COW'S MILK SPECIFIC IgE, IgA AND IgG4 AS A PREDICTOR OF OUTCOME IN ORAL IMMUNOTHERAPY

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ABSTRACT

Cow's milk allergy (CMA) is one of the major food allergies among infants and young children in Western populations. CMA is defined as an immunologic adverse reaction to cow's milk proteins. It affects 2-3% of infants. In 85% of the children, CMA resolves spontaneously within the first 5 years of life. Those with persistent CMA have a lifelong threat of anaphylaxis. 20 patients were recruited for a trial of cow's milk (CM) oral immunotherapy (OIT). Blood samples were collected at baseline and at multiple time points during the trial. Specific IgE, IgA and IgG4 to casein, β -lactoglobulin (BLG) and α -lactalbumin (ALA) were measured in children treated with OIT using Enzyme-Linked Immunosorbent Assay (ELISA). We observed that cow's milk specific IgE decreased by 5 to 10 times from baseline to maintenance therapy, where children are able to consume an appreciable amount of CMP. Importantly, in one patient there was no detectable sIgE 3 months post-OIT. In some subjects, CM sIgE was variable. As the role of sIgA and sIgG4 is poorly understood, we measured sIgA and sIgG4 to three major CMP components in a subset of subjects. Specific-IgA increased significantly over time in 7 of 9 patients to casein, in 6 of 9 patients to BLG and ALA. Specific IgG4 to casein increased in 8 of 9 patients, and in all 9 patients in case of BLG and ALA. In summary, as patients underwent oral immunotherapy to milk, sIgA and sIgG4 increased in parallel to the decrease in IgE in most subjects tested. Our results support the observation that sIgA and sIgG4 are more important prognostic markers in specific oral tolerance induction as they are more consistent compared to sIgE. Thus, CMP specific IgA and IgG4 may help predict responses to CM OIT more accurately.

ABRÉGÉ

L'allergie IgE-médiée au lait de vache se retrouve parmi les allergies alimentaires les plus fréquentes chez les jeunes enfants des pays industrialisés. Elle se définit par une réaction immunitaire inappropriée aux protéines retrouvées dans le lait de vache. Elle affecte 2-3% des enfants. Dans 85% des cas, l'allergie au lait de vache rentrera dans l'ordre spontanément avant l'âge de 5 ans. Par contre, ceux qui présentent une allergie persistante au lait de vache s'exposent à un risque à vie d'anaphylaxie. Nous avons recruté 20 patients dans le cadre d'un projet de recherche sur l'immunothérapie orale (ITO) au lait de vache. Un prélèvement sanguin a été effectué au début et à différents moments au cours de l'étude chez les sujets participants. Les IgE et les IgA spécifiques (IgEs/IgAs) pour la caséine, la β -lactoglobuline (BLG) et l'α -lactalbumine (ALA) ont été mesuré par technique ELISA, de même que les IgEs, les IgAs et les IgG_4 spécifiques (IgG_4s) pour le lait de vache chez les sujets ayant reçu l'ITO. Chez ceux ayant toléré une quantité suffisante de lait vache dans le cadre du processus d'ITO, nous avons observé une diminution de 5 à 10 fois des niveaux de base des IgEs aux protéines du lait de vache. En particulier, l'un des sujets présentait des niveaux indétectables d'IgEs après 3 mois de traitement. Il est à noter que certains sujets ont présenté des niveaux variables d'IgEs aux protéines du lait de vache au cours de l'étude. Nous avons ensuite mesuré chez un groupe de sujets les niveaux d'IgAs et d'IgG_4s pour les 3 protéines allergènes principales du lait de vache afin de définir leur rôle lors du processus d'ITO. Les IgAs ont augmenté significativement au cours de l'étude chez 7 sujets sur 9 pour la caséine et 6 sujets sur 9 pour la BLG et l'ALA. En ce qui a trait aux IgG_4s, les niveaux ont augmenté chez 8 sujets sur 9 pour la caséine et chez tous les 9 sujets pour la BLG et l'ALA. En conclusion, chez la majorité des sujets ayant reçu le traitement d'ITO au lait de vache,

l'augmentation des niveaux d'IgAs et d'IgG_4s s'est associée à une baisse parallèle des niveaux d'IgEs. Ces résultats supportent le fait que les niveaux d'IgAs et d'IgG4s sont des marqueurs pronostiques importants des résultats de l'ITO et ce, de manière plus consistante que les niveaux d'IgEs pris isolément. C'est pourquoi, le dosage des IgAs et des IgG_4s pour les protéines allergènes du lait de vache peut contribuer à prédire de façon plus adéquate la réponse clinique au traitement d'ITO par le lait de vache.

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PREFACE AND CONTRIBUTION OF AUTHORS

Dr. Bruce Mazer, the candidate's supervisor, along with Dr. Moshe Ben-Shoshan, conceived the idea for this project and designed this study.

Dr. Mazer made extensive edits and provided significant suggestions on all parts of my thesis.

Dr. Ben-Shoshan, Dr. Sarah De Schryver, and Duncan Lejtenyi performed the oral immunotherapy procedure in Allergy clinic at the Montreal Children's Hospital. They have also carried out the Skin prick test (SPT) in CMA individuals.

Dr. Sarah De Schryver has contribution in the introduction part of IgA section, as it was taken from a manuscript prepared by both Dr. Sarah and the candidate of this thesis.

Tanvir Rahman, the candidate and the author of this thesis was responsible for carrying out almost all of the laboratory experiments described in this thesis: isolation of PBMC's, cow's milk protein specific IgE, IgA, and IgG4 ELISA's. Furthermore, the candidate performed all statistical analyses. He produced all figures and tables presented in this thesis. Finally, the candidate was responsible for writing the thesis in its entirety.

LIST OF ABBREVIATIONS

CMA	Cow's milk allergy
CMP	Cow's milk protein
BLG	Beta-lactoglobulin
ALA	Alpha-lactalbumin
OIT	Oral Immunotherapy
ECIT	Epicutaneous immunotherapy
SLIT	Sublingual immunotherapy
SOTI	Specific oral tolerance induction
POIT	Post oral immunotherapy
IgE	Immunoglobulin E
IgA	Immunoglobulin A
IgG4	Immunoglobulin G4
HM	Heated milk
BSA	Bovine serum albumin
TF	Transferrin
LF	Lactoferrin
CT	Cholera toxin
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-10	Interleukin-10
TGF- β	Transforming growth factor-β
IFN-γ	Interferon- γ

INTRODUCTION

Rationale

Food allergy is an increasing problem in the Western population. IgE mediated food allergy affects 2-4.5% of children[1]. Cow's milk allergy (CMA) is one of the major food allergies, affecting 2% of all infants[2]. CMA can be categorized into IgE mediated "Acute onset", non-IgE mediated "Delayed onset" or both. Age of onset and clinical scenario of CMA may vary depending on the type of allergy[3]. As like other allergic diseases, IgE is involved in the pathogenesis of food allergy[4-9]. Lower sIgE was reported to be associated with less severe allergic reactions and early tolerance induction in children with CMA[9-11]. Recently, a lesser diversity and lower affinity of IgE binding to CM peptide epitopes was found to be associated with a better outcome in CMA oral immunotherapy (OIT)[12-14]. Hence, CMP-sIgE is an important marker to predict the outcome of OIT in CMA. The other two CMP-specific immunoglobulins we studied are IgA and IgG4. The role of CMP-sIgA in CMA is not clearly understood. However, CMP-sIgA has been reported to be associated with both better and worst outcomes in CMA[15, 16]. In addition, few studies have evaluated the role of CMP-sIgA in CMA. We sought to inquire the role of CMP-sIgA, if it has any role in tolerance induction in this clinical trial. Recently, it was reported that increased levels of CMP-sIgG4 were associated with the induction of tolerance in CMA patients[14, 17], whereas a low level of CMP-sIgG4 was related to persistent CMA[18]. Additionally, increased diversity & higher affinity of IgG4 binding to CM peptide epitopes was reported in successfully CM tolerized subjects[7, 13]. It is important to know the role of CMP-sIgG4 in this CM tolerance study.

Hypotheses

Specific IgE to casein, β- lactoglobulin, and α- lactalbumin will be significantly higher in CMA patients compared to non-CMA healthy controls. Successful OIT to CMA will be associated with decreased CMP-sIgE. Successful oral tolerance induction to CM will be associated with increased CMP-sIgA. The level of CMP-sIgA and -sIgG4 will be significantly different in CMA patients compared to non-CMA healthy controls. Successful OIT will be associated with increased level of CMP-sIgA and -sIgG4 in CM tolerized subjects.

Project Objectives

To determine if CMP-sIgE has any significant difference at baseline in CMA children compared to non-CMA healthy controls. To determine if successful OIT is associated with a decreased baseline CMP-sIgE level. To determine if CMA children and non-CMA healthy controls have any significant difference in baseline CMP-sIgA. To determine if successful OIT is associated with an increase in CMP-sIgA antibodies in CM tolerized subjects. To determine if CMP-sIgG4 has any significant difference at baseline in CMA children compared to non-CMA healthy controls. To determine if successful OIT is associated with an increase in CMP-sIgG4 antibodies in CM tolerized subjects.

LITERATURE REVIEW

A short introduction to CMA: Pathogenic CMP's, clinical features & diagnosis

Cow's milk consists of two portions- whey (20%, approximately 5g/L) and coagulum (80%, approximately 30g/L). Casein (also known as Bos d 8) makes up the coagulum, whereas β- lactoglobulin (Bos d 5) and α- lactalbumin (Bos d 4) comprises the whey portion. Studies suggest that, though casein being the largest fraction of cow's milk protein (CMP), β- lactoglobulin (BLG) & α- lactalbumin (ALA) are also important in the induction of allergenicity and antigenicity in cow's milk allergy (CMA). Lactoferrin (LF), immunoglobulins, transferrin, bovine serum albumin (BSA), and proteose-peptone are among the minor CMP's [17, 19-22]. Although previous studies have found casein as the most allergenic and antigenic in nature compared to other CMP's, it is possible that patients might be sensitized to more than one CMP at the same time. Multiprotein sensitized patients have been reported to be associated with a poor prognosis in CMA[23].

Clinical reactivity to cow's milk can be mild, moderate, or severe. Mild symptoms include skin rash or urticaria, circumoral itchiness, abdominal pain, bloating, and angioedema. Moderate symptoms include diarrhoea, vomiting, breathing difficulties, and rhinitis. Severe reactions include wheeze, hypotension, cyanosis, and arrhythmia. Fatal cases have also been recorded in CMA[24, 25].

The diagnosis of CMA is largely based on previous history of accidental CM exposure and subsequent anaphylactic reactions, CMP-sIgE, and skin sensitivity to CM. Double blinded placebo controlled food challenge (DBPCFC) is regarded as the "gold standard" in diagnosis of CMA[3, 6, 8, 9].

Current and prospective treatments of CMA

Up to 85% of CMA resolves naturally within the first 3-5 years of life[26]. However, approximately 15% of the CMA population retains their allergic status even after 8 years of age[8]. Persistent CMA has been reported to be associated with the increased risk of development of allergic rhinitis and atopic asthma, an event known as "Atopic March"[8]. Due to the ubiquitous presence of CM in food products, children with CMA are in constant threat of accidental exposure, which may lead to life-threatening anaphylactic reactions. Currently, the avoidance of cow's milk and dairy products remains the mainstay of the treatment. There are also soy-based and extensively hydrolysed milk–based formulas as the replacement of cow's milk. Anaphylactic reactions following accidental exposure are treated with intramuscular (IM) epinephrine and anti-histamine. Avoidance of milk is not an ideal treatment option. Moreover, cow's milk is an indispensable part of the food chart in early years of life. Avoidance of milk may cause decreased bone and tooth growth, ultimately leading to a compromised overall development[27, 28]. Avoidance of CM may also lead to decreased bone mineralization[29] and predisposes to fractures in early years of life[30]. It is thus necessary to develop a treatment option for CMA. Inhalant allergen specific immunotherapy (e.g. pollen) dates back to 1911[31]. Success in inhalant allergen specific immunotherapy (SIT) also intrigued the food allergen SIT. SIT to CMA is still under trial. Different methods like sublingual immunotherapy (SLIT)[32], epicutaneous immunotherapy (ECIT)[33], oral immunotherapy (OIT)[34-36], anti-IgE monoclonal antibody (Omalizumab®) in conjunction with OIT[36] to CM have been trialed to induce tolerance in CMA patients. Among all modes of immunotherapy to CMA, OIT prevailed

over SLIT & ECIT with the cost of more adverse reactions[37]. The goal of SIT is to accelerate tolerance induction, as well as to alleviate the burden of unwanted anaphylactic reactions, & thus improving the quality of life.

Pathophysiology of cow's milk allergy

Cow's milk allergy is an immediate type or Type-1 hypersensitivity reaction. Type-1 hypersensitivity reactions occur within minutes to hours after antigen exposure. After the first ingestion or exposure to CM, dendritic cells recognize and process the CM allergen into simplified peptides. These peptides are then presented to CD4+ naïve T cells. In CMA patients, these CD4+ naïve T cells differentiate into Th2 cells under the influence of pro-inflammatory cytokines IL-4 & IL-5. Th2 cells are known as pro-inflammatory cells by virtue of their ability to produce IL-4, IL-5, and IL-13. Th2 cells then interact with B cells by direct cell-to-cell contact and indirectly via IL-4 and IL-13. This will in turn lead the immunoglobulin class switch recombination with the production of IgE. IgE exists as a dimer consisting two heavy chains and two light chains. Like other immunoglobulin molecules, IgE consists of antigen binding Fab (Fraction of antigen binding) and cell surface-receptor binding Fc (Fraction of complement binding) fragments. IgE recognizes and binds to FcεRI on the surface of mast cells and basophils[38]. This process is known as sensitization[38-40]. Subsequent exposure to milk leads to the cross-linking of allergen with the preformed specific antibodies on the mast cell and basophil surface causing their degranulation. The degranulation causes release of several chemical mediators including histamine, serotonin, prostaglandins, and bradykinin. These chemical mediators are responsible for the clinical manifestations in CMA[41]. IL-4 in turn takes part in a "positive feedback" loop causing further increase in

the production of Th2 and differentiation of IgE producing plasma cells. These Th2 cells will eventually produce more IL-4 to continue with the inflammatory cascade. The other pro-inflammatory cytokine IL-5 attracts eosinophils to the site of inflammation, leading to tissue damage. This inflammation and damage will encourage further inflammation facilitating a vicious cycle[42].

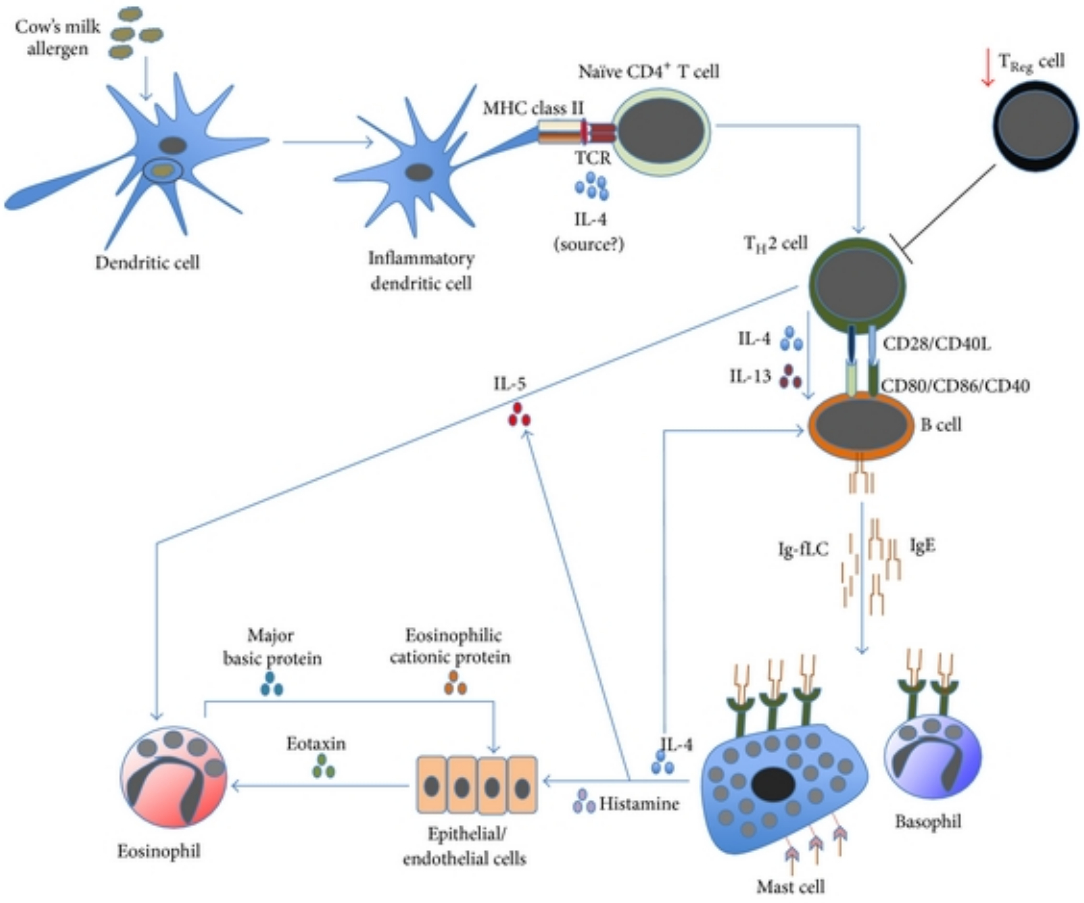

Figure 1: Depiction of the pathogenesis of Cow's milk allergy. CMP's are recognized, processed and presented by the Dendritic cells with MHC class II to TCR of naïve CD+ T- cells. It turns naïve CD4+ T- cells into Th2 Helper cells secreting IL- 4, IL- 5, and IL- 13. This Helper T-cell subset communicates through its CD28/CD40L to CD80/CD86/CD40 of B lymphocytes in a cell-to-cell direct manner. Indirect communication pathway acts via IL- 4, and IL- 13. This communication induces IgE class switching and differentiation of IgE producing plasma cells. These IgE molecules and some immunoglobulin light chains; Ig- fLC binds to the surface of Mast cells. Further antigen exposure causes cross linking of CMP-antigen with preformed IgE on mast cells causing mast cell degranulation, releasing inflammatory mediators and subsequent allergic cascade in Type- 1 hypersensitivity reaction (Adapted from Jo et al., 2014)[42].

Role of cow's milk protein specific IgE in CM OIT

Longo et al.[43] evaluated the safety and efficacy of specific oral tolerance induction (SOTI) against CMA. Ninety-seven severely CMA (CM-sIgE>85kUA/L) patients were recruited (age, 5-17 years) at the beginning of the trial. Double-blinded placebo-controlled food challenge (DBPCFC) was performed to confirm CMA in these patients. Finally, 60 patients were selected for the trial. They were divided into OIT recipient or group A and observation control or group B (who were on milk free diet and followed up for 1 year at regular intervals). The duration of OIT was over a period of 40 weeks. Blood samples were collected at the beginning, at 6 months and at 12 months of OIT. Serum casein, BLG, & ALA specific IgE were measured using ImmunoCAP assay (Phadia, Uppsala, Sweden). CMP-sIgE decreased significantly baseline (*p<0.05) in 50% of group A (15/30) patients at the end of OIT. No significant change was observed in observation controls or group B. At the end of 1 year, 36% of group A (11/30) patients successfully completed the protocol, 54% (16/30) tolerated moderate amounts of milk, 10% (3/10) had left the study due to adverse reactions. Martorell et al.[44] also reported significant decrease in CMP-sIgE levels in successfully CM tolerized patients. They studied CMA in young children (n=60, age: 24-36 months). The total population was divided into active treatment recipient and observation control groups. Serum was collected at baseline and at 1-year post-OIT. Serum sIgE to whole CM, casein, BLG, and ALA were determined using ImmunoCAP assay. In the active treatment group, sIgE to whole CM and casein decreased significantly baseline (*p<0.05) after 1 year (median, 15kU/L to 7kU/L to whole CM and 11.4 kU/L to 2.61kU/L to casein) of OIT. The control group did not show any significant change from the baseline to the end of 1 year. This

study showed 90% success in the actively treated patients, whereas 23% of the control group achieved natural tolerance. Skin sensitivity to whole CM was also significantly decreased baseline at the end of the OIT in active intervention group (****$p < 0.0001$) compared to the observation control group[44]. In another randomized double-blinded placebo-controlled trial (n=28, age: 6-14 years), Salmivesi et al.[45] reported increased levels of CMP-sIgE in failed OIT recipients. CMA patients who failed to respond to their protocol and left the study had significantly higher levels of CMP-sIgE compared to successful OIT recipients. CM-sIgE was higher (>70IU/L) in 2 out of 16 successful OIT recipients. CM-sIgE was 70IU/L & 313 IU/L in children who discontinued OIT. In 14 of 18 tolerant subjects, the consumption of CMP increased significantly from a median of 6mg (range, 0.05-162mg) to 6400mg. The control placebo group, who were also CMA, went through open-label OIT. After the completion of the open-label OIT, they were able to drink 200ml (6400mg) of CM daily. The level of CM-sIgE in this group was found less than 70IU/L. The patient progress was further tracked 12 and 6months post-OIT respectively in the double-blinded OIT and open-labeled OIT groups. Both groups were able to consume CM or CM products equivalent to 200ml of CM or 6400mg of CMP.

Another cohort by Ito et al.[7] also emphasized on the usefulness of sIgE to determine the prognosis of CMA. Their cohort included 83 suspected CMA children (median age, 3.5 years) with concomitant atopic dermatitis (85%) and asthma (32%). An open oral milk provocation test and prior history of allergic reactions further subdivided the population into two groups. CMA was confirmed in 61 subjects. Remainders were declared non-CMA as they did not show any reaction to CMA & were used as controls. There was no significant difference in total IgE levels between CMA & non-CMA groups. However,

CMA patients showed significant differences in sIgE compared to the non-CMA individuals (***p< 0.001, ***p< 0.001, *p<0.05 respectively for CM, casein and BLG). No significant difference in sIgE was found in case of ALA between groups. BLG and ALA specific IgE was not detectable in 19 and 30 CMA patients respectively. This yields the clinical sensitivity values of 98%, 69%, & 51% respectively for Casein, BLG, & ALA in that study population. CMA children who became tolerant after 5 years of age showed less CMP-sIgE compared to patients with persistent CMA. Carmen et al.[9] described evolution of tolerance to CMA in infants (mean age, 4.8 months). The mean age of diagnosis was 3.3 months (range, 1-8 months). In total, 170 suspected CM sensitive infants were recruited over a span of 4 years. Open controlled challenge test were performed in all but 9 infants who did not have history of severe allergic reaction to CMA. On the first day a total of 17ml (2, 5, & 10ml) dose was reached. On subsequent days, a maximum of 100ml dose was reached. Parents of infants who could tolerate 100ml of CM, were suggested to continue the same amount for the next 15 days before they were declared tolerant. Specific IgE to whole CM, casein, BLG, & ALA was measured in CMA and tolerant infants. A statistical significance of ***p<0.001 was achieved between CMA and tolerant groups. Saarinen et al.[8] studied the natural course of IgE mediated CMA in infants. They studied if CMA infants were prone to develop any future allergic diseases. Diagnosis of CMA was confirmed using elimination challenge test in 118 infants (mean age: 7 months) from a huge cohort of 6209 new born full-term infants. Recruits were followed up at every 6 to 24 months until achievement tolerance. CM-sIgE was measured at diagnosis, 6 and 12 months after the diagnosis of CMA. Development of tolerance was significantly earlier in CM-sIgE negative patients

compared to those who were positive (****p <0.0001). Additionally, IgE positive CMA children developed other allergic diseases more frequently including egg allergy, allergic rhinoconjunctivitis, birch pollen allergy, & allergy to animal dander compared to IgE negative CMA & non-CMA healthy controls. Shek et al.[5] reported a negative correlation between a decreased CM-sIgE level & the development of tolerance in a retrospective study. Sixteen of the 49 CMA patients enjoyed natural tolerance to CMA when they grew older. A trend of decreased sIgE was observed in tolerant subjects compared to persistent CMA patients (median, 4.63 vs. 29.1kU/L in tolerant vs. persistent CMA, *p=0.06*). Early development of tolerance evidenced by a decrease in sIgE was more pronounced in younger (< 4 years) than older (> 4years) age groups. By using a logistic model they were able to show a direct correlation between the probability of developing tolerance and a decrease in CMP-sIgE level. The probability of developing tolerance increased along side with the percent decrease of CM-sIgE over a period of 12 months. A probability to achieving tolerance of 0.94 was recorded when CM-sIgE fell 99% from baseline over 12 months[5]. Morisset et al.[46] also reported a significant decrease (*p<0.05) in sIgE level in successful OIT participants (n= 27, age range, 1.1–6.5 years) when they could tolerate 200ml of CM compared to those who avoided CM (n= 30)[46]. Patriarca et al.[47] also suggested that a successful OIT was associated with a decrease in sIgE to CMP. Nineteen out of 24 (79%) OIT recipients completed the oral desensitization protocol in 3-12 months. Successfully desensitized subjects were able to drink 120 ml of milk on a regular basis. sIgE level to CM was measured at 6, 12 and 18 months after the commencement of OIT. CM-sIgE decreased significantly from baseline during the follow-up (**p< 0.01) in successful OIT recipients. Staden et al.[48] also

observed a significant decrease (*p<0.05) from baseline CMP-sIgE to the end of the protocol in successful OIT recipients. The decrease of sIgE was more pronounced in OIT tolerant group compared to those who outgrown CMA naturally (***p<0.001 vs. *p<0.05 in OIT tolerant vs. naturally tolerant). In addition, ~~the~~ baseline CM-sIgE was higher in failed OIT recipients compared to successful participants. Zapatero et al.[49] reported a decrease in CMP- sIgE baseline in desensitized patients at the end and at 6 month post-OIT. Though all CMP- sIgE decreased with OIT, significance was only recorded in case of casein (*p=0.012 at the end of OIT, *p=0.019 at 6 month post-OIT). In their short duration OIT protocol (range, 11-17 weeks), 16 of 18 patients (88%) could tolerate 200-250 ml of CM on a regular basis.

In contrast to the studies mentioned above, Skripak et al.[34] did not observe a decrease in CM-sIgE level in successful OIT recipients. They recruited 20 patients aged from 6-21 years by DBPCFC. They were divided into OIT recipient and placebo group at the ratio of 2:1. At baseline, median milk threshold dose of 40mg was recorded in both recipient and placebo groups. With the progression of OIT, the treatment group showed more consumption of CMP compared to the placebo group. OIT recipients could tolerate a median cumulative dose of 5140mg of CMP (range, 2540-8140mg) compared to the placebo group who reacted at 40mg. There were more adverse reactions recorded in active treatment group (45.4%) compared to the placebo group (11.2%). There was no significant difference in CM-sIgE level pre- and 3-6 month post-OIT in successful OIT recipients. The placebo group also did not show any significant change in sIgE level.

Another OIT but shorter in length by Pajno et al.[35] aimed to introduce a shorter, patient-friendly and robust protocol for cow's milk OIT. Their weekly up-dosing regimen

took only 18 weeks to complete. The patient population was at least 4 years of age or older (n=30, range, 4-10 years). Children diagnosed as CMA based on certain criteria were randomly selected to participate either as active OIT recipients or controls. Active OIT group was treated with whole CM, whereas the control group was given soymilk formula. The dose escalation continued till the maximal tolerated dose, preferably 200ml. Dose escalation was halted at any undesirable reaction. Serum samples were collected before randomization, at one intermediate time point of 13 weeks (8ml), and at the end of 18 weeks (200ml). Specific IgE to CM, Casein, BLG, & ALA was measured. There was no significant difference in CM-sIgE at 8ml or 200ml dose compared to baseline in both the active OIT and control groups. Three children who showed the highest clinical reactivity to CM during desensitization, revealed 85% higher sIgE baseline (mean, 34.8 kU/L vs. 66.6 kU/L). Ten (77%) out of 15 active OIT participants achieved full tolerance to 200ml of milk, 1 achieved partial tolerance (64ml), and the remainder (15%) could not complete due to adverse reactions[35]. Meglio et al.[50] also could not find a significant drop in CM, casein, and BLG-specific sIgE level from the beginning to the end of OIT. Fifteen of the 21 (71.4%) children could tolerate 200ml of milk at the end of OIT. Moreover, 8 of 15 OIT recipients did not show any adverse reaction during the escalation phase of OIT aimed to reach at least 200ml of milk. This study suggests that successful OIT recipients with mild or no symptoms may end up without showing any change in sIgE level during the course of OIT. Kaneko et al.[51] cohort also did not find any significant difference in baseline CMP-sIgE in between successful and discontinued OIT participants. It was a slow dose-up OIT, where doses were increased bi-weekly until a maximum dose of 100ml of CM was reached. Eight of 10 (80%) children (age, 4-14

years) tolerated the highest dose of CM successfully, whereas 2 children left the study due to repeated adverse reactions at low CM doses (5-20 ml).

Among the other modes of immunotherapy including SLIT & ECIT, CMP-sIgE did not change significantly baseline overtime[33, 37].

In summary, successful oral tolerance induction to CMA was accompanied by a significantly decreased CMP-sIgE from baseline to the end of OIT. CMP-sIgE also decreased from baseline in cases where natural tolerance to CMA took place. Additionally, in failed OIT recipients, the baseline CMP-sIgE was higher compared to the successful OIT recipients. These findings suggest the role of CMP-sIgE as a good prognostic marker in CMA. At the same time, findings from some other studies doubted sIgE as a good prognostic marker in CMA. In those studies, successful oral tolerance induction to CMA was not associated with a decrease in CMP-sIgE from baseline. Again, in one study[45] CMA patients with a high baseline CMP-sIgE level successfully completed the OIT protocol, whereas with the same sIgE level some failed to respond to therapy. These self-opposing findings of CMP-sIgE in oral tolerance induction prevent it from becoming a universal prognostic marker in CMA.

Cow's milk allergy and specific IgE in animal models

CMA has also been studied in mouse models. Li et. al[52] described CMA in a C3H/HeJ mouse model. The mice were sensitized with intragastric administration of cow's milk along with Cholera toxin (CT) as an adjuvant. Booster doses were applied at weekly intervals for 5 weeks. Significant increases (**$p < 0.01$) in CM-specific IgE were noted by 3 weeks following the first dose. The sIgE peaked at the 6th week post sensitization. A re-challenge after 6 weeks with intragastric CM initiated severe systemic anaphylactic reactions within 15-30 minutes in CM-sensitized mice. Increased vascular permeability evidenced by extravasation of Evan's blue dye in CM- sensitized mice footpad, increased plasma level of histamine, increased mast cell degranulation in mouse ear tissue were observed supporting systemic anaphylaxis due to CMA in CM-sensitized mice. Additionally, histology of the intestine of CM-sensitized mice showed marked vascular congestion, edema of the lamina propria, sloughing of enterocytes from the tip of intestinal villi. In the lungs of CM-sensitized mice, marked accumulation of inflammatory cells, mucus cells, and accumulation of mucus were noted. Such findings were not found in control non-CM-sensitized (CT-sham) mice. Culture of splenocytes from CM-sensitized mice with CMP revealed a significant up regulation of Th2 cytokine IL-4, and IL- 5 after 72- hours when compared to unstimulated cells. On the other hand, the level of Th1 cytokine IFN-γ did not change significantly in between CMP-stimulated and unstimulated cells. This study suggests an association of the development of CMA with CMP-specific IgE. At both 3- and 6-weeks post sensitization, sIgE increased baseline in parallel with the worsening of systemic anaphylactic reactions in CM-sensitized mice. Morafo et. al[53] compared C3H/HeJ and Balb/c mice in a model of

CMA. The mice were sensitized weekly for 5 weeks by intragastric administration of CM plus CT. They were challenged after 6 weeks following initial sensitization. Systemic anaphylactic reactions were seen in 87% of C3H/HeJ but no Balb/c mice reacted. Plasma histamine levels, IL-4 and CM-sIgE levels were increased in C3H/HeJ mice but not in Balb/c. These studies suggest an association of CM-sIgE in the pathogenesis of CM-sensitivity in mouse models. Studies by Adel et al.[54] using Balb/c mice confirmed their lack of response.

From the studies stated above, development of CM sensitivity in mouse models was associated with a concomitant presence of CMP-sIgE. Failure to induce CM sensitivity was accompanied by absent CMP-sIgE responses. CMP-sIgE was closely related to the development of CM sensitivity in mouse models.

Role of cow's milk protein specific IgG4 in CMA

IgG4 is the least abundant subtype of immunoglobulin G. Recent studies have suggested its role as an anti-inflammatory blocking antibody, which may inhibit the action of IgE. Increases in IgG4 have been documented in immunotherapy studies including OIT to CMA[55-57]. Studies suggesting CMP-sIgG4 as a marker of CMA has also been reported[58]. These self-contradictory reports on CMP-sIgG4 demand further investigations to unveil the definitive role of CMP-sIgG4 in CMA.

Savilahti et. al.[11] studied specific IgG4 profiles in subjects (a) who had outgrown CMA by the age of 3 years, (b) by 3-8 years and (c) who remained allergic until 8 years of age. Lower β−lactoglobulin specific IgG4 was detected in group (c) compared to group (a), (b) & non-CMA control subjects. In case of casein specific IgG4,

no difference was noted in between groups. Specific IgG4 to BLG increased significantly in group (a) who were milk-tolerant by 3 years of age over group (c) who had persistent CMA at 8 years of age. Another study[18] by the same group suggested that BLG-sIgG4 might be helpful in differentiating CMA associated eczema from eczema with a suspected association to cow's milk. They found that infants with well-documented CMA have lower BLG-sIgG4 levels compared to milk food challenge negative infants. Skripak et al.[34] described a significant increase in CM-sIgG4 in their active treatment group (n=14, median age 9 years). Specific IgG4 was increased by a median of 767% (range, 29-1321%) from baseline in active OIT recipients (*p* value=0.002), whereas no change in sIgG4 level was observed in the placebo group (n=7, median age 11 years). There was a positive correlation between sIgG4 and the tolerated milk doses, suggesting sIgG4 as an useful prognostic factor in CMA. Pajno et al.[35] also reported significant increases in sIgG4 from baseline to the end of OIT in active participants (p=0.003) compared to placebo group. Another cohort by the same group[59] reported significant increases in CM-sIgG4 from the beginning (****p<0.0001) to the end (****p<0.0001) of OIT in CMA outgrown patients. Another OIT to CMA by Lee et al.[60] reported significantly increased CMP-sIgG4 levels in successfully desensitized CMA infants compared to failed OIT recipients. Successful recipients could consume 200ml of cow's milk on a daily basis. The level of sIgG4 was lower in infants with persistent CMA. Bedoret et al.[36] conducted a rapid CM OIT, with consumption of 2000 mg of CMP/day in only 7- to 11- weeks. They described a 15-fold increase in sIgG4 from baseline to the end of OIT in 10 out of 11 patients. At the end of 24 weeks, successful recipients were able to tolerate at least 8000 mg of CMP per day. Kim et al.[61] reported that dietary baked-milk

accelerated the resolution of CMA in children. Baked-milk tolerant subjects (n=88, median age: 6.6 years) were challenged to progressively less heated forms of milk during their visits. Initially, 65 of 88 children (74%) successfully passed the baked-milk (e.g. muffin) challenge. Out of 65 children, 39 (60%) enjoyed tolerance to CM in next five years. Median casein sIgG4 level increased significantly baseline during the final visit in these patients (0.6 to 1.3mg_A/L, ***p<0.001). BLG-sIgG4 did not change significantly from baseline to final visit in baked-milk tolerant group. In addition to increase in casein-sIgG4; casein IgE/IgG4 and BLG IgE/IgG4 decreased significantly baseline at final visit (p=0.001 and ***p<0.001, respectively). Noh et al.[6] studied CM-sIgG4 in cow's milk specific atopic dermatitis. DBPCFC was performed to confirm the diagnosis of CMA (n=60, mean age: 13.9±8.8 years). CM-sIgG4 was found higher in non-CMA patients compared to those who were confirmed as CMA (66.1 ± 13.3 vs. 40.2 ± 35.3 Immunoglobulin binding unit or IBU). Ruiter et al.[17] reported that, CM-sIgG4 increased significantly baseline (*p<0.05) in 4 of 6 CMA children who enjoyed natural tolerance by the age of four years compared to those who remained persistently CMA even after 6 years of age. Duchen et al.[62] reported significantly lower (*p<0.05) BLG-sIgG4 in CMA subjects at 4 years of age compared to their age matched healthy controls, suggesting the importance of sIgG4 in immune tolerance. Ito et al.[7] studied CMP-sIgG4 level in CMA and non-CMA healthy controls. They found that, specific IgG4 was increased to all three major cow's milk proteins in healthy non-CMA individuals compared to CMA patients, suggesting its role as a good prognostic marker in CMA.

The above mentioned studies help to consider CMP-sIgG4 as a good prognostic marker in CMA, whereas Hochwallner et al.[58] reported that CMA was associated with

a high level of CMP-sIgG4. They measured sIgG4 to 6 major fractions of CM (αs1-casein, αs2-casein, β-casein, κ-casein, β-lactoglobulin or BLG, α-lactalbumin or ALA) in 25 CMA patients (4months-70years). Specific IgG4 to all CMP's but BLG was increased in all CMA patients compared to those who have outgrown CMA. Høst et al.[63] reported that, persistent CMA in infants is associated with significantly high levels (*p<0.05)of BLG-sIgG4 compared to non-CMA healthy infants, suggesting sIgG4 as a marker of allergy in CMA.

In summary, it is still uncertain if CMP-sIgG4 is a good prognostic marker in CMA. Studies indicating both a pro- and anti-inflammatory role of CMP-sIgG4 were observed. Additionally, there are a lot of factors which vary from patient to patient including, age, sex, BMI, immune status leaving the definitive role of CMP-sIgG4 yet to be determined.

Cow's milk peptide epitope mapping shows broader diversity and increased affinity of IgG4 and **IgE binding respectively in successful and failed OIT recipients**

Immunoglobulin binds to specific epitope-binding sites of the CM peptides. Mapping of the epitope-binding sites was important to further dissect the role of sIgE and sIgG4 in CMA. Each of the CMP's has specific epitope binding sites. Previously with SPOTS membrane-based immunoassays (Genosys Biotechnologies, Woodlands, Texas, USA) and now Peptide microarray-based immunoassay is being used to map CM epitopes[64, 65]. αs1- casein, αs2- casein, β-casein, κ-casein, BLG consists of 199, 207, 209, 169, 162 AAs respectively[65]. Different groups have identified AA sequence 28-50, 17-36, 39-48, 173-194 as an IgE epitope-binding site for αs1- casein. IgE epitope

binding sites showed variability from children to adults. For αs2-, β-, and κ-caseins IgE epitope binding sites were respectively AAs 182-189, 181-189, 34-53[65-69]. Among all the fractions of CMP, αs1- casein was reported to be the most allergenic in CMA[70].

Wang et al.[64] studied the relationship between IgE epitope binding and the outcome of OIT to CMA. As a sample population, they chose three distinct groups: allergic to all forms of milk, tolerant to heated milk (HM), those who have outgrown CMA. IgE epitope binding was fewest in the tolerant group compared to the allergic group (median; outgrown vs. allergic: 3.5 vs. 17, p=0.062). The HM- tolerant group showed significant differences in terms of epitope binding (median, HM-tolerant vs. allergic: 3 vs. 17, *p=0.019). In other words, IgE epitope binding patterns were quite similar in both subjects who had outgrown CMA and patients in the HM-tolerant group. On the other hand, IgG4 epitope binding patterns in the HM- tolerant group showed more similarity to the allergic group. Further, they tried to correlate epitope-binding patterns with severity of clinical reactivity during challenge defined as "Anaphylaxis grade". Anaphylaxis grade 4 to 5 was associated with a median IgE epitope binding of 89.5, whereas grades 1 to 2 correlated with median IgE epitope binding of 4.5 (p=0.02). HM-tolerant subjects mimicked the same pattern as observed in lower grades. On the contrary, IgG4 epitope binding did not reveal any correlation with clinical reactivity during challenge. Combining the HM tolerant and the outgrown group against the allergic group, they tried to figure out areas of significance in the peptides termed as "informative epitopes". Epitope binding differences exceeding 30% were considered significant. After analysis, they have found 8 areas of informative epitopes located mostly in αs1-casein. This finding is consistent with the findings by previously used SPOTS membrane bound

immunoassays [12, 67, 68]. Again, a competition assay showed higher IgE binding affinity to CM peptides in discontinued OIT group compared to successful OIT recipients[64]. Savilahti et al.[13] study consisted of 32 children aged between 6-17 years. Twenty-six out of 32 CMA patients completed the OIT protocol. The aim of the study was to investigate the diversity and affinity of IgE & IgG4 epitope binding in the settings of OIT. Samples were collected at baseline and at the end of OIT. CM sIgE levels were decreased in both successful OIT (mean, 11 kU/L to 8 kU/L) and those who discontinued therapy (mean, 85kU/L to 57kU/L). Specific IgE levels were higher in the discontinued group both at the beginning and at the end of the trial. On the other hand, sIgG4 increased both in successful (mean, 0.2-2.9 AU/ml) & discontinued (mean, 1.9 to 6.5AU/ml) group. This increase in sIgG4 in discontinued group might be associated with fewer side effects compared to those who never show an elevation of sIgG4 level baseline. At both the initiation & termination the discontinued group showed a broader diversity and higher intensity of IgE binding to peptides compared to the successful group. Nonetheless, IgE binding decreased over time in both groups. The intensity of the IgE binding to peptides was stronger than IgG4 in the discontinued group compared to the successful group. Decreased IgE binding was noted most evidently in αs1-casein in the successful OIT group. There was also more overlap in between IgE & IgG4 epitope binding sites in successful OIT patients than discontinued ones. Another small cohort led by the same group observed change in CM-sIgE and IgG4 profile based on epitope binding properties in subjects with persistent CMA (n=11) and those who recovered CMA by the age of three years. Serum samples were taken at the time of diagnosis, 1 year after diagnosis, and at follow up (mean age, 8.6 years). CMA outgrown group

showed less IgE epitope binding overtime, whereas in persistent CMA group peptide recognition by IgE either increased baseline or remained same over time. In case of IgG4 epitope binding, it increased in the CMA outgrown group overtime. No significant increase was observed in the persistent CMA group[14]. Chatchatee et al.[67] segregated persistent CMA patients (n= 9, median age, 12 years) from those who were likely to outgrow CMA by 3 years (n= 8, median age, 2 years) of age on the basis of distinguished epitope binding sites in αs1-casein (AA 69-78, 173-194). Persistent CMA had a higher sIgE to CM at baseline compared to likely to be outgrown group (<30kU/L). Those who had persistent CMA recognized the above-mentioned epitope binding sites. The intensity of IgE binding to epitopes was also increased in persistent CMA patients. These findings were not seen in those who were likely to outgrow CMA[67]. Jarvinen et al.[68] reported persistent CMA subjects (n=11, age, 4-18 years, CM-sIgE>100kU/L) recognized less IgE binding epitopes compared to those who were likely to outgrow CMA (n=8, age <3 years, CM-sIgE <30kU/L). Persistent group recognized 11 IgE epitope-binding sites on BLG and ALA compared to likely to outgrow CMA group who could recognize only 3. This suggests, less epitope recognition by IgE antibodies is associated with more chance to develop natural tolerance to CMA. Also, CM OIT may accelerate the induction of tolerance to CM in these patients. In a sentence, CMP-sIgE epitope binding decreased and CMP-SIgG4 epitope binding increased significantly in successful CM OIT recipients compared to those who remained persistently allergic to CM.

A short introduction to Immunoglobulin A

Immunoglobulin A (IgA) is a major serum immunoglobulin and the predominant antibody in mucosal secretions. In humans, there are two subclasses of IgA, termed IgA1 and IgA2. Like all immunoglobulins, the monomeric structural unit of IgA comprises of two identical heavy chains and two identical light chains arranged into two Fab regions and an Fc region, separated by a flexible hinge region. The major difference between the two subclasses lies in the hinge region, which is greatly extended in IgA1[71].

The shorter hinge region in IgA2 renders this subclass of IgA more resistant to bacterial proteases than IgA1, providing a distinct advantage in the mucosal environment[72].

Serum IgA is produced by plasma cells in the bone marrow, lymph nodes and spleen and is predominantly (90%) monomeric IgA class 1[73].

Secretory IgA (S-IgA) is the product of local synthesis at mucosal surfaces in respiratory, gastro-intestinal and genito-urinal tract as well as in colostrum, saliva and tears. Secretory IgA is mainly polymeric with similar levels of IgA subclasses 1 and 2[71, 74, 75].

Polymeric IgA antbodies are monomeric IgA antibodies joined together at the Fc region by a polypeptide called the J chain. This dimeric IgA binds the polymeric immunoglobulin receptor (pIgR) present on the basolateral membrane of epithelial cells and is transported across the epithelium and onto mucosal surfaces. The pIgR is cleaved to release pIgA bound by a glycoprotein called the secretory component (SC) from the pIgR[75].

S-IgA and serum IgA are therefore molecules with different biochemical and immunochemical properties produced by cells with different organ distributions.

Different methods of immunization can induce serum or secretory IgA responses or a combination of both[72].

The main role of secretory IgA has been well documented. S-IgA inhibits adherence of pathogenic microorganisms to the mucosal wall. It is a hydrophylic, negatively charged molecule because of the predominance of hydrophylic amino acids in the Fc region of IgA, and abundant glycosylation of both IgA and SC[71]. As such, IgA can surround microorganisms with a "hydrophilic shell" that is repelled by mucosal surfaces. Additionally SIgA interacts with bacterial products such as enzymes and toxins, and neutralizes their action and it can neutralize viruses intracellularly[72, 74]. These qualities make IgA antibodies perfect for guarding mucosal surfaces. Because no inflammation is triggered via these mechanisms, IgA is considered as a non-inflammatory antibody. This is further accentuated by the fact that IgA is a poor activator of complement. On the contrary, the role of serum IgA, is relatively unknown and contradictory reports have been published to that matter. Induction of IgA mediated cellular effector functions requires interaction with specific Fc receptors (FcαR) on the cell surface. The most important receptor, FcαRI (CD89) is expressed on myeloid cells including monoytes, neutrophils and macrophages and can be up-regulated by certain cytokines. Activation of these cells via FcαRI is a key factor in immunolgical defense because it can mediate cytokine release, respiratory burst and phagocytosis. The receptor binds both secretory and serum IgA, as well as the different subclasses[74]. Previous studies have shown that serum IgA can mediate either pro- or anti-inflammatory effects in innate immune cells[76-80] This dual capacity might play a role in maintaining a balance between pro-inflammatory and anti-inflammatory activities.

Food proteins and microbial flora are abundant in the intestinal tract. Locally produced IgA interacts with these antigens and the resulting immune complexes are either taken up by phagocytes or transcytosed back to the lumen via pIgR. This process is called immune exclusion and is an effective way to clear the mucosal surfaces of immune complexes.

IgA deficient individuals provide further support for the immune exclusion role of IgA. The majority of those patients do not present with severe symptoms but are predisposed to allergies and auto-immune disease which might be due to a diminished epithelial barrier function, leading to inappropriate responses against dietary components and indigenous bacterial flora[81].

Role of cow's milk protein specific IgA in CMA

The role of CMP-sIgA in CMA is not yet clearly understood. Many studies suggest it as a good prognostic marker in oral tolerance induction[16, 82, 83], whereas others deny it, rendering its role in CMA yet to be determined.

Secretory IgA plays a key role in oral tolerance. High intestinal IgA in infancy was associated with a reduced risk of IgE mediated allergies[16] and low levels of sIgA in colostrum appear to increase the risk for cow's milk allergy[82, 83]. Selective IgA deficiency has been reported to be associated with increased risks of allergies and infections[84]. IgA antibodies in colostrum and human milk may prevent antigen entry at the intestinal surface of breastfed infants. Decreased secretory IgA levels in the gut have been found associated with food allergy in mouse models. Additionally, higher antigen-

specific secretory IgA in feces was associated with the development of tolerance to BLG in a mouse model[85].

There are not too many cohorts that studied the role of CM-sIgA in CM OIT. Mostly, CM OIT worked with CMP-sIgE and sIgG4 as previously mentioned in this literature review. Different studies mentioning CM-sIgA in CMA or CM OIT are briefly described below:

Savilahti et al.[14] reported CMP-sIgA increased baseline at follow up in persistent CMA patients. Persistent CMA patients also showed high signal intensity IgA binding compared to those who recovered early. This suggests the role of sIgA as a pro-inflammatory antibody associated with a bad prognosis in CMA. Savilahti et al.[18] also reported in another cohort that, CM-sIgA are not significantly different between eczematous infants with or without confirmed CMA suggesting CM-sIgA is not a solid marker in CMA. Ruiter et al.[17] did not find any significant difference in CM-sIgA levels in between CMA, atopic but non-CMA, and non-atopic individuals. Sletten et al.[86] observed no significant difference in sIgA level in between reactive CMA, tolerant CMA, and non-CMA healthy individuals. Though they reported a significant increase in CMP-specific IgA to IgE ratio in tolerized CMA individuals compared to reactive CMA patients (*p<0.05), it was mostly due to a decrease in sIgE level in the CM tolerized group. Böttcher et al.[87] observed that infants who developed allergies had higher salivary levels of total and allergen specific IgA whereas high secretory IgA levels in saliva seemed to protect sensitized infants from developing allergic symptoms during the first 2 years of life.

There are some other studies as well who mentions CM-sIgA as a good prognostic marker in CMA. Jarvinen et al.[88] studied if there is any relationship between maternal CM avoidance diet and the development of CMA in their infants. Casein and BLG- specific IgA level was significantly lower in CM restricted mothers compared to those with no CM restriction (p=0.019 & p=0.047 respectively). Casein and BLG-specific IgA was more frequently detectable in infants from no CM elimination diet mother compared to mothers with CM restriction. CMP-specific IgA was detected less frequently in infants with CMA compared to those who were non-CMA. Savilahti et al.[11] also suggested a high baseline serum BLG-sIgA in children may predict the early development of clinical tolerance to CMA. Children who achieved tolerance to CM by the age of 3 years showed higher baseline BLG-sIgA level compared to those who became tolerant at a later age or remained persistently CMA beyond 8 years of age. CM-specific salivary sIgA showed a positive correlation with the serum BLG-sIgA at diagnosis (*p<0.05) & 1 year (*p<0.03) in subjects who became tolerant to CMA by the age of 3 and 8. Such correlation was not observed in subjects with persistent CMA beyond 8 years of age.

In summary, reports are available suggesting CM-sIgA both as a pro- and anti-inflammatory marker in CMA. Based on the above discussion, it is evident that, intensive research is needed to elucidate the role of specific IgA in CM OIT.

MATERIALS & METHODS

First Canadian double-blinded placebo-controlled OIT to CMA

Treatment of CMA by OIT has been carried out in the USA & in many European countries. Arguably, it lacks studies that are rigorous, blinded, and with adequate controls. Data was also lacking to conclude the tolerance as temporary or permanent. Moreover, there is no such Canadian trial, depriving our children from a potential treatment strategy to outgrow CMA. The goal of this novel technique is to offer an ideal, realistic, and safe treatment option to our CMA population based on certain clinical and immunological parameters. By analysing different serological and cellular markers, it might also be possible to predict the outcome of OIT in CMA patients at the very beginning of the therapy. Thus, it may help determining the ideal candidates to CM OIT.

Research methods

Twenty boys and girls, aged between 6 to 19 years, diagnosed as CMA were recruited in the study. The patients described in this thesis were recruited from the allergy clinic at the Montreal Children's Hospital. This is a multicentre trial and patient recruitment from other Canadian provinces and territories (e.g. British Columbia, Chicoutimi) has started. Inclusion criteria included: (a) IgE mediated 2 mild symptoms (pruritus, urticarial, flushing, or rhinoconjunctivits) and/or 1 moderate (angioedema of face and lips, throat tightness, vomiting, diarrhoea, crampy abdominal pain, cough, nasal blockage), and/or 1 severe symptom (bronchospasm, wheezing, hypoxia, cyanosis, low blood pressure, shock or circulatory collapse), (b) Skin prick test (SPT) wheal diameter to milk >3 mm than that of normal saline and/or CM-sIgE> 0.35kU/L.

Before initiating desensitization procedure, a single-blinded challenge with fresh cow's milk was carried out in all OIT recipients. The dose started from 0.1ml of fresh CM to up to a total of 150ml of CM (doses of 0.1,0.3,1.0,3.0,10.0,30.0,45.0,60ml. Subjects remained under observation up to 2 hours after the last dose to monitor any objective symptom of CMA. Patients with positive symptoms of CMA were recruited in the study. The negative challenge subjects were excluded from the study. Subsequently, they were divided into OIT recipient (n=10, who will receive the OIT as the first group) and observation control (n=10, will receive OIT after the first group ends and will be in regular follow up before OIT) groups.

A Paediatrician clinically examined all the patients at every OIT visit before initiation of the therapy. All necessary medications (e.g. I/M Epinephrine, anti-histamine) and emergency equipment's were ready to support the patient at any time if needed. The continuous presence of a paediatrician and a trained nurse were ensured throughout the procedure. The desensitization procedure was adapted from Martorell et al.[44], as it was reported to be associated with a higher resolution of CMA and low incidence of adverse reactions.

Timepoints for sample collection

Blood and saliva samples were collected at the time of challenge, 6ml, 25ml, 125ml, and 200ml. The 200ml milk dose is marked as the completion of OIT. The gap between each dose was roughly 4-6 weeks depending on the patient's response to the dose escalation. When the final dose of 200ml was reached, a re-challenge with 300ml of

milk was performed after 1 month. If the patient successfully passes the challenge, he or she will be followed up at 1, 3, 6, 12 months post-300ml dose **(Figure 2)**.

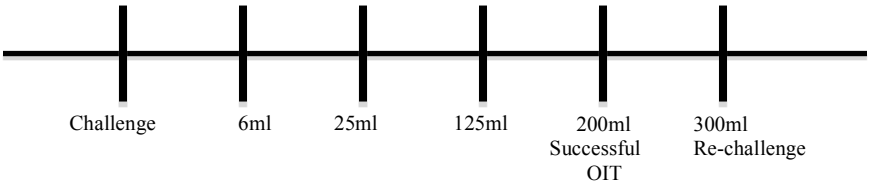

Figure 2: Schematic showing the escalation phase of CM OIT. Samples were collected at the time of challenge, 6ml, 25ml, 125ml, & 200ml. The 200ml of milk dose indicates a successful OIT. After 1-month post oral immunotherapy (POIT), a second challenge test with 300ml of milk was performed to confirm the state of desensitization. Subsequently, samples were collected at 1, 3, 6, 12 months post-300ml dose (not shown in the schema).

Collection of blood & saliva

Blood samples were collected in both heparinized and non-heparinized tubes to isolate plasma and serum respectively. Peripheral blood mononuclear cells (PBMC's) were also isolated from the green heparinized tube.

Saliva was collected according to the protocol described by Hensen et. al[89].

Serum/plasma separation & isolation of Peripheral Blood Mononuclear Cells (PBMC's)

Isolation of serum/plasma from whole blood

Whole blood was taken into a 50ml tube. Centrifugation was done at 3000rpm for 10 minutes (Acceleration 9, Deceleration 9, 3000rpm, temperature 24° Celsius). Plasma was isolated and preserved at -80°C. The procedure was followed to isolate serum from the clotted blood.

Ficoll paque density gradient centrifugation of heparinized blood

The 50ml tube was filled up till 15ml with Ficoll. The whole blood was poured very slowly on top of the surface of the Ficoll, so that it doesn't get mix with the Ficoll. Rest of the tube was filled up till 50 ml with whole blood (if the quantity of blood is less than 35ml, 1X sterile PBS was added to reach that 35ml volume). Strict precaution was taken to keep the Ficoll-blood column undisturbed. Acceleration was set at 5, deceleration at 1, at 400g for 35 minutes at 24°Celsius. Ficoll-blood containing tube was counterbalanced with the same weight.

The tube was centrifuged for 45 minutes while the break was off. After 45 minutes when the machine stops, the tube was carefully taken out so that the thin layer of PBMC or "Buffy coat" does not get disrupted. The middle layer or interphase of the fluid column was aspirated carefully with Pasteur pipette. The 50ml tube

containing the interphase was filled up with 1X PBS till 50ml. Centrifugation was done at Acceleration 9 Deceleration 9, 1500rpm for 5 minutes at 24°Celsius. Tube was taken out the centrifuge machine after 5 minutes. The supernatant was discarded without disturbing the cell pellets at the bottom of the tube. 5ml of 1X sterile PBS was added to the tube to prepare for cell counting. 50ul of the cell suspension was taken for cell counting. The 50ml tube was filled up again with 1X sterile PBS for final wash before freezing. After wash again the supernatant was discarded and freezing medium was added depending on the cell count. 1ml of freezing medium was used for maximum 5 million of cells. The vials were shifted right away into a Styrofoam box or Mr. Frosty to store them temporarily at -80 degree Celsius. The tubes were shifted in liquid nitrogen tank the following day for future experiments.

Cell counting

Cell counting was done either with automated Beckman-Coulter cell counter or manually with improved Neubaeur counting chamber.

Measurement of total IgE in serum by ELISA

Total IgE was measured using Enzyme linked immunosorbent assay (ELISA). 50ul/well of anti-IgE capture antibody (Bethyl, Montgomery, TX, USA) 1 in 1000 dilution was used for coating on Costar™ Corning 96- well half area micro titre plate (Thermo Fisher Scientific Inc., Waltham, MA, USA). The plate was sealed (ProGene, St. Laurent, QC, Canada) & incubated overnight at 4°C. The following day capture antibody was discarded and the plate was washed three times with washing buffer (1X PBS + 0.05% Tween 20; Sigma-Aldrich, St. Louis, MO, USA). 100ul of blocking buffer was

added on every well and the plate was incubated at room temperature for 30 minutes. After the incubation, 50ul/well of standards (50ng/ml to 0.78ng/ml) and samples were added into designated wells. The plate was sealed and incubated for 1 hour at room temperature with shaker on. The plate was washed 5 times with washing buffer. After that, 50ul/well of biotinylated anti- IgE antibody (Bethyl, Montgomery, TX, USA) diluted in blocking buffer was added onto each well (1 in 20,000 dilution). Again, the plate was sealed and incubated for 1 hour at room temperature. After incubation, the plate was washed again with washing buffer for 3 times. Then, 50ul of Horseradish peroxidase streptavidin or SAV-HRP (BioLegend, San Diego, CA, USA) was added to each well and incubated for one hour at room temperature. The plate was washed again for 5 times with washing buffer and soaked with extra care, as any unbound SAV-HRP must be cleared off. After the final wash, 50ul of TMB (BioLegend, San Diego, CA, USA) was added to each well. 1N Phosphoric acid (H_3PO_4) was added as stop solution immediately after the appearance of the darkest standard color. Optical density (OD) was measured at 450nm using an ELISA plate reader (Tecan group Ltd., Mannedorf, Switzerland).

Measurement of CMP-specific IgE & IgA in serum

We collected both sera and plasma from our patients. Samples were collected at the baseline or pre-challenge and in every timepoint of the immunotherapy schedule. Samples were preserved immediately at -80°C until analyzed. Casein, BLG and ALA (Sigma Aldrich, Germany) specific IgE & IgA were measured using Enzyme-Linked Immunosorbent Assay (ELISA). Optimization experiments were done to figure out the best coating concentration of the milk allergens. All three allergens were coated in duplicates at a concentration of 20ug/ml (1ug/well) in double bicarbonate buffer in Costar

Corning 96-well half area microtitre plate (Thermo Fisher Scientific Inc., Waltham, MA USA). The plate was incubated at 4°C overnight. Additionally, to obtain the standard curve, 50ul/well of anti-IgE and anti- IgA capture antibody (Bethyl, Montgomery, TX, USA) was added 1 in 1000 and 1 in 100 dilution respectively. The next morning, capture antibody was discarded and the plate was washed three times with washing buffer (1X PBS + 0.05% Tween 20; Sigma-Aldrich, St. Louis, MO, USA). 100ul of blocking buffer was added to every well and was incubated at room temperature for 30 minutes. The standard concentration was prepared for IgE was from 50ng/mL to 0.78ng/mL (Phadia, Uppsala, Sweden). For IgA standard (500 to 7.8ng/mL) was prepared from human reference serum (Bethyl, Montgomery, TX, USA). Standards and serum samples (1:5-1:10) were added in duplicates in their assigned wells. Subsequently, plates were incubated at 37°C for 1 hour in the incubator. After the incubation, plate was washed to remove any unbound antibody. After that, 50ul/well of biotinylated anti-IgE (Bethyl, Montgomery, TX, USA) and HRP-conjugated anti-IgA (abcam, Cambridge, UK) antibody was added into each well at 1 in 20,000 and 1 in 10000 dilution respectively. Again, the plate was sealed and incubated for 1 hour at 37°C. For IgA ELISA, 50ul/well of TMB (BioLegend, San Diego, CA, USA) was added after five times of washing. The reaction was stopped using 1N Phosphoric acid (H_3PO_4) and OD was recorded by a ELISA plate reader. In case of IgE ELISA, there was one more step compared to IgA ELISA. 50ul of Horseradish peroxidase streptavidin (BioLegend, San Diego, CA, USA) was added into each well and incubated for one hour at 37°C. Following the final incubation, the plate was washed for five times. TMB and stop solution was added as

previously mentioned. Optical Density (OD) was measured as soon as the darkest standard color appeared.

Measurement of CMP-specific IgA in saliva by ELISA

Detection of salivary CMP-sIgA was performed following the same protocol as serum.

Measurement of CMP-specific IgG4 in serum by ELISA

Specific IgG4 was measured against casein, BLG, ALA in serum samples of patients. It was not possible to measure sIgG4 quantitatively as the capture antibody and detection antibody was not a match. We tried two different manufacturers but it turned out that the capture and detection antibody cannot be paired. As a result, it yielded either excessive or dim signals, which was not appreciable. In this situation, we measured sIgG4 in "Arbitrary Units". Costar™ Corning 96-well half area micro titre plate (Thermo Fisher Scientific Inc., Waltham, MA USA) was coated with 20ug/ml of CMP. The plate was sealed (ProGene, St. Laurent, QC, Canada) and incubated overnight at 4°C. The following day capture antibody was discarded and the plate was washed three times with washing buffer (1X PBS + 0.05% Tween 20; Sigma-Aldrich, St. Louis, MO, USA). 100ul of blocking buffer was added in every well and was incubated at room temperature for 30 minutes. Serum pool was used to generate the standard concentrations. Previous study has shown an increase in sIgG4 to casein 3 months post-exposure to heated-milk[90]. We made a serum pool containing serum of the OIT recipients at the 15th week of immunotherapy, which corresponds to the 125 ml milk dose. The serum pool was diluted 1 in 20 times. This concentration was considered as the highest concentration and assigned a value of 100 AU/ml. Subsequently; further halving dilutions were performed

for the remaining wells. After the incubation, 50ul/well of standards and samples were added into the designated wells. One pair of well was filled up only with blocking buffer as blank. The plate was sealed and incubated for 1 hour at 37°C. After the incubation, the plate was washed 5 times with washing buffer. After that, 50ul/well of biotinylated anti-IgG4 (abcam, Cambridge, UK) antibody diluted in blocking buffer was added onto each well (1 in 1000 dilution). The plate was sealed again and incubated at 37°C for 1 hour. After 1 hour, the plate was washed for three times. Then, 50ul of Horseradish peroxidase streptavidin (BioLegend, San Diego, CA, USA) was added to each well and incubated for one hour at 37°C. After the last incubation, the plate was washed 5 more times and 50ul of TMB (BioLegend, San Diego, CA, USA) was added to each well. 1N Phosphoric acid (H_3PO_4) was added as stop solution immediately after the appearance of the darkest standard color. Optical density was measured immediately at 450nm using an ELISA plate reader (Tecan group Ltd., Mannedorf, Switzerland).

Statistical analysis

Statistical analyses were performed using GraphPad prism 6.0. Differences between groups were assessed using one-way ANOVA with Tukey's Honest Significant Difference Test for the analysis of specific immunoglobulins at different milk doses. Unpaired *t* test was done when comparing two groups. Results are presented as mean ± SEM (Standard error of the mean). Statistical significance was defined as a *p* value less than 0.05.

RESULTS

In this trial, 9 of 20 recruited CMA patients completed 200ml of cow's milk dose so far. We studied serum samples of OIT recipients, observation controls and non-CMA healthy controls. Samples were collected at different milk doses during the OIT as previously mentioned.

Measurement of total IgE to assess the overall allergic status

First, we measured total IgE to have an overview of the IgE status of our patients. All patients showed total IgE levels higher than normal (mean+2SD in children, 93-328 kU/L)[91]. As total IgE does not reflect any specific allergic sensitivity, we measured cow's milk protein specific IgE. A subject who is allergic to house dust mites but not CM was used as a negative control. The negative control showed an appreciable amount of total IgE, which was comparable to CMA patients **(Figure 3)**.

CMP specific IgE

No significant difference at baseline sIgE levels between OIT recipients and observation controls

Specific IgE to the three main cow's milk proteins, casein, β-lactoglobulin and α-lactalbumin were measured in OIT recipients (n=10) and CMA controls (n=8) who were part of a one year observational cohort who did not receive OIT **(Figure 4)**. No significant difference was observed in between groups.

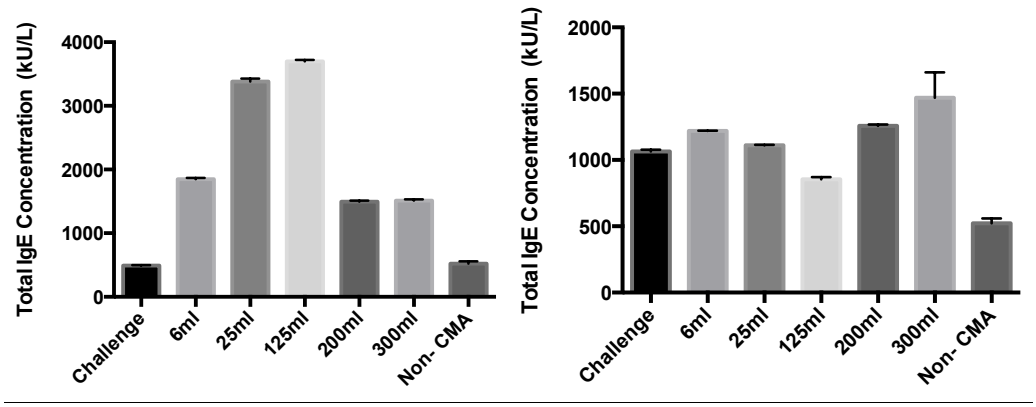

Figure 3: Measurement of total IgE levels in patients with cow's milk allergy. Total IgE showed a trend of increasing from the baseline along with the progression of OIT. Non- CMA atopic control also showed a considerable amount of total IgE level.

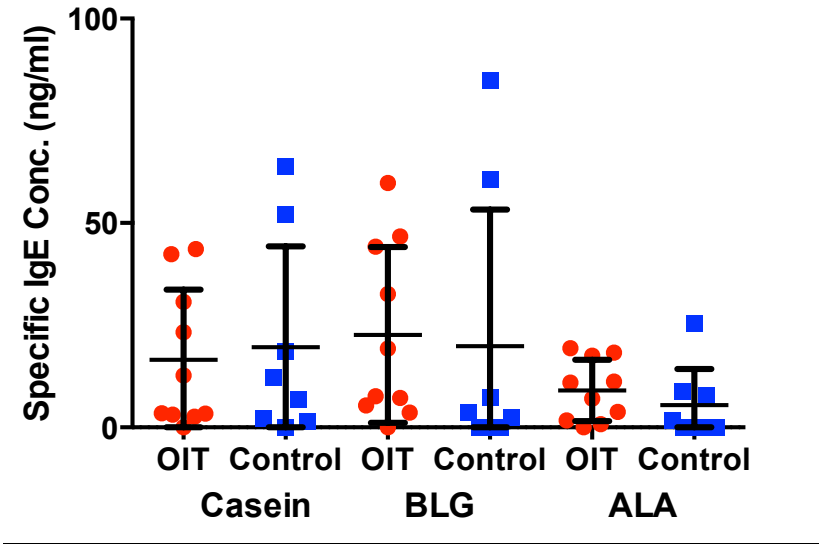

Figure 4: CMP- specific IgE level is not different between OIT and observation control groups. There was no significant difference (unpaired *t* test, $p>0.1$) between baseline specific IgE to casein, BLG, & ALA between OIT recipients **(n= 10)** and observation controls **(n= 8)** who will be followed for one year without OIT.

Specific IgE to cow's milk proteins are significantly higher in CMA patients compared to non-CMA subjects

Cow's milk protein specific IgE was measured in CMA and non-CMA healthy controls. CMA patients (n=18) showed significant differences in specific IgE levels at baseline to all three major CMP's, casein, β - lactoglobulin (BLG), & α- lactalbumin (ALA) compared to non- CMA individuals (n=7). As expected, the difference was significantly high between two groups. In case of casein and β - lactoglobulin the difference was very significant (****$p < 0.0001$). All of the CMA individuals showed detectable sIgE to casein and BLG. ALA sIgE was undetectable in 5 out of 18 (28%) patients **(Figure 5)**.

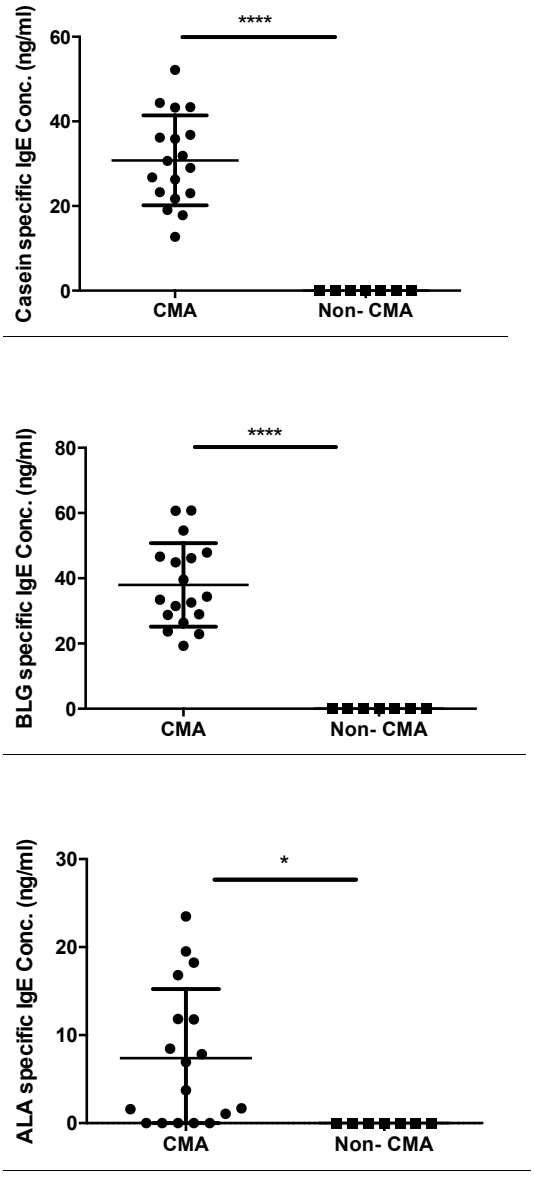

Figure 5: CMP-sIgE level at baseline is significantly different between CMA and non-CMA healthy controls. There was significant difference in sIgE levels at baseline in CMA patients compared to non- CMA individuals (unpaired *t* test, ****$p < 0.0001$ for casein, ****$p < 0.0001$ for BLG, *$p < 0.05$ for ALA). CMP-sIgE was undetectable in non-CMA healthy controls.

Specific IgE to CMP increased and **then decreased with the progression of OIT**

Specific IgE to casein, BLG, and ALA were measured during the course of OIT. The purpose was to see if the specific IgE to CMP's decreased over time with the progression of OIT. For all 3 CMP's, sIgE increased from initiation of therapy until the 25ml dose of milk and then started to decrease after the dose of 125ml was reached **(Figure 6)**. There was a wide variation in sIgE from patient to patient. Because of the small sample size and high variability, statistical significance was not achieved.

Specific IgE to CMP's decreased significantly baseline in patients who completed the OIT successfully

Two OIT recipients completed the whole treatment program and were followed for 12 months. Specific IgE to casein, BLG, and ALA decreased towards the end of the OIT in both of the patients. Patient GS002 showed 2.64-fold decrease in casein specific sIgE level at 6 months post oral immunotherapy. sIgE to BLG and ALA decreased 7.36-fold and 1.87-fold respectively at the end of 1 year. Patient ID: TS003 showed undetectable sIgE to casein at 3 months post-OIT. This was maintained during the 6^{th} and 9 months visit. At the end of the therapy sIgE had decreased 18.8-fold below baseline. sIgE to BLG, and ALA decreased 32.25- and 2.4- fold 3 months post-OIT **(Figure 7)**.

As the trial is still ongoing, we do not have complete data on all subjects. However, I can illustrate certain trends with the data that is available. sIgE to casein and BLG fell below baseline at the dose of 125 ml in Patient ID: AP001 and sIgE to ALA was undetectable at the same dose in this patient. For three other recipients (Patient ID: BL004, JY013, EB015) casein-sIgE decreased below baseline at 125 ml, at 3 months post-OIT, and at 200-ml. sIgE response to BLG and ALA was more variable in these patients. In case of

two other recipients (Patient ID: MG016, & SB017), sIgE to all three CMP's did not decrease from baseline till 200 ml of OIT.

Patient ID: MC010 demonstrated no or minimal detectable sIgE level at baseline (data not shown). The sIgE to casein did not increase during OIT nor did the sIgE to BLG, and ALA. However, the patient had a true, severe reaction on oral challenge and progressed well on OIT. We will discuss this patient further in the section of sIgG4.

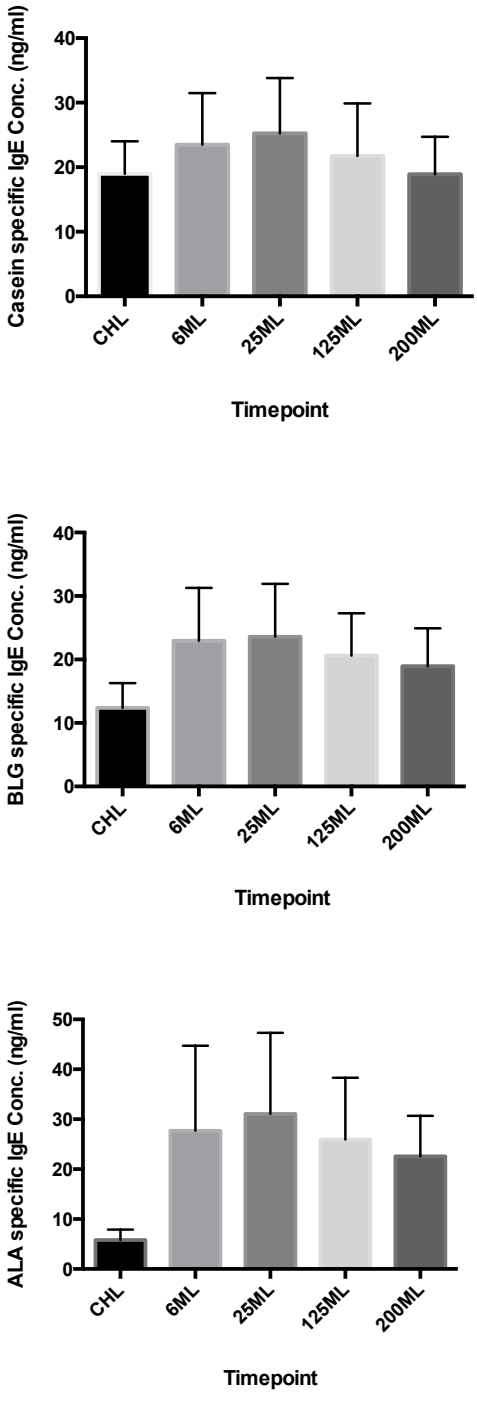

Figure 6: CMP-specific IgE level increased and **then decreased with the progression of OIT.** CMP-sIgE was measured in all 9 patients who completed the OIT till 200ml of milk dose (one way ANOVA, $p>0.1$). A trend was observed from the challenge to 200ml of milk dose. P value was not statistically significant among groups.

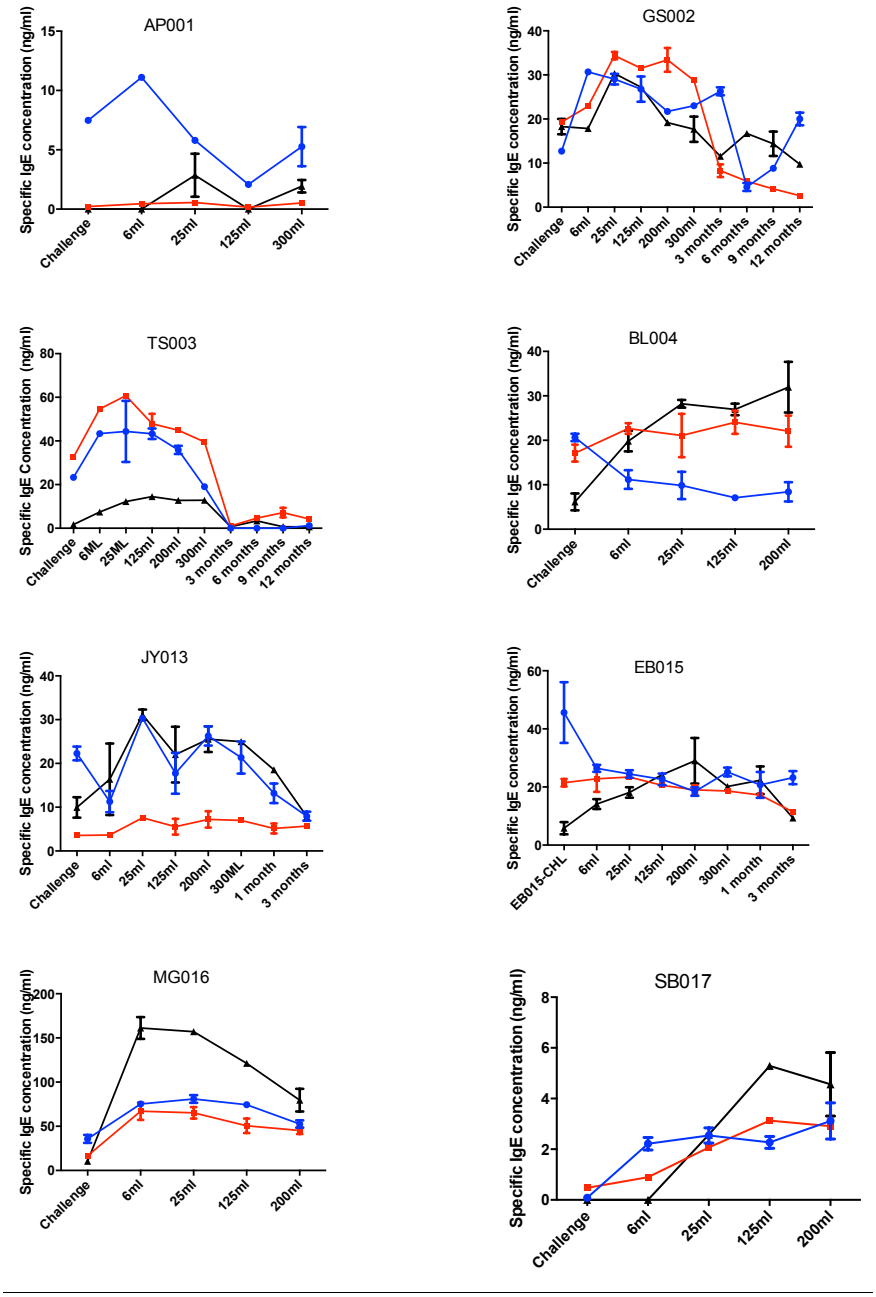

Figure 7: CMP-sIgE level went below baseline in successful OIT patient's 1-year post-OIT. Specific IgE level to casein, BLG, & ALA of individual patients at different time points were measured. Two of the patients (Patient ID: GS002, TS003) competed OIT successfully show significantly decreased sIgE 1-year post-OIT. Blue, red, and black curves represent casein, BLG, and ALA respectively.

CMP specific IgA

Specific IgA levels in OIT recipients and observation controls

To determine if sIgA at baseline to CMP's in OIT recipients is comparable to the observation control group, sIgA was measured to casein, BLG, and ALA. There was no significant difference in between two groups **(Figure 8)**.

IgA to casein is significantly higher in healthy control, non-CMA individuals compared to CMA patients

Casein, BLG, and ALA specific IgA was measured in all CMA patients (n= 20) at baseline and in healthy control non-CMA individuals (n= 8). Specific IgA to casein was significantly higher in non-CMA healthy controls compared to CMA patients (****p <0.0001). No significant difference was observed in case of sIgA to BLG and ALA in between CMA patients and non-CMA healthy controls **(Figure 9)**.

Specific IgA to CMP's showed a trend to increase with OIT

sIgA to casein, BLG, and ALA increased with the course of OIT. BLG levels increased and then plateaued from 25ml to 300ml of milk. In case of casein and ALA, sIgA showed a trend to increase from baseline to 300ml of milk dose **(Figure 10)**.

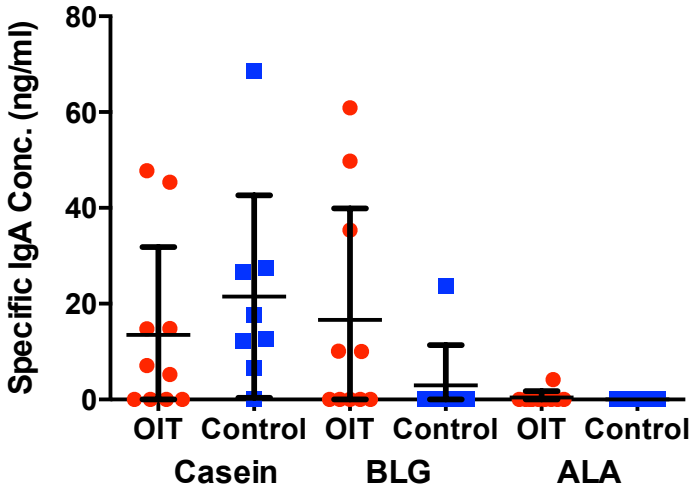

Figure 8: Baseline CMP-specific IgA level is not different between OIT and **observation control groups.** OIT recipients (n=10) and observation controls (n=8) did not show any significant difference at baseline CMP-sIgA (unpaired *t* test, $p>0.1$).

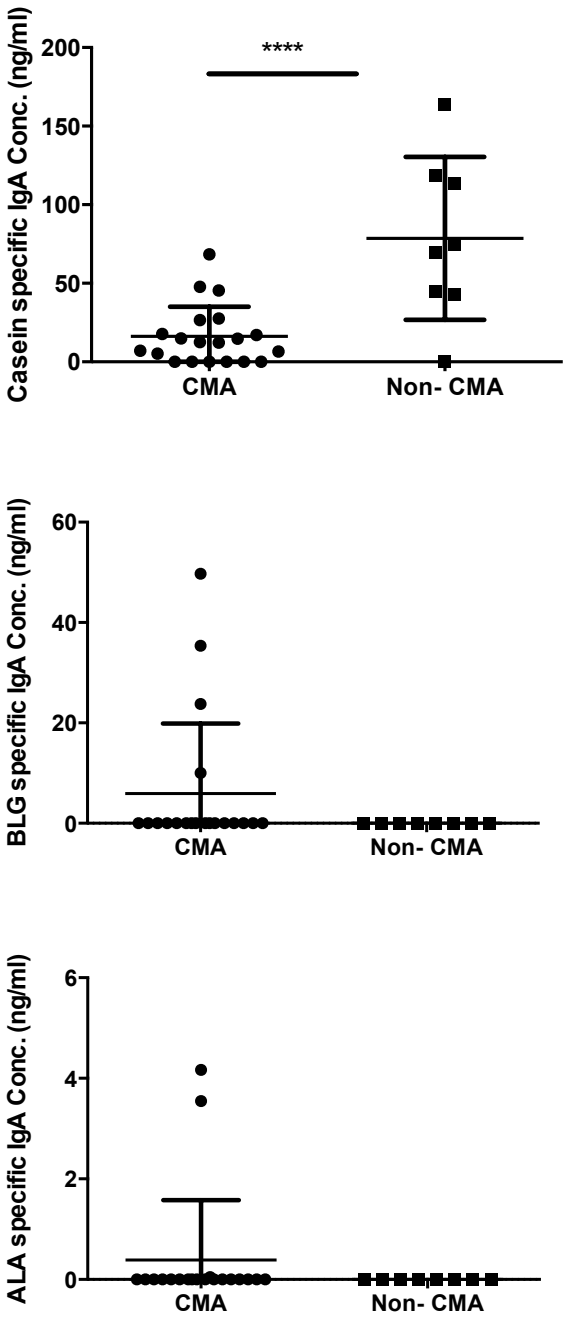

Figure 9: Specific IgA level to casein is significantly higher in non-CMA healthy individuals compared to CMA- patients (unpaired *t* test, ****$p < 0.0001$). This was not seen in BLG and ALA.

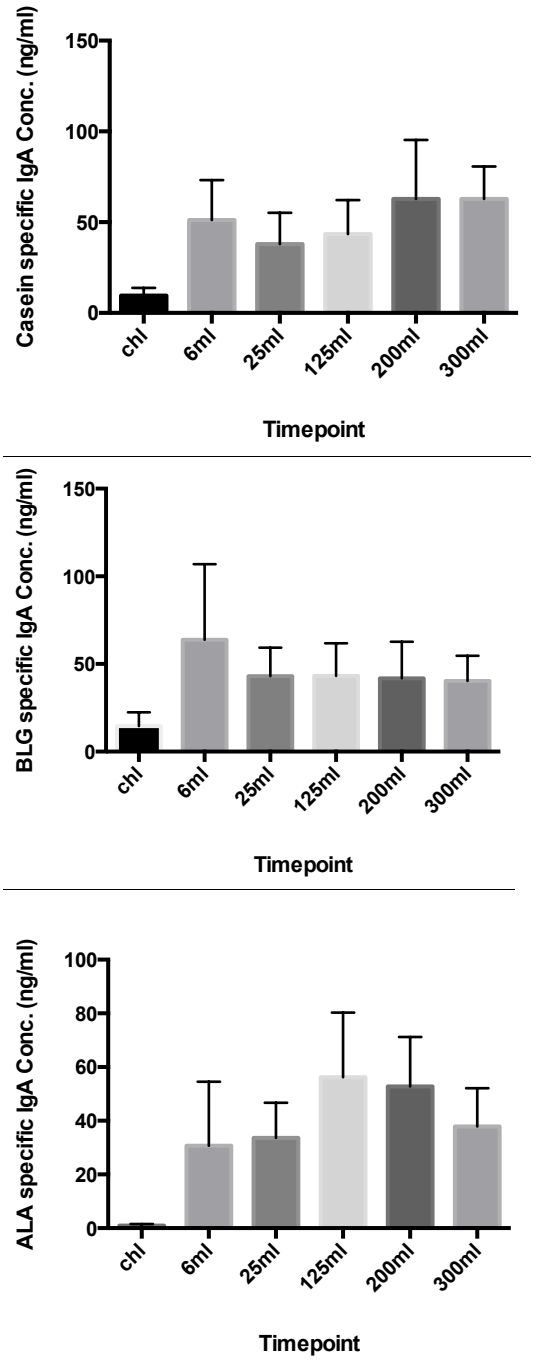

Figure 10: Casein- and **ALA- sIgA level showed a trend to increase with OIT.** CMP-sIgA was measured in all 9 CMA patients who completed the 300ml milk challenge. Casein and ALA specific IgA showed a trend to increase from baseline to 300ml of milk. In case of BLG, the increase from baseline remained static with the course of OIT (one way ANOVA, in all cases *p* value was >0.1).

Comparison of sIgA at baseline and at 300ml

The role of sIgA has long been a matter of debate, as opinions exist describing its role as a pro- and anti-inflammatory immunoglobulin. Our hypothesis was to see if a successful OIT could lead to increases in sIgA levels. We compared the sIgA level prior to therapy (e.g. baseline or the initial oral milk challenge). The second was following the 300ml milk challenge at completion of OIT. sIgA to casein increased significantly from pre-therapy to the 300ml challenge dose (**$p < 0.01$). Similar results were observed with ALA (*$p < 0.05$). No significant difference was noted in case of BLG, where specific IgA did not change from pre-therapy to 300ml **(Figure 11 and 12)**. This indicates that increases sIgA to casein and ALA correlate with successful OIT, whereas BLG does not increase significantly.

	Maximum sIgA level to Casein	Maximum sIgA level to BLG	Maximum sIgA level to ALA
Low dose Responder (6-25ml)	22.2% (n= 2)	33.3% (n= 3)	11.1% (n= 1)
High dose responder (125-300ml)	55.5% (n= 5)	22.2% (n= 2)	55.5% (n= 5)
No response	22.2% (n= 2)	44.4% (n= 4)	33.3% (n= 3)

Table 1: Specific IgA level peaks at different milk doses in different patients. Data are shown as percentage of patients along with the number of the patients in the parenthesis. In case of casein and ALA, it took higher doses to reach the peak in the sIgA curve, whereas for BLG, lower doses were found to induce higher sIgA.

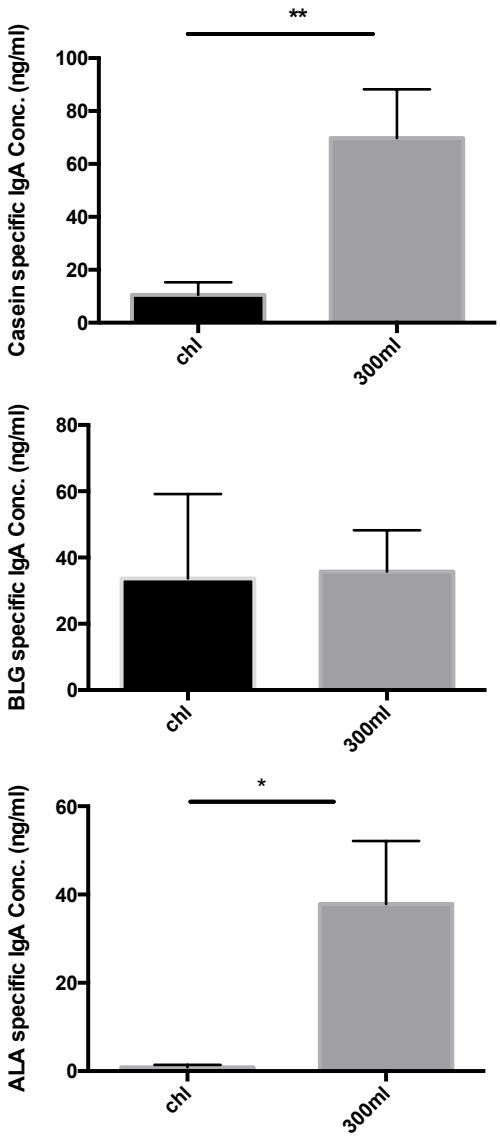

Figure 11: Specific-IgA level to casein and ALA increased significantly baseline with OIT (unpaired *t* test, $^{**}p < 0.01$ & $^{*}p < 0.05$ respectively).

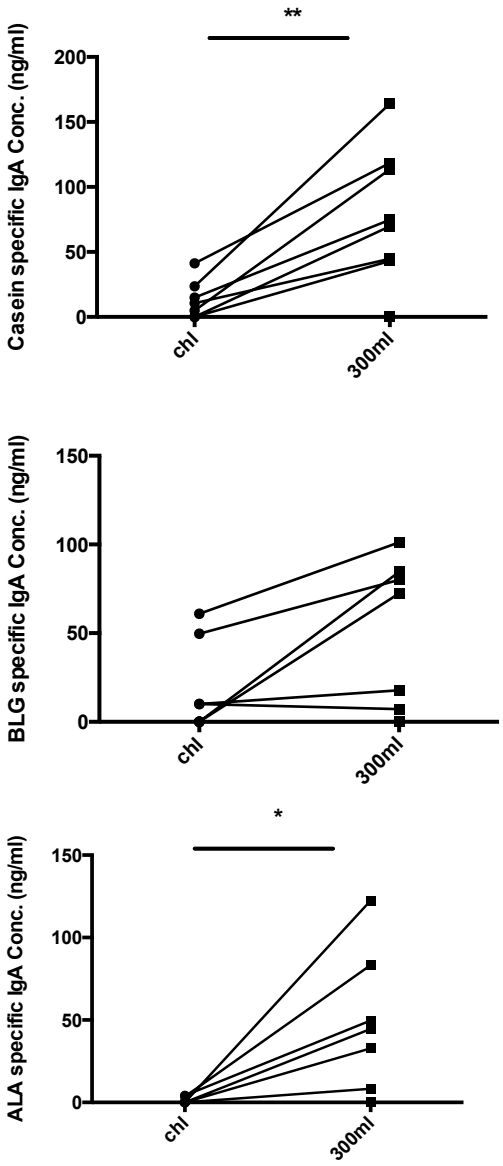

Figure 12: Specific-IgA level to casein and ALA increase significantly from pre-therapy to 300ml challenge (unpaired *t* test, $^{**}p < 0.01$ & $^{*}p < 0.05$ respectively).

CMP specific IgG4

Specific IgG4 levels to CMP's has no significant difference between OIT recipients and controls

Specific IgG4 level at baseline in CMA patients (n=8) and in CMA control subjects (n=8) was measured. There was no significant difference in between two groups **(Figure 13)**.

Specific IgG4 response to β−lactoglobulin is significantly different in CMA patients compared to non-CMA subjects

CMP- specific IgG4 was measured at baseline in CMA patients (n= 10) and in non-CMA individuals (n=8). Significant difference in between groups was noted in case of BLG. In this group BLG specific IgG4 was significantly higher in non-CMA group compared to CMA patients **(Figure 14)**. Before, while analysing the sIgA to CMP's, it was found that non-CMA individuals had higher casein- & ALA-specific IgA compared to CMA group. BLG-specific IgA did not show a significant difference in between CMA- & non-CMA. Interestingly, regarding sIgG4 to CMP's, significant difference was only noted in case of BLG. Putting everything all together, sIgA to casein and ALA; sIgG4 to BLG was found significantly increased in non-CMA individuals compared to CMA patients.

Specific IgG4 to CMP increased significantly baseline due to OIT

To assess if there is any change in the level of sIgG4 from the beginning towards the end of the OIT, sIgG4 to casein, BLG, and ALA was measured **(Figure 15)**. Specific

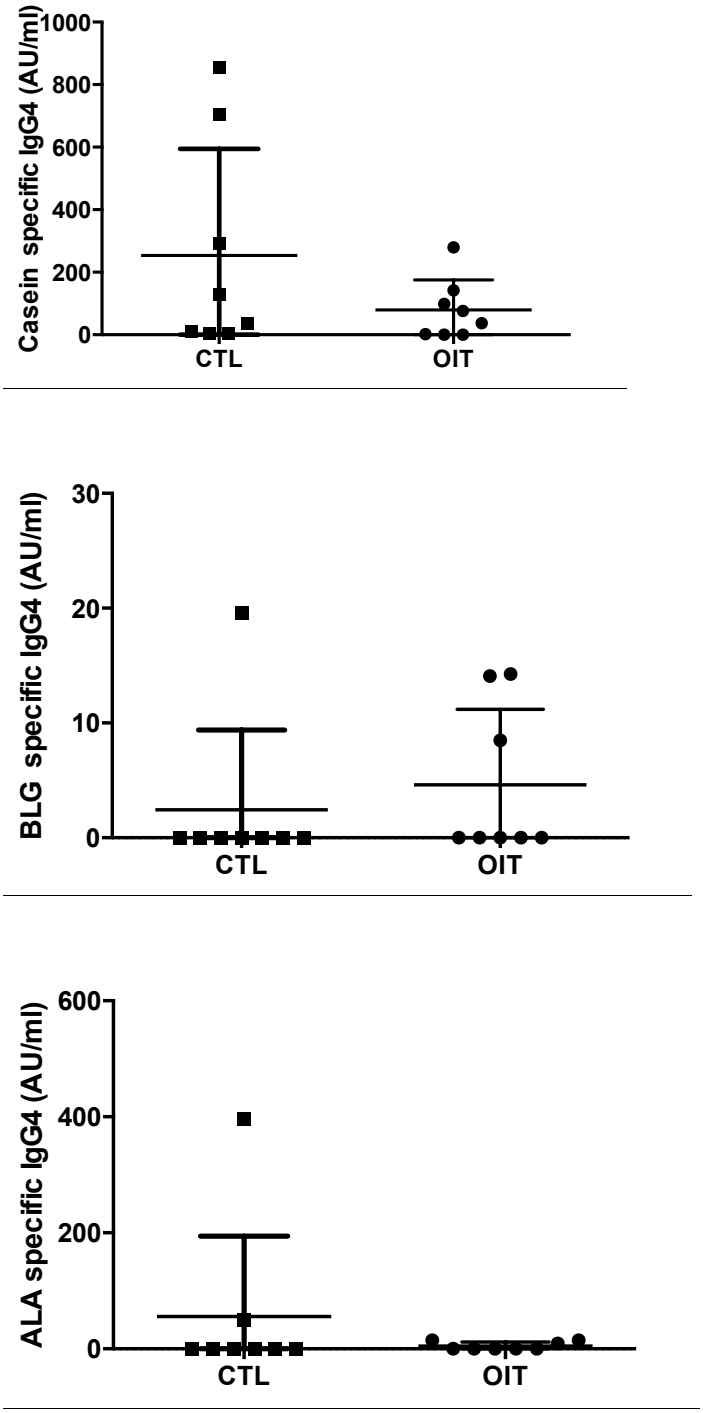

Figure 13: Baseline CMP-specific IgG4 level is not different between OIT and **observation control groups (in both groups, n=8).** No significant change was noted in between groups (unpaired *t* test, $p>0.05$).

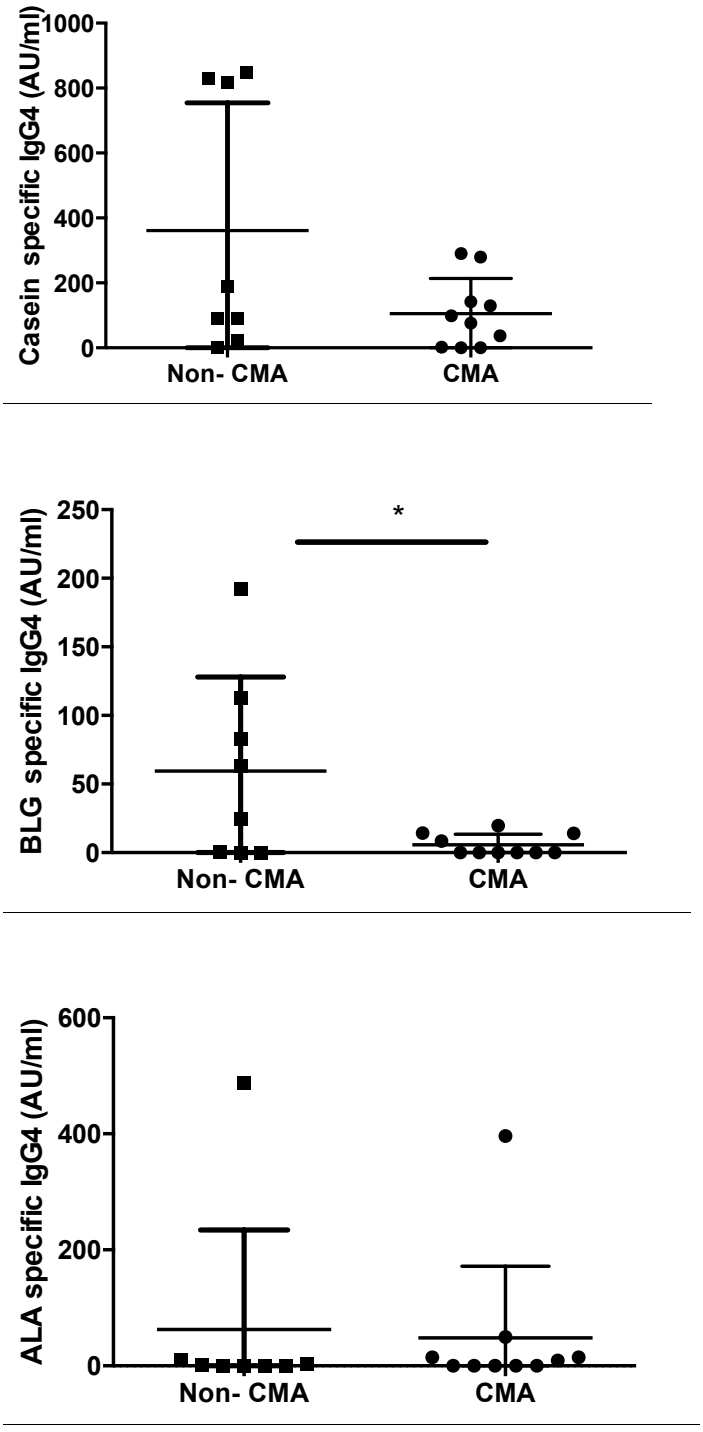

Figure 14: BLG specific IgG4 was significantly different in CMA vs. non- CMA individuals. In non- CMA subjects BLG- specific IgG4 was significantly elevated (Unpaired *t* test, *$p < 0.05$). Casein and ALA- specific IgG4 did not show any difference in between groups ($p>0.05$).

IgG4 to all three CMP was increased significantly (n= 9) from baseline at 200ml of milk consumption (*p <0.05, ***p <0.001, ***p <0.001 respectively for casein, BLG, and ALA).

Early sIgA responders also showed high IgG4 level to all three CMP's

It was observed that, the patients whose sIgA reached the plateau during the early milk doses, revealed higher level of specific IgG4 at 200ml as well **(Table 1)**. In the graph showing casein specific IgG4, the green dots represent the patients (Patient ID: GS002, SB017), who showed their highest sIgA to casein during the early escalation phase (6-25ml of milk) of the OIT. The finding was more pronounced in case of BLG. All three patients (Patient ID: JY013, EB015, MG016), who exhibited the highest sIgA in response to lower doses, displayed higher level of sIgG4 to BLG. These patients are marked with the purple dots on the plot. Regarding the sIgA to ALA, only one patient (Patient ID: MG016) out of nine responded with the highest sIgA to ALA. This one patient also remains among the high sIgG4 responders to ALA, marked as the orange dot on the plot **(Figure 15)**. These data suggests that, early sIgA responders are also among the highest sIgG4 producers to CMP's. Additional to these findings, one of the OIT recipients (Patient ID: MC010) revealed very low sIgE to casein only, no detectable sIgE to other two CMP's. Also, there was no detectable sIgA to any CMP's at any timepoint. Interestingly, this patient responded with the highest sIgG4 level among all the OIT recipients (n= 9). This patient is denoted by a red dot in the graph. The total course of the sIgG4 progression from challenge till 300ml in case of this patient is depicted separately **(Figure 16).**

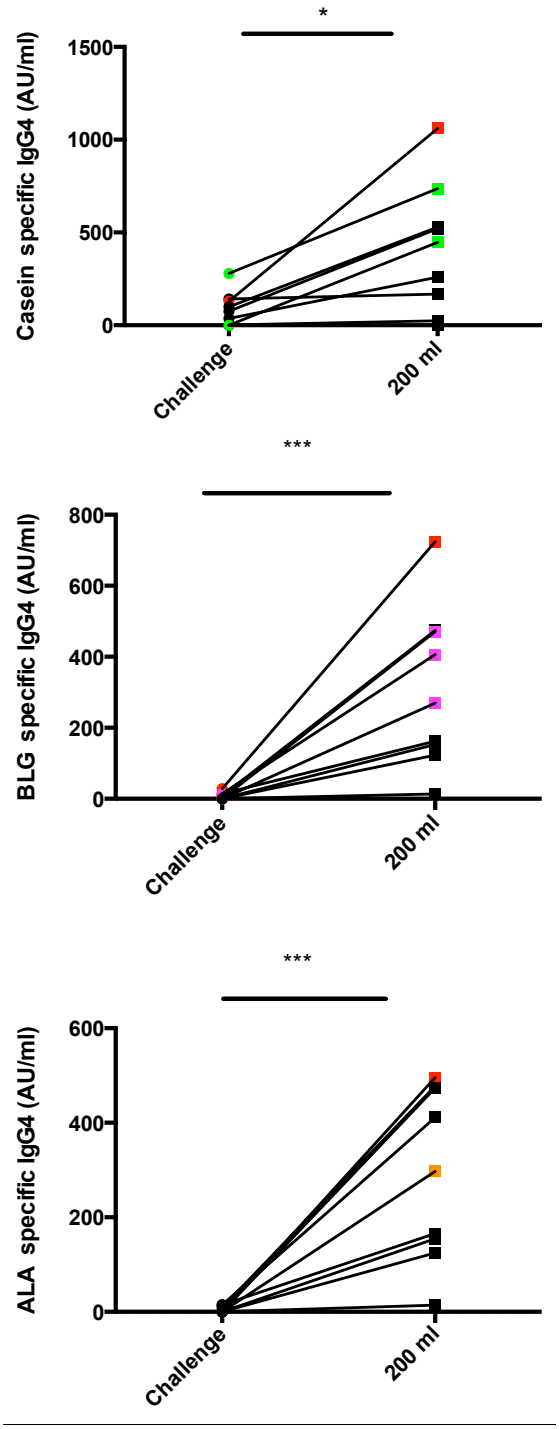

Figure 15: CMP-Specific IgG4 level increased significantly from challenge to 200ml with OIT (Unpaired *t* test, $*p < 0.05$, $***p < 0.001$, $***p < 0.001$ respectively for casein, BLG, and ALA). Specific IgG4 to casein increased in all but one patient. The response of sIgG4 in case of BLG and ALA is more robust. Five of 9 patients started as undetectable at the baseline and increased significantly baseline in case of BLG. In case of ALA, 6 of 9 patients displayed the same result.

CMP-sIgG4 data of Patient ID: MC010

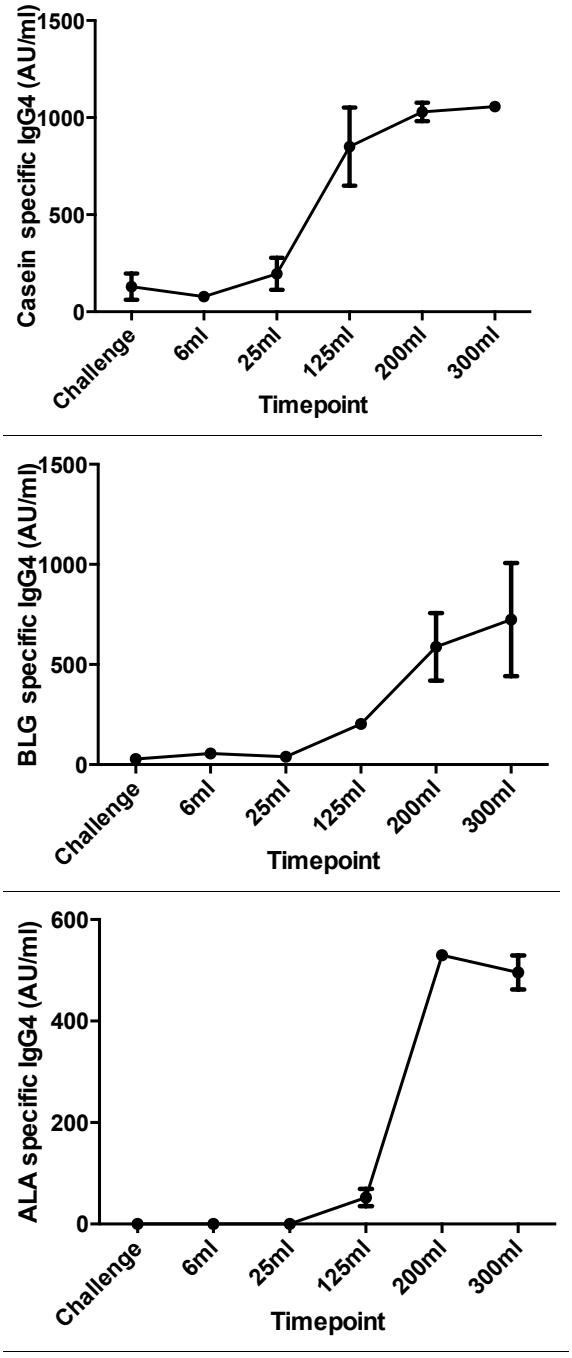

Figure 16: A rise in specific IgG4 level to casein, BLG, and ALA was observed in a patient who showed almost no detectable sIgE and sIgA to the CMP's. Baseline sIgG4 to casein and BLG was at very low level when compared to 300ml milk dose. For ALA, sIgG4 was undetectable until 25ml dose. It began to increase when the patient reached 125ml and peaked at 200ml of OIT.

DISCUSSION

Nine of 12 (75%) patients completed the OIT successfully by reaching 200ml of cow's milk so far, which is in line with previous trials[43-45]. The main objective of this thesis was to investigate the CMP-specific immunoglobulin levels along with the course of OIT, which may help predicting the ideal candidates for OIT. Successful tolerance to CM was accompanied by decreased level of CMP-sIgE at the end of the therapy in most of the patients (67%). Baseline specific IgE to all three CMP's did not show any significant difference between OIT recipients and observation controls as we have expected. CMP-sIgE was significantly higher in CMA patients when compared to non-CMA healthy individuals. CMP-sIgE at first showed a trend to increase and then decrease towards baseline in parallel with the progression of OIT. Those patients who completed the protocol completely showed a significant deceased baseline CMP-sIgE at 3-6 months post oral immunotherapy. In one of our patients (TS003), CMP-sIgE was undetectable at 3-months post oral immunotherapy (POIT) and remained so till 9 months POIT. At 12 months POIT, sIgE was detectable but was in very low amount compared to baseline in that patient. Additionally, CMP-sIgE in our patients (n=5) also showed a tendency to fall towards baseline even before reaching 200ml dose. On the contrary, in other patients (n=3) sIgE remained persistently higher from baseline to 200ml of CM dose. One patient (MC010) only showed detectable casein-sIgE during the recruitment. Thereafter, no casein, BLG, and ALA-sIgE were detectable in that patient. Above-mentioned data on sIgE indicates both an increase and decrease in sIgE level with OIT. The decrease in sIgE in successful OIT patients (n=5) consolidates the findings of previous studies referring the decrease in CMP-sIgE with a successful OIT [43, 44]. Discontinued OIT patients

(n=3), whose CMP-sIgE did not show a tendency to decrease supports the findings by Pajno et al.[35]. In their OIT trial, CMA patients tolerating 200ml of milk showed no significant difference in CM-sIgE level at the end of OIT from baseline[35] These contradictory findings on CMP-sIgE in CMA oral tolerance induction does not allow CM-sIgE to establish itself as a consistent marker in successful oral tolerance induction to CMA.

We have also looked for CMP-sIgA level in serum and saliva from our CMA patients. CMP-sIgA showed no significant difference at baseline between OIT recipients and observation control groups. We tried to seek if CMP-sIgA level varies between CMA and healthy non-CMA controls at baseline. We have noticed a significantly higher level of casein-sIgA in healthy non-CMA controls compared to CMA patients. Our findings suggests that decreased baseline casein-sIgA is associated with the development of CMA in children. Previously, low colostral IgA and low IgA in mothers milk were reported to be associated with the development of CMA in infants[82, 83].

Further, we measured casein, BLG, and ALA specific IgA in our patients from the beginning to the end of the trial or last received dose. Casein- and ALA-sIgA significantly increased baseline to 300ml of CM dose. On the other hand, BLG-sIgA remained almost static from the beginning to the end of OIT. Our data suggests that, BLG-sIgA might be of little importance in terms of oral tolerance induction in CMA. Savilahti et al.[11] reported high baseline BLG-sIgA was associated with the induction of tolerance in children below 3 years of age. In our cohort, we recruited older CMA children (age, 6-19 years). As previously mentioned, natural tolerance to CMA has been reported to occur in 85% patients within first 5 years of life[26]. It is thereby possible

that, BLG-sIgA may behave differently in different age groups. Based on the above discussions, it can be said that increased level of casein and ALA sIgA might be considered as important markers in successful oral tolerance induction in children with CMA.

Casein- and BLG-sIgA did not show any significant difference from baseline to 200ml of CM dose **(supplementary data-Figure 19)**. Significant increase was noted in case of casein at 300ml of milk dose. Expression of IgA is associated with an increased level of tolerogenic cytokine IL-10 & TGF-β[80]. Therefore, it can be hypothesized that, a significant up regulation of IL-10 & TGF-β took place at 300ml of CM dose compared to baseline. Increase in sIgA level thus points a shift from the pro-inflammatory to anti-inflammatory cytokine profile.

We have also seen an increase in salivary CMP-sIgA from baseline in our OIT recipients as they continue to tolerate increasing amounts of milk. One patient has been provided with his salivary CMP-sIgA data **(supplementary data- Figure 20),** as we have his salivary samples almost from the beginning of the OIT to 1month post 300ml re-challenge. Finding from this patient suggests salivary CMP-sIgA levels were also up regulated due to OIT. We are still in the process of collecting more salivary samples at each time point during our patient visits. Other patients salivary CMP-sIgA may add up more information's to this finding.

Along with the rise of CMP-sIgA, we have also observed significant rise in CMP-sIgG4 from baseline in our OIT recipients. At first, we tried to see if there was any significant difference at baseline CMP-sIgG4 between OIT recipients and observation controls. CMP-sIgG4 was not different between OIT recipient and observation control

groups as expected. We also tried to look for if there was any difference at baseline CMP-sIgG4 in CMA and non-CMA healthy controls. BLG-sIgG4 was found significantly increased in non-CMA healthy controls compared to CMA patients. Although, some healthy controls did show very high casein-sIgG4, statistical significance was not achievable due to small sample size and wide range of subjective variations. ALA-sIgG4 did not show any difference between healthy controls and CMA individuals at baseline. Henceforth, casein and BLG specific IgG4 might be taken into consideration as a marker of tolerance in CMA. Our findings somehow resembles with the findings by Savilahti et al.[18], where they reported a low BLG-sIgG4 level was associated with an increased risk of eczema associated with CMA. CMP-sIgG4 increased significantly baseline after the completion of OIT in all our OIT recipients. This may suggest the role of sIgG4 as a good prognostic marker in oral tolerance induction. We have already mentioned in our study that, sIgE decreased in 6 of 9 patients in this cohort. Again, now we see that, increase in sIgG4 is associated with a successful outcome in CM OIT. Meiler et al.[92] reported that, IL-10 secreted by T-reg cells promote IgG4 and suppress IgE production. This might be possible that we find high IL-10 from T-reg cells in successful OIT patients at the end of their trial. On the contrary, Bedoret et al.[36] suggested a Th1/Th2 cytokine mechanism as the reason of change in specific immunoglobulin pattern in CM OIT. They reported that, a shift from Th2 to Th1 cytokine profile is responsible for the decrease in sIgE and increase in sIgG4 in their CM OIT[36]. Noh et al.[93] reported an increase in IL-10 producing regulatory B cells (Br1) in milk tolerant subjects compared to milk allergic ones. On the other hand, Lee et al.[94] reported a possible role of TGF-β producing regulatory B cells (Br3) in maintaining the tolerance to

CM. Study of these regulatory B- and T-cell subtypes along with the serological findings will further help to understand the mechanism of immune tolerance in CMA.

High sIgA responders during the early phases of OIT were among the high sIgG4 responders as well, when compared to late sIgA responsders **(Result section, Table 1 and Figure 15)**. As previously mentioned, TGF-β is a regulator of IgA[80]. Again, it has been reported by Satoguina et al.[95] that, IL-10 derived from T-regulatory-1 (Tr1) cells up regulated the production of IgG4. Hence, high TGF-β and IL-10 level might be responsible for the high CMP-sIgA and high CMP-sIgG4 responses during the early periods of OIT.

In one of our patients, casein, BLG, & ALA specific IgE & IgA was almost undetectable. This patient showed a very strong IgG4 response. It might be possible that, this patient is reactive to other minor CMP's like transferrin, lactoferrin & serum albumin, which demands further research.

Three (Patient ID: CAL007, AT008, ERL023) of 12 patients quit the study due to significant adverse reactions during the early phase of trial. CAL007, AT008, ERL023 discontinued the OIT respectively at 6ml, 20ml, and 4ml. CAL007 **(Supplementary data-Figure 18A)** and AT008 **(Supplementary data-Figure 18B)** both had a low sIgE level at baseline. More specifically, CAL007 did not show any sIgE to BLG at all. This goes against the finding of the studies that say discontinued OIT are associated with a higher sIgE[45]. In our study these patients do not reveal the same scenario on sIgE. Again, these two patients had no detectable sIgA to any of the three major CMP's at any given timepoint. It suggests that, absence of CMP-sIgA at baseline and at subsequent higher doses might be associated with a worst outcome in OIT. In support to this, our

findings on decreased casein-sIgA at baseline in CMA patients can be added. CAL007 had also a low level of CMP-sIgG4 at baseline and did not change at the time of termination of OIT at 6ml dose. This points towards a decreased expression of IL-4, which has been found responsible to induce class switch of B-cells to produce both the pro-inflammatory IgE and anti-inflammatory IgG4[96]. In case of AT008, casein and BLG specific IgG4 remained almost static from challenge to 20ml of CM dose. ALA-sIgG4 showed a trend of increase though. The last patient to quit the OIT was ERL023 **(Supplementary data-Figure 18C)**. This patient showed a very high CMP-sIgE at baseline and at termination (4ml). The specific IgA was low in case of casein, almost undetectable in case of BLG, and undetectable in case of ALA. At the termination CMP-sIgA level showed a trend to increase from baseline in case of casein and BLG, while the ALA-sIgA was still undetectable. In case of CMP-sIgG4, it was very high both at the beginning and the immature termination of OIT at 4 ml. As previously mentioned increased level of sIgE and sIgG4 is associated with an increase in IL-4[96]. Hence an increased IL-4 response from this patient can be expected from cellular studies. Summarizing the serological markers in discontinued OIT patients, CMP-sIgA is a more robust marker compared to sIgE and sIgG4, as it has been found consistently undetectable or at very low level at the beginning of the study. On the contrary, the level of sIgE and sIgG4 level at baseline was not consistently high or low in discontinued patients. We have seen discontinued patients with both a high and low profile sIgE and sIgG4 at baseline. Though it is too early to conclude anything with such a small sample size of discontinued patients, it might be of use to keep these findings in consideration while studying further dropouts.

In summary, our study successfully induced oral tolerance to CM in 9 of 12 patients. None of our control CMA patients achieved natural tolerance one year after recruitment. sIgE cannot consistently be used as a biomarker of successful outcome for OIT. sIgA and sIgG4 proved to be more consistent biomarkers in successful oral tolerance induction. We have seen a significant rise in sIgA level towards the end of OIT. To the best of our knowledge, this increase in sIgA was not observed in other CMA OIT studies.

LIMITATIONS & FUTURE DIRECTIONS

Despite of promising results, this study has limitations. One of the limitations is the sample size. We believe that, with time this issue will be resolved. Another limitation is that, we could not measure CMP-sIgG4 quantitatively due to not having matched pairs of coating and detection antibodies; we therefore used arbitrary units. This also prevented us from calculating a CMP-specific IgG4 to IgE ratio. Due to inter-patient variability, an IgG4 to IgE ratio might be a better prognostic marker. As a future direction we will study regulatory T- and B-cell subtypes and their cytokines (e.g. IL-10, TGF-β) to better understanding the mechanism of immune tolerance in CM OIT.

FINAL SUMMARY AND CONCLUSION

In this thesis, the potential of using CMP specific IgE, IgA, and IgG4 as biomarkers were examined in CMA patients in the setting of oral immunotherapy. We observed that, CMP-sIgE decreased with OIT in most of our CMA patients. CMP-sIgA and -IgG4 increased with time in OIT recipients. In patients where CMP-sIgA was high early in the course of OIT, also showed significantly greater rise in CMP-sIgG4 compared to those subjects, in which sIgA increased later in therapy. These observations suggest that up regulation of CMP-sIgA and -IgG4 may be a key factor in tolerance induction to CM. The highest response to CMP-sIgA and -IgG4 was found at doses of 300ml and 200ml of CM respectively. In those who completed OIT, statistically significant increases in sIgA and sIgG4 to all milk components were only reached at the 200-300ml dose levels. We observed an absence or very low level of sIgA in patients who discontinued OIT. This further supports the role of sIgA as a marker of tolerance induction in CM OIT.

Lastly, the results presented here provide strong evidence that a high level of CMP-sIgA and -IgG4 is associated with oral tolerance to milk, whereas CMP-sIgE is more variable.

SUPPLEMENTARY DATA

Undetectable CMP-sIgE in CMA patients

	Absent casein-sIgE at baseline	Absent BLG-sIgE at baseline	Absent ALA-sIgE at baseline
Number of patients (n)	4	5	5

Table 2: Data showing number of patients in whom CMP-sIgE was undetectable at baseline. This suggests that, all CMA patients might not be reactive to all three major CMP's.

Association with other allergies

Patients recruited in our study found to have allergies other than CMA.

	Egg	Peanut	Treenut	Sesame	Animal meat, Fish	Pollen	HDM	Animal dander	Wheat, Oat, Rye
Number of patients (n)	9	8	2	5	4	8	1	1	1

Table 3: Data showing number of CMA patients in our study who have other concomitant allergies at the same time. Egg allergy (n=9) and peanut allergy (n=8) were the most commonly found food allergy in our CMA patients.

Skin prick test findings

Skin sensitivity to CM decreased in parallel with the resolution of sign symptoms of CMA and decrease in CMP-sIgE **(Graph 1)**.

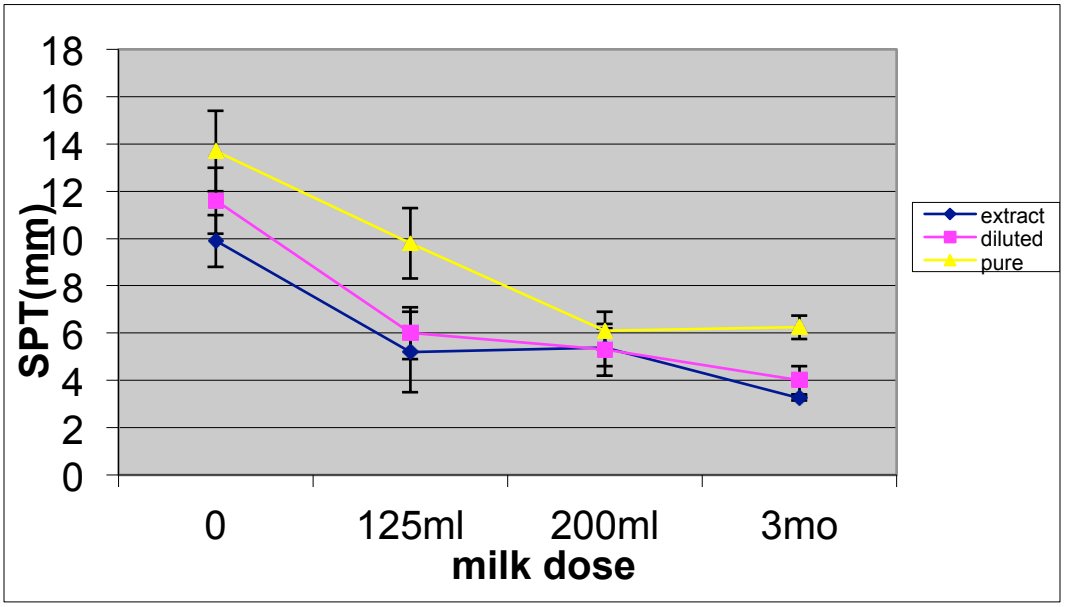

Figure 17: SPT wheal diameter to CM decreased significantly baseline (*p <0.05) at 3 months post-OIT in CMA patients.

CMP-specific immunoglobulin profile in discontinued OIT patients

CAL007

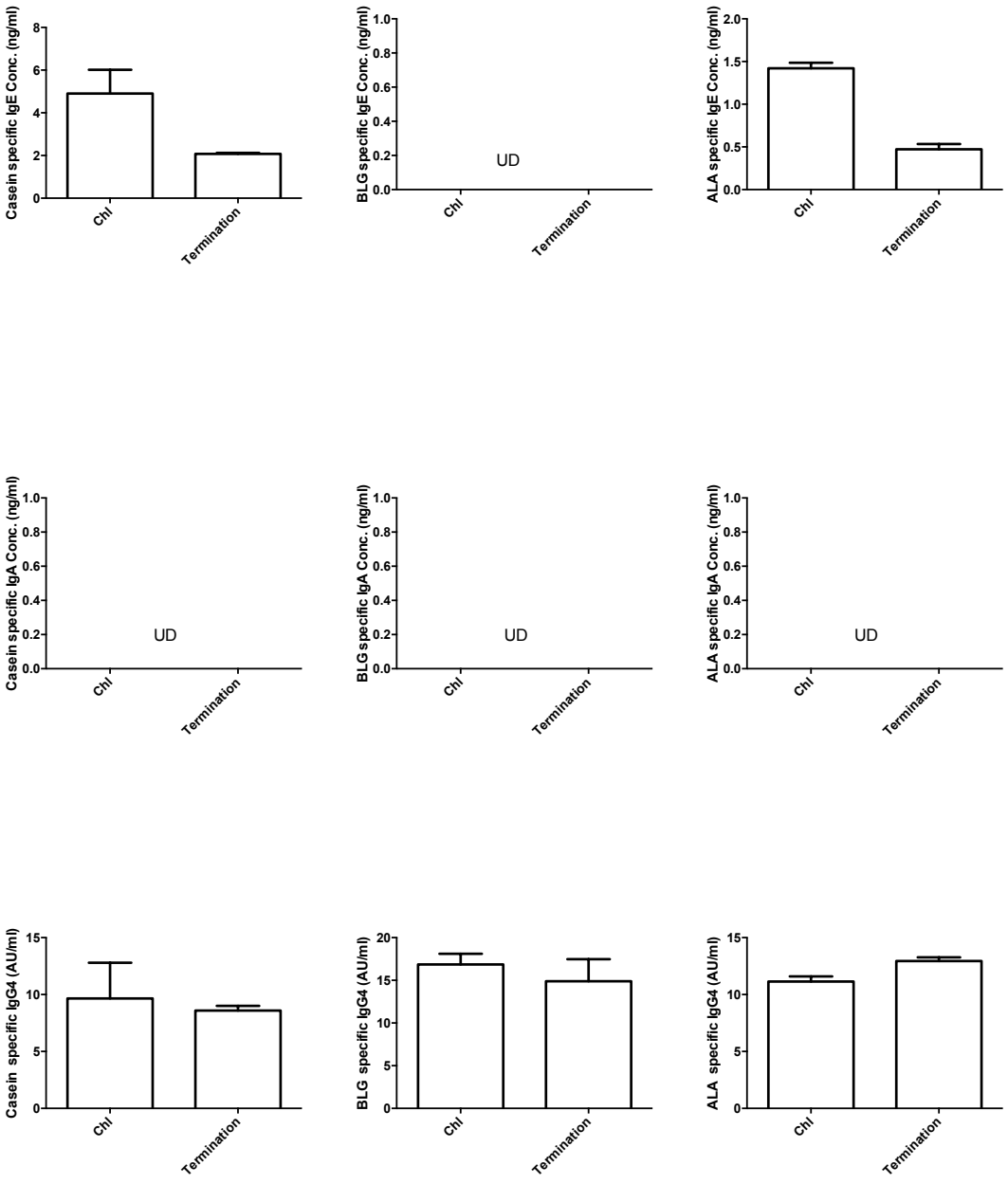

Figure 18(A): CMP-sIgE, -IgA, and -IgG4 level in a failed OIT recipient (CAL007). CMP-sIgA was undetectable from the beginning (challenge) to the termination of OIT (6ml).

AT008

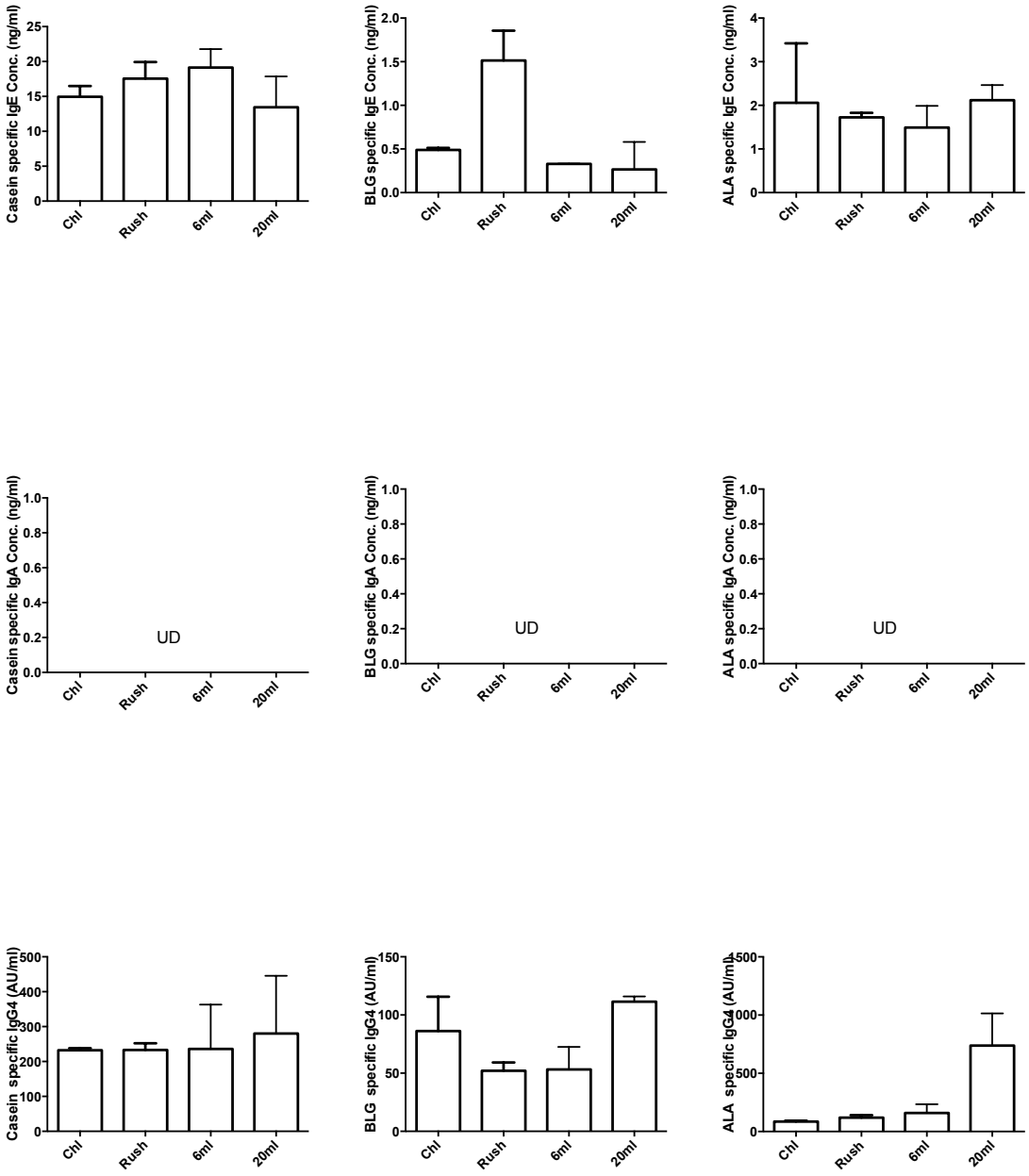

Figure 18(B): CMP-sIgE, -IgA, and -IgG4 level in a failed OIT recipient (AT008). CMP-sIgA was undetectable from the beginning (challenge) to the termination of OIT (20ml).

ERL023

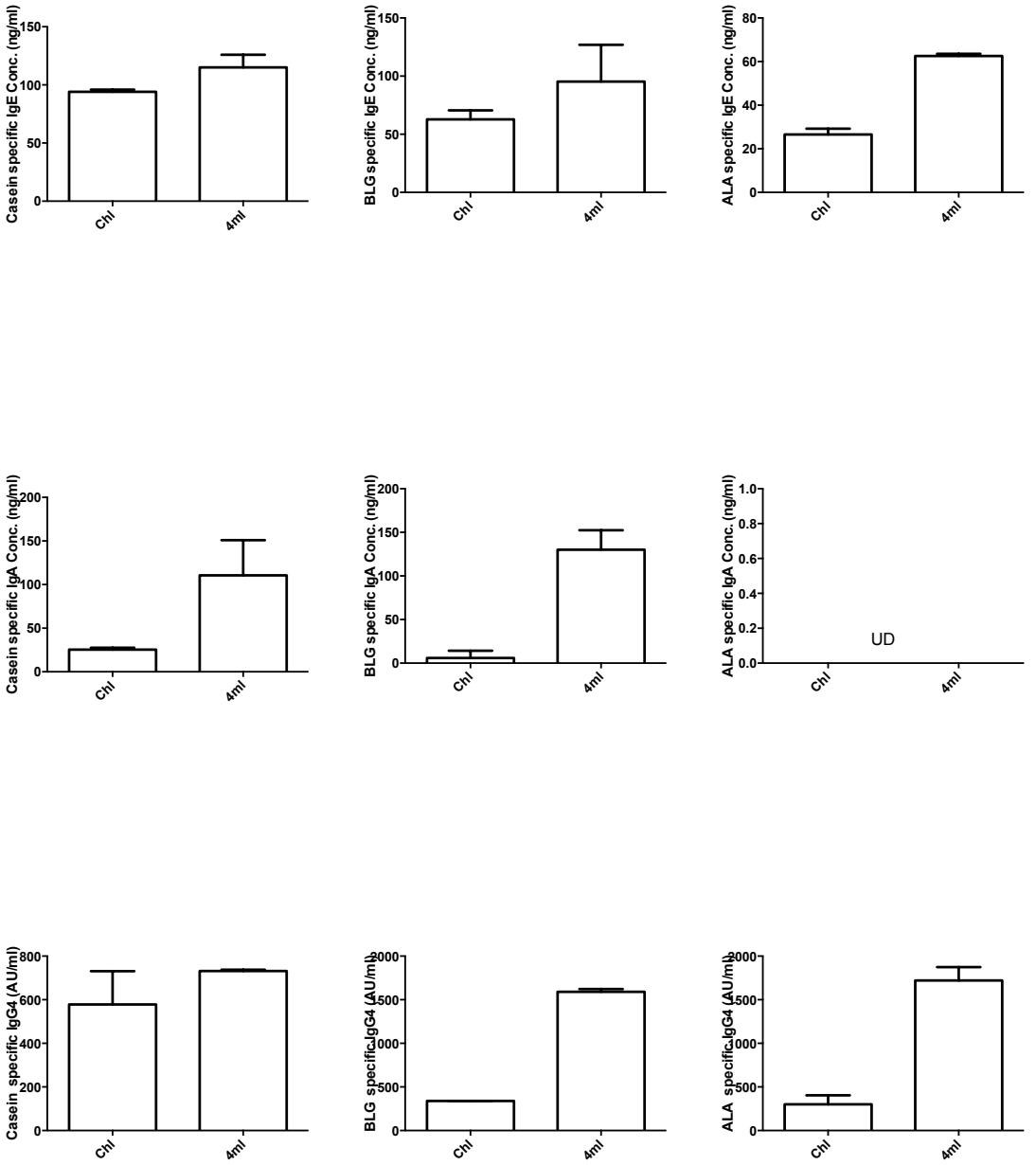

Figure 18(C): CMP-sIgE, -IgA, and -IgG4 level in a failed OIT recipient (ERL023). CMP-sIgA level was variable in this patient. In case of casein- & BLG-sIgA, there was an increase from baseline to the termination (4ml) of OIT, whereas ALA-sIgA was undetectable from the challenge to the termination of OIT.

CMP-sIgA from challenge to 200ml did not show any significant difference to casein and BLG

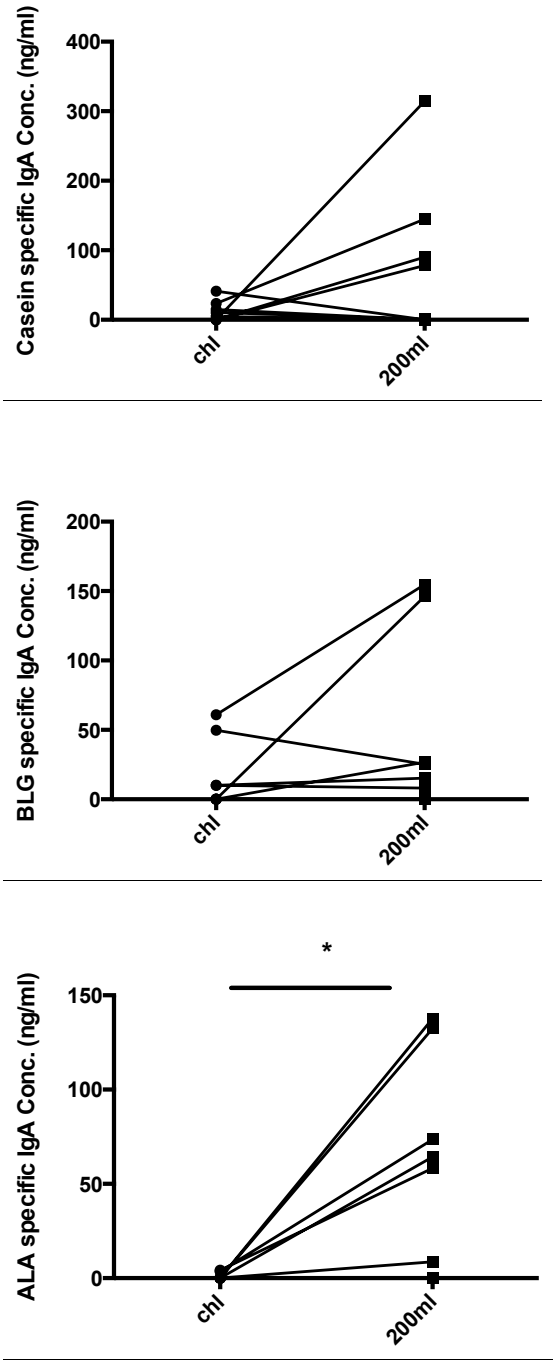

Figure 19: Casein-, and BLG-sIgA level did not increase significantly baseline at 200ml of cow's milk. They significantly increased when a final dose of 300ml was reached.

Salivary CMP-sIgA increased from baseline in a patient with OIT

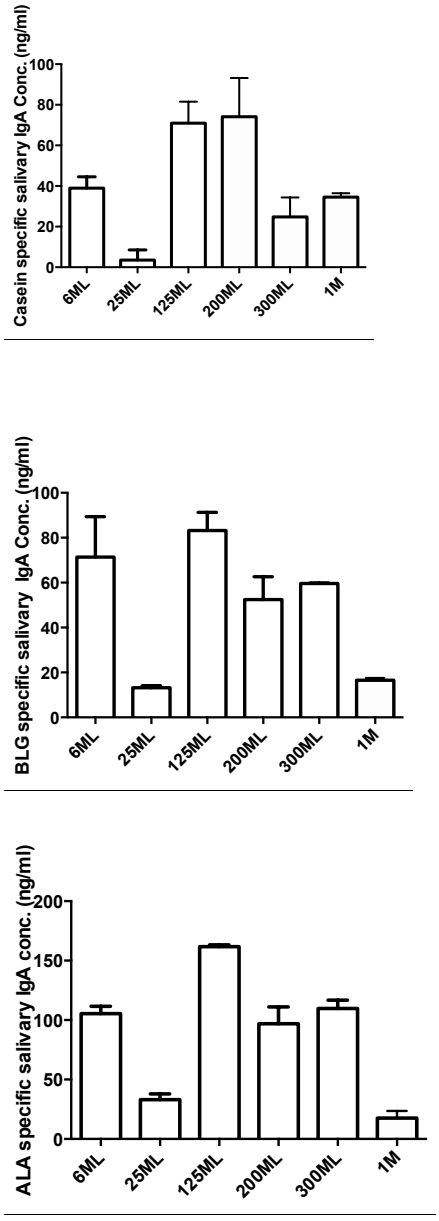

Figure 20: CMP-sIgA level in saliva increased from baseline due to OIT in one of our CMA patients (BL004).

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