects who tolerate less than the 200 ml upper dose will be instructed to continue their maximum tolerated dose for the 12 month follow-up period, at which time another blinded milk challenge will be performed to determine if their tolerance increased.

3.3.4 Long Term Follow-up: to assess the persistence of milk desensitization from clinical, cellular and molecular perspectives, participants will be asked to return to the research center once a year for five years following the end of the Maintenance Phase. At these visits, participants will be asked about their dairy consumption, any reactions they may have experienced in the interval between the last visit and changes in their quality of life (as assessed by questionnaire). Participants will also have the following analyses done: SPT, salivary IgA, serum milk-specific IgE, IgA and IgG4 concentrations, Glycosylation state, Vitamin D levels, and B and T cell regulatory cell phenotyping. Participants will be reminded of the importance of continuing the consumption of dairy products in the maintenance of desensitization. Participants who have completed the study will be asked to reconsent to the extension. Given that these participants may not have scheduled visits to the hospital, to lessen their inconvenience, a delegated member of the study team might make a home visit in order to obtain informed consent

3.3.4 Control Subjects: Subjects randomized to no treatment will be followed every 3-6 months as is standard in our clinic for milk allergy. They will have skin testing and blood testing as on routine follow-ups, but they will be required to fill out symptoms diaries. At the end of the study period, oral desensitization will be offered to the control patients who had not spontaneously achieved tolerance.

3.3.4 Documentation: Subjects and families will be asked to complete daily home diaries to document that daily doses were taken, as well as to report accidental ingestions, problems with dose administration, or related symptoms. Follow-up visits in addition to or in conjunction with the weekly visits for dose escalation will be at 3, 6, 9, 12 months from completion of OIT. Each visit will include a medical history and physical examination, SPT, serum milk-specific IgE, IgA and IgG4 concentrations, Vitamin D levels, and B and T cell regulatory cell phenotyping will be measured at enrollment and at follow-up visits. (see Appendix C). Biweekly or monthly Telephone follow-up of these patients will be done prior to and following reaching the maximum tolerated dose. The telephone follow-up will review symptoms

and protocols for how to treat potential reactions.

3.3.5 Adverse reactions to OIT: All patients and their caregivers (if applicable) will be provided self-administered epinephrine, along with instructions and indications for administration and education about the nature of possible reactions to the desensitization therapy. Home diary forms will be provided to record the dose, date and time taken, symptoms occurring after the dose or any other time, and medications taken each day (Appendix D).

Safety measures: The patients' caregivers will be instructed **verbally and in writing** about the recommendations to be followed after desensitization and how to **treat** possible allergic reactions. They will also be given telephone and pager numbers of investigators and nurses for direct consultation 24/7. Parents will be instructed to contact the research team should any symptoms suggestive of an allergic reaction be present (appendix E, bottom of page).:

Withdrawal from therapy: Subjects will be carefully monitored during the in hospital rush therapy phase, as well as during the home OIT phase. Instructions for treatment of reactions will be provided as above. Patients who are enrolled will be informed that in previously published studies, minor allergic reactions are common in these protocols (mild itching, abdominal discomfort) but resolve with increased time on therapy. Subjects will be withdrawn if they cannot tolerate the low dose, rush desensitization phase, if they have more than 2 or more moderate or severe reactions during the home (maintenance) OIT phase, if they experience a rare or unforeseen complication such as severe flares of eczema or chronic gastrointestinal symptoms (e.g. development of eosinophilic esophagitis) and if the parents or subjects wish to voluntarily terminate therapy.

3.3.6 : Variables measured. The following variables will be measured in each patient:

A. The primary outcome measure will be the presence of desensitization defined by the ability to tolerate 200 ml of milk 1 year after the start of the trial. This is a qualitative variable with three categories: total desensitization (200 mL of cow's milk); partial desensitization (20–200 mL); and failed desensitization (<20 mL milk). Only patients presenting total desensitization were regarded as being successfully desensitized.

B. Secondary outcome measures will be:

1. Number and severity of adverse reactions occurring after oral exposure to milk during the desensitization and follow-up phases.
2. Minimum dose of milk that triggered symptoms during the desensitization protocols or challenge tests.
3. Days until desensitization was achieved.
4. Indices of desensitization :
 - a. Change in skin prick test weal size, comparing tape transferred, cpoputor measured diameters and areas of the skin tests to milk extract.
 - b. Levels of sIgE, sIgA and sIgG4 before desensitization, during follow-up and at the end of the study.
 - c. T and B reg before desensitization, during follow-up and at the end of the study.

Sample Preparation and storage: Blood draws will be performed according to the Study Protocol Appendix C. 7-10 ml of blood will be drawn into heparinized tubes (green stopper) and 2-3 ml in red stopper tubes. Serum will be separated and frozen at -80 until processing for sIgE, IgA and IgG4 levels. Peripheral blood mononuclear cells, including T and B cells, will be isolated by Ficoll-Hypaque centrifugation. They will be resuspended at a concentration of 5×10^6 / ml, and frozen in RPMI + DMSO medium in liquid nitrogen until use for measurement of T-and Breg.

3.3.7 : Measurement of Milk specific IgE, IgA and IgG4: Milk specific IgE, IgG4, and IgA antibodies will be measured by Immunocap (Phadia, Upsalla, Sweden). This automated, matrix-based ELISA system is able to quantify specific antibodies with high accuracy, and is considered the gold standard for specific IgE measurement (62). It is ideal for pediatric samples as it requires approximately 120 μ l of blood per sample. The Immuncap system has been in use at the Montreal Children's Hospital since 2000.

3.3.8 Measurement of Regulatory T-cells: Regulatory T-cells will be measured from peripheral blood samples by flow cytometry. By definition, Treg are defined as CD4+ cells that co-express the surface marker CD25 and the intracellular marker Foxp3. 7-10 ml of blood will be procured from subjects and lymphocytes will be isolated by Ficoll-Hypaque density centrifugation. The isolated cells will be cultured at 1×10^6 / ml with milk proteins alpha, beta and kappa-casein as detailed in Noh et al (41). Following 48 hours of culture, the cells will be stained with fluorescent antibodies (CD4 APC, Foxp3 – Alexa 488, CD25 PE) and casein induced Treg populations will be determined by flow cytometry (LSRII, Becton Dickinson, Toronto, ON). Controls will include medium alone and an irrelevant antigen (gliadin). The Treg will be expressed as a percent of the total CD4+ T-cells, and the changes in Treg in response to culture with casein fractions will be followed over time. We predict that successful OIT will increase Tregs in response to milk protein exposure.

3.3.9 Measurement of Regulatory B-cells: Regulatory B-cells will be measured from peripheral blood samples by flow cytometry. By definition, Breg are defined as CD19+ cells that co-express the surface marker CD5 and the intracellular marker Foxp3. 5-10 ml of blood (as above) will be procured from subjects and lymphocytes will be isolated by Ficoll-Paque density centrifugation. The isolated cells will be cultured at 10^6 / ml with milk proteins alpha, beta and kappa-casein as detailed in Noh et al(41). Following 48 hours of culture, the cells will be stained with fluorescent antibodies (CD19 APC, Foxp3 –Alexa 488, CD5 PE) and casein induced Breg populations will be determined in an LSRII. Controls will include medium alone and an irrelevant antigen (gliadin). The Breg will be expressed as a percent of the total CD19+ B-cells, and the changes in Breg in response to culture with casein fractions will be followed over time. We predict that successful OIT will increase Bregs in response to milk protein exposure. We will also evaluate intracellular regulatory cytokines in CD19+ cells, specifically the presence of IL-10, which is expected to increase in subjects following successful OIT to milk.

4.0. Statistical Considerations

A: Sample Size calculation: Given that previous studies have attributed at least 50% improvement in the ability to tolerate milk(24) with an α of 0.05 and a power (1- β) of 0.80, a sample of 42 cases and 42 controls will be recruited.

B: Statistical Analysis: Statistical analyses will be performed using paired t-tests. A p value of <0.05 will be considered significant. Descriptive statistics of the variables including means, standard deviations and frequencies were computed for all study variables. The concentrations of milk –specific IgE, IgG and IgA, the size of the skin tests , T reg and Breg expression at the different study time-points will be compared with their baseline values using the one-sample paired t-test and to the control using a 2 sample t-test .

Multiple regression models will be used to assess factors associated with response to desensitization, including baseline demographic characteristics; gender; age; baseline SPT and specific IgE, Treg and Breg numbers, and vitamin D level.

5. Ethical Considerations

In the consent form given at study enrolment, participants will be advised that they will be randomly assigned to either oral immunotherapy or observation for a one year period. Participants will also be advised that their data will be shared only among the OIT study team. Participants in the observation control group who will not receive OIT treatment, will be told that they may to undergo such therapy after a year of observation and a positive oral food challenge.

Appendix A Scale for grading reaction Severity

Score	Symptom
Mild	Pruritus, Urticaria, Flushing, Rhinocconjunctivitis
Moderate	Angioedema, Throat tightness, Gastrointestinal complaints (cramping, pain, vomiting, diarrhea) Respiratory symptoms (Cough, Mucous production)
Severe	Wheeze, Respiratory Distress Hypoxia, Cyanosis, Hypotension Circulatory collapse (Shock)

Appendix B Desensitization protocol

Study Time Point	Milk Dilution	Volume (ml)
Day 1	1/100	1
	1/100	2
	1/100	4
	1/100	8
	1/10	1.6
Day 2	1/10	1.6
	1/10	3.2
	1/10	6.4
	1/10	12
	Undiluted	2.5
Week 2	Undiluted	4
Week 3	Undiluted	6
Week 4	Undiluted	8
Week 5	Undiluted	10
Week 6	Undiluted	12
Week 7	Undiluted	15
Week 8	Undiluted	20
Week 9	Undiluted	25
Week 10	Undiluted	30
Week 11	Undiluted	40
Week 12	Undiluted	50
Week 13	Undiluted	75
Week 14	Undiluted	100
Week 15	Undiluted	125
Week 16	Undiluted	150
Weeks 17-52	Undiluted	200

Appendix C Study Design

Visit 1: Explanation, Informed Consent, Skin testing, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg)

Visit 2: Physical Exam, Blinded Placebo Controlled Food Challenge

Visit 3, 4: Physical exam, 2 Day in hospital Rush initiation of Oral Immunotherapy

Visit 5: Two weeks after rush initiation, Physical exam, symptoms diary, first dose escalation

Visit 6-21: (or more if necessary) Vital Signs, Symptom Diary, Escalation of Oral Immunotherapy

Visit 22, one week after completion: Physical Exam, Symptom Diary, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg)

Visit 23, One month after completion of OIT escalation: Physical Exam, Symptom Diary, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg)

Visit 24: One month after cessation of OIT: Physical Exam, Symptom Diary, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg) Blinded Placebo Controlled Food Challenge

Visit 25: Three months after cessation of OIT: Physical Exam, Symptom Diary, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg)

Visit 26: 6 Months after completion of OIT: Physical Exam, Symptom Diary, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg)

Visit 27: 9 Months after completion of OIT: Physical Exam, Symptom Diary, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg)

Visit 28: 12 Months after completion of OIT: Physical Exam, Symptom Diary, Basic Blood testing + Immune Tolerance Evaluation (CBC, biochem, specific IgE, IgG4, IgA, Treg, Breg)

AVAILABLE FACILITIES AND EQUIPMENT

The study will be based at the McGill University Health Center- Montreal Children's Hospital and Montreal General Hospital. We have access to a Clinical Investigation Unit and specialized nurses that have experience with food challenges and treatment of anaphylaxis. Ms. Turnbull has coordinated several previous food allergy studies, and Mr. Lejtenyi is the highly experienced in the required assays, as laboratory coordinator for clinical research since 2004. The antibody testing by Immunocap will be performed in the clinical laboratory at the Montreal Children's Hospital. The cell culture studies for enumeration of Treg and Breg will be performed in Dr. Mazer's laboratory at the Meakins Christie laboratories. We have all the necessary cell culture facilities and an LSR-II multiparameter flow cytometer that can perform up to 11 color cell analysis. We have all the assays in place for the culture of T-cells and B-cells and for measurement and quantification of B-reg and Treg. The Food Allergy Epidemiology Unit at the Montreal General Hospital, of which Dr. Ben-Shoshan is a member, has extensive experience in coordinating, maintaining and analysing large studies.

CMA is a very common problem in children with important nutritional impact. Moreover, milk is among the most common foods associated with severe allergic reactions and anaphylaxis, and is extremely difficult to avoid. Treatment of CMA is currently based on strict avoidance, which is difficult to adhere to due to the ubiquitous nature of dietary dairy products. Treatment with oral immunotherapy (OIT) has been performed in the USA and Europe, but there are few rigorous, blinded clinical trials, with adequate controls and outcomes. Moreover, there is no current research into OIT for CMA in Canada, depriving our population of a potential cure for this very common problem. A well defined clinical trial with adequate controls and immunological outcomes is essential for understanding the ideal patients and the safest and most efficacious methodology and for dissemination of successful milk specific OIT protocols to practitioners for more general use. Successful treatment will improve the quality of life for the individuals suffering from milk allergy, as well as increase the margin of safety that can be precarious due to the high prevalence of milk and milk products. They will also be able to benefit from the important nutritional impact that includes a ready source protein, and calcium and vitamin D, which are crucial components for growth in early childhood.

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