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Individual cow's milk allergens as prognostic markers for tolerance development?

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Abbreviations

DBPCFC Double-Blind, Placebo Controlled Food Challenge

CM Cow's milk

CMA Cow's milk allergy

CMP Cow's milk proteins

Ig Immunoglobulin

SIT Specific immunotherapy

Abstract

Background:

Cow's milk allergy (CMA) is one of the most common causes of food allergy in the first years of life. Fortunately, the majority of children with CMA develop clinical tolerance with time. However, no good individual markers exist to predict whether this will occur. Therefore, a prognosis to identify children with persistent CMA at diagnosis would be helpful.

Objective: In this study we sought to assess whether measurement of IgE to individual allergens of cow's milk (CM) would separate patients with persistent CMA from those who became clinically tolerant to CM over time.

Methods: 52 patients ranging from three months to 114 months of age with proven CMA by DBPCFC were followed over time. From these 52 patients, 32 (61.5%) patients became tolerant in the analyzed time period. All patients were re-challenged at least once, some were re-challenged twice or three times. Serum was analyzed prior to each challenge for specific IgE-, IgG- and IgG4-binding to crude CM protein as well as to individual allergens of CM.

Results: The individual likelihood of outgrowing CMA significantly correlates with a low level of CM-specific IgE as well as a low level of specific IgE to α -lactalbumin, β -lactoglobulin (Bos d5.0102), κ -casein and α_{s1} -casein. No significant correlation was found for IgE-levels to total casein, lactoferrin, β -casein, and β -lactoglobulin (Bos d5.0101) as well as IgG and IgG4 levels to α -lactalbumin, β -lactoglobulin and total casein.

Conclusions:

CM-specific IgE is a good prognostic marker for persistence of CMA. Additionally, component-resolved diagnostic showed similar results. However, in our view the rising laboratory costs do not justify a measurement on a daily basis. Additional determination of specific IgG or IgG4 levels was not useful in predicting tolerance development in our study population.

Introduction

Cow's milk allergy (CMA) is the most common cause of food allergy in infancy and childhood. It affects 2% to 3% of infants (1,2), who are typically sensitized to distinct cow's milk (CM) proteins: four proteins in the casein fraction (α_{s1} -, α_{s2} -, β -, and κ -casein), as well as two whey proteins: α -lactalbumin and β -lactoglobulin. These proteins are considered to be major CM allergens (3). Caseins account for about 80% of the total protein content in cow's milk, and whey proteins contain the rest.

Fortunately, the vast majority of children with CMA outgrow their CMA before school age (4,5). However, it is not clear for which individuals this will occur. Therefore, a prognosis to identify children with persistent CMA at diagnosis would be helpful, e.g. in order to recommend oral immunotherapy. The outcome of DBPCFC, the gold standard for diagnosis of clinically relevant food allergy, provides no prediction about the course of CMA over time (6,7).

James and Sampson excluded clinical factors such as symptoms at initial exposure and size of skin-prick test reactions as predictors of acquired CM tolerance, finding that the initial casein- and β -lactoglobulin-specific IgE were lower and decreased significantly in their patients (8). Similarly, lower levels of CM-specific IgE in children who became tolerant were observed in another study (9).

Moreover, a small number of studies have analyzed the Ig epitope-binding pattern in CMA and observed differences in the IgE epitope recognition between individual patients with (persistent or resolved) CMA (10,11,12). Especially the IgE recognition of the casein fraction seems to be associated with persisting CMA (13,11,12,14).

Recently, Savilahti et al analyzed the dynamics of Ig epitopes in patients with persisting CMA and compared these with patients who became CM tolerant (13). They found that IgE epitope-binding patterns were stable over time in patients with persisting CMA, whereas in patients who recovered, early IgE epitope-binding decreased, but the signal of IgG4 binding increased over time. They stated that IgE and IgG4 bindings to a panel of α_{s1} -, α_{s2} -, β -, and κ -casein regions were able to predict an outcome with significant accuracy.

Indeed, IgG antibodies are discussed as being immunopathologically involved in food allergy. It is assumed that specific IgG4 plays a role in the induction of tolerance by allergen-specific immunotherapy (SIT), which was first observed in respiratory and insect venom allergies, where patients who became tolerant after SIT showed an increase in allergen-specific IgG4 and a decrease in allergen-specific IgE (15). Recently, similar results have been observed

during oral immunotherapy in food-allergic patients (16,17). However, the role of IgG4 in (natural) tolerance development in food allergy is hardly understood and available data is contradictory (18,19).

Regarding this, we not only analyzed different IgE but also the IgG and IgG4-levels to α -lactalbumin, β -lactoglobulin, and casein with the aim to correlate whether recognition of specific IgE-, IgG- and IgG4-binding to CM as well as to individual allergens of CM would separate patients with persistent CMA from those who became clinically tolerant to CM over time.

Material and Methods

Study Population

We examined retrospectively serum samples from 52 children with CMA who had at least two consecutive food challenge tests. The diagnosis CMA was first proven by double-blind, placebo-controlled, oral food challenge tests (DBPCFC). Children were followed over time. All patients were re-challenged at least once, some were re-challenged twice or three times. Once an individual patient developed tolerance, no further challenge tests were performed on this patient.

Furthermore, additional information from all study participants' data was collected at baseline and included: sex, positive family history for allergies, duration of breastfeeding and presence of atopic dermatitis. Our database includes no information about a milk-free diet of the breastfeeding mother.

The study was approved by the local institutional review board.

Blood sampling

Venous blood samples of all patients were collected at all time points prior to oral food challenge and obtained serum probes were aliquoted and stored at -20° C until further analysis.

Fluorescence enzyme immunoassays

Determination of total IgE, CM-specific IgE, IgG and IgG₄ to alpha-lactalbumin (nBos d 4), beta-lactoglobulin (nBos d 5) and casein (nBos d 8) were done by fluorescence enzyme immunoassay using ImmunoCAP 250 System[®] (ThermoFisher Scientific). Specific IgE titers were quantified in protein units designated as kilounits of antibody per liter (kU/l) with a lower detection limit of 0.35 kU/l and an upper limit of 100 kU/l as proposed by the manufacturer. Higher values are reported as greater than 100 kU/l.

Allergen microarray analysis

In parallel, ImmunoCAP ISAC[®] IgE assay (VBC-GENOMICS, Vienna, Austria; Phadia, Uppsala, Sweden) was undertaken for the following cow's milk natural purified proteins: α -lactalbumin-IgE (Bos d 4), serum albumin-IgE (Bos d 6), Immunoglobulin-IgE (Bos d 7), casein-IgE (Bos d 8), lactoferrin-IgE (Bos d lactoferrin), α -casein-IgE (Bos d 8 α_{s1}), β -casein-IgE (Bos d 8 β), κ -casein-IgE (Bos d 8 κ), β -lactoglobulin-IgE (Bos d 5.0102), β -lactoglobulin-IgE (Bos d 5.0101).

Protein microarray procedure

At room temperature the ImmunoCAP ISAC[®] slides were incubated for one hour with solution A (prepared as it states in the ImmunoCAP ISAC[®] test protocol) and magnetic stirring. After self-drying, 25 μ l of the control (KS11, VBC-GENOMICS, Vienna, Austria; Phadia, Uppsala, Sweden) and patients' sera were spotted on different slides' fields and

incubated for two hours in a humid chamber. The slides were then washed for 10 minutes with Solution A and followed by a purified water wash for five minutes, both with magnetic stirring (washing steps). When completely dried, the chips with already fixed patients/control IgE antibodies were once more incubated in the humid chamber, this time each field with 25µl of the Detection Antibody Solution (prepared as it states in the ImmunoCAP ISAC® test protocol). After repeating the washing steps as previously described, the slides were dried at room temperature and scanned immediately afterwards with the CapitalBio™ LuxScan 10K microarray scanner. The laser used was the green (wavelength = 532nm) and the scanning option ISAC-1.

Protein microarray analysis

The images were analyzed using MIA-Microarray Image Analyzer software also provided by VBC-GENOMICS (Vienne, Austria) / Phadia (Uppsala, Sweden). The results are given in ISU/l (ISAC Standardized Units/litre) and presented in 4 classes: undetectable (<0.3 ISAC kU/l), low (≥ 0.3 -<1 ISAC kU/l), moderate to high (≥ 1 -<15 ISAC kU/l) and very high (≥ 15 ISAC kU/l).

Oral Food Challenge

Clinical reactivity to CMA was investigated by DBPCFC as described in detail previously (7). In brief, patients received placebo or verum on two different days. The clinical dietician performed the randomization and preparation of the challenges. A maximum of seven gradually increasing titration steps were given at intervals of 30 min, ranging from 0.1 to 100 ml of pasteurized CM (corresponding to 3, 10, 30, 100, 300, 1,000, 3,000 mg cow's milk protein). The cumulative dose during DBPCFC was 144 ml CM (corresponding to 4.4 g protein) if no reaction was observed. Provocation was stopped if objective clinical symptoms such as urticaria, exacerbation of eczema (at least 10 point increase in SCORAD score),

angioedema, wheezing, allergic rhinitis or conjunctivitis, vomiting and/or diarrhea were observed or the highest dose was reached.

Bioinformatic analysis

The association of immunologic parameters and baseline characteristics (e.g. sex) to challenge outcome was evaluated by time-to-event analysis. The interval between initial diagnosis (baseline) and tolerance development (first negative DBPCFC) was used as the dependent variable in a univariate Cox proportional hazards model. All non-tolerant participants were right-censored upon their last positive re-challenge.

Immunologic parameters used as independent variables in the analysis were dichotomized at their baseline median (CM-specific IgE) as a robust cut-off for our analysis or at their detectability (individual CM allergens: class undetectable, i.e. ≤ 0.3 ISU/l, vs. detectable, i.e. > 0.3 ISU/l).

Data was analyzed using the SPSS Statistics 18.0 (Statistical Package for Social Science, Chicago, IL, USA) and R (R Development Core Team (2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.) Package survival: Terry Therneau and original Splus- $\rightarrow$ R port by Thomas Lumley (2011). Survival: Survival analysis, including penalised likelihood. R package version 2.36-9.

Results

Description of the study population

The diagnosis of CMA for 52 included children was first proven by DBPCFC at an age

between three and 114 months, median 22 months (baseline), and the first re-challenge was performed at 9-142 months, median 44 months. Some children had a second or third re-challenge (**Table 1**). Of these initial 52 patients, 32 outgrew their CMA during the observed time. Once an individual patient developed tolerance (individual “tolerance time”), no further challenge tests were performed. Prior to all challenge time points (median 14 days) serum probes were taken and analyzed (**Table 2**).

41 children were breastfed (duration median 4.6 month); from 11 children the information about breastfeeding is missing.

90% of our study population suffered from atopic dermatitis.

Atopic dermatitis, sex, positive family history for allergies, and breastfeeding duration showed no association with the observed tolerance times (data not shown).

Analysis of individual CM markers stratified on baseline data

Significant differences in the nonparametric maximum likelihood estimate of the survival function were detected for using baseline specific serum IgE levels to CM-specific IgE, α -lactalbumin, β -lactalbumin (Bos d5.0102), α_{s1} -casein and κ -casein as predictor (**Table 3; Figures 1, 2**). **Figure 1** shows the Kaplan-Meier survival curve for the population after dichotomization according to the initial CM-specific IgE level using the median (5.0 kU/l) as cutoff. For the two groups, every step in the curve represents a child who became tolerant; every cross (vertical tick) depicts a child who had not become tolerant by his last recorded challenge; however, further re-challenges were not performed during the study. A Cox proportional hazards regression model revealed that children with an initial CM-specific IgE level below the median of 5.0 kU/l were four times more likely to become tolerant than children with a CM-specific IgE level above the median in our cohort (**Figure 1, Table 3**).

Similarly, after dichotomization according to the baseline value of α -lactalbumin-, β -lactalbumin- (Bos d 5.0102) and κ -casein-specific IgE according to being detectable (value > 0.3 ISU/l), CM allergic children with an initial undetectable concentration of α -lactalbumin, β -lactalbumin (Bos d 5.0102) or κ -casein, have a 7.2, 2.7, and 3.8 times enhanced likelihood of becoming tolerant (**Figure 2, Table 3**). No significant differences in the survival curves of CM allergic children could be obtained using their initial specific IgE against other CM proteins as predictor: casein, lactoferrin, β -casein, β -lactoglobulin (Bos d 5.0101) and serum albumin, nor specific IgG against α -lactalbumin, β -lactoglobulin, casein, or IgG4 against α -lactalbumin-IgG4, β -lactoglobulin-IgG4 and casein-IgG4.

Analysis of individual CM markers correspondent to each challenge test

Figure 3 shows the specific IgG and IgG4 levels of casein, β -lactoglobulin and α -lactalbumin in mg/l. CM-specific IgE levels in kU/l are presented as a reference. Every box plot depicts the minimum, the 25%, 50% (median) and 75% quantile as well as the maximum. Measurements were performed at the baseline, first and second repetitive DBPCFC (corresponding to the rows as marked on the right). CM-specific IgE levels were at all times higher in children with persistent CMA compared to children who became tolerant to CM. Interestingly, no significant differences were observed for all analyzed IgG and IgG4 parameters of casein, β -lactoglobulin and α -lactalbumin.

Discussion

Our data shows that the assessment of individual CM allergens may in part enable discrimination between transient versus persistent CM allergic children. In children who resolved their CMA early, the CM-specific IgE levels were lower at diagnosis (baseline). The Kaplan-Meier calculation showed that if the CM-specific IgE level was lower than the

median, the half-life tolerance time (the time, after which 50% of the observed children became tolerant) was calculated at 18 months (**Figure 1**). However, if the level of CM-specific IgE was above the median, the half-life tolerance time was about 33 months.

The median of CM-specific IgE in our population was 5.0 kU/l. We chose the median for dichotomization due to its robustness. Another publication described comparable CM-specific IgE levels in a population of CM allergic patients (6). In this study a 50% predicted probability was depicted with 2-6 kU/l (6, Fig. 1).

Next to the correlation between transient and persistent CMA and the level of CM-specific IgE, significant observations can be made by analysis of α -lactalbumin-, β -lactoglobulin- (Bos d5.0102), κ -casein- and α_{s1} -casein-specific IgE. Looking at the level of α -lactalbumin-specific IgE (**Figure 2**), differences in the tolerance time between 18 and 60 months were depicted, depending on the detectability of α -lactalbumin-specific IgE (hazard ratio: 0.14).

Corresponding to the already known phenomena that CM-specific IgE levels in children who acquire clinical tolerance to CM decrease over time (**Figure 3**), a decrease of all specific IgE levels to individual CM proteins was observed in our study population (data not shown). In general, this was more prominent in children who became tolerant earlier.

As exposure to CM protein may clearly influence the results (26), blood samples for analyses were collected prior (median 14 days) to oral provocation tests.

One could argue that the results might differ in children with and without atopic dermatitis. Although 90% (47 children) of our study population suffered from atopic dermatitis, there were mainly immediate type reactions such as urticaria, vomiting, stridor, conjunctivitis, wheezing etc. following the food challenge test. Skin reactions alone (without any other organ symptoms involved) were observed in only five children. Comparing CM allergic children with or without atopic dermatitis, we found no difference regarding the duration of tolerance development (data not shown).

In addition to IgE the role of IgG in tolerance development is still being discussed. IgG production is a normal physiological response to ingested dietary antigens; it is known to be found in a large proportion of healthy subjects, and is not considered as a marker of allergy (20,21,22,23,24). Shek et al (18) determined specific IgG1 and IgG4 levels in patients with IgE- and non-IgE-mediated CMA, and showed significantly enhanced levels of α - and β -casein-specific IgG1 and IgG4 in the IgE-mediated group. In contrast, in a recent comparison of hen's egg-allergic children and hen's egg-sensitized but tolerant children, no difference in the levels of hen's egg-specific IgG or IgG4 could be found (25). Moreover, it is known that during oral immunotherapy and the development of clinical tolerance food-specific IgG is increasing (16,17).

In the present data, no differences in casein-specific IgG and IgG4 levels in children with persistent CMA compared to children who became tolerant have been observed (**Figure 3**).

Our study population was on a cow's-milk-free diet due to their CMA. An intervention, such as oral immunotherapy (16,17), did not take place, as ours was simply an observational study looking at the natural course of CMA. Nevertheless, our observations are in contrast to the study of Savilahti et al (13), who stated that among patients who recovered early from CMA, the signal of IgG4 binding increased over time.

A possible bias may lie in the fact that we investigated serum samples from the early stages of CMA (the youngest children were three months old at diagnosis) and later stages of CMA and included children in all age groups. These age differences reflect the detected IgG-levels due to differences in the maturing status of the former native immune system. Furthermore, in this retrospective study the patient's parents decided on the time interval between visits in our outpatient clinic and the performance of repetitive food challenges. A prospective study design with a predefined schedule is desirable (and from a statistical point of view, a schedule with a high frequency of re-provocation is preferable in order to measure the time to tolerance as precisely as possible), as it is known that patients with CMA have the potential for

recovery at any age (5).

In summary, already at the time of diagnosis we found significant differences in the levels of specific IgE to individual CM allergens in our study population of children with CMA. Children became tolerant earlier if their specific IgE-antibody levels against the two whey proteins from CM, α -lactalbumin-specific IgE and β -lactoglobulin-specific IgE (Bos d5.0102), as well as against two of the four casein fractions κ -s1- and α -casein-specific IgE was low. However, CM-specific IgE also showed to be a good prognostic marker for persistence of CMA. Therefore, in our view, the additional value of a component-resolved diagnostic does not justify rising laboratory costs on a daily basis. Furthermore, we found no benefit from additional detection of CM-specific IgG or IgG4. Taken all together, our results are helpful in the diagnosis and management of CMA. Although there are many diagnostic tools available to assess the likelihood of tolerance development an oral food challenge is still necessary for final confirmation.

Conflict of Interests

Kirsten Beyer was reimbursed for attending a symposium/got a fee for speaking and fees for consulting of Danone, Novartis, Phadia, Infectopharm, Hipp, UCB, Meda Pharma, and ALK. The funds for research and for a member of staff in her laboratory are from GRF, EU, Danone and Bea-Foundation. The other authors have no conflict of interests to declare.

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Figures

Figure 1: Time between initial diagnosis of CMA and development of tolerance (Kaplan Meier curves), stratified on baseline CM specific-IgE (dichotomized at baseline median, 5 kU/l).

Figure 2: Time between initial diagnosis of CMA and development of tolerance (Kaplan Meier curves), stratified on baseline α -lactalbumin-specific IgE, β -lactoglobulin-specific IgE Bos d5.0102 and κ -casein-specific IgE (dichotomized at baseline corresponding to detectability of individual CM allergens, ISAC class undetectable (≤ 0.3 ISU/l) vs class detectable (> 0.3 ISU/l)).

Figure 3: Box plot analysis showing the minimum, 0.25, 0.50 (median), 0.75 quantile and maximum levels of casein-specific, β -lactoglobulin-specific, α -lactalbumin-specific IgG and IgG4 levels in mg/l and CM-specific IgE levels in kU/l. Measurements are split in rows according to baseline, first and second repetitive oral food challenge, OFC).

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Table 1: Patients' ages and number of serum samples at examined time points

Descriptive statistics						
Time points of food challenge	(n)			Age (month) Minimum - Maximum	Age (month) Mean	Standard deviation (month)
		F	M			
Baseline/ at diagnosis	52	27	25	3 - 114	21.6	24.9
1RE-DBPCFC	52	27	25	9 - 142	43.5	29.8
2RE-DBPCFC	10	5	5	30 - 92	55.1	17.1
3RE-DBPCFC	4	3	1	42 - 72	61.0	13.7

1RE-DBPCFC = First Repetitive DBPCFC

2RE-DBPCFC = Second Repetitive DBPCFC

3RE-DBPCFC = Third Repetitive DBPCFC

Table 2: Mean and standard deviation of analyzed parameters of all patients' serum samples at examined time points

	Unit	Baseline	1RE-DBPCFC	2RE-DBPCFC	3RE-DBPCFC
sIgE					
Total IgE	kU/l	436.9±924.2	442.0±1371.5	380.9±661.4	154.7±75
CM-IgE		21.7±41.6	10.8±19.2	15.7±31.4	3.0±2.2
α-Lactalbumin-IgE (Bos d 4)	SU/l	1.9±3.7	1.3±2.5	2.8±4.6	1.3±0.5
Serum Albumin-IgE (Bos d 6)		1.6±7.5	0.2±0.7	0.0±0.0	0.0±0.0
Immunoglobulin-IgE (Bos d 7)		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Lactoferrin-IgE (Bos d lacto)		0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Casein-IgE (Bos d 8)		2.4±6.4	1.3±3.6	1.3±2.7	0.2±0.4
α-Casein-IgE (Bos d 8α _{s1})		2.2±5.4	1.3±4.2	1.3±3.0	0.1±0.3

β -Casein-IgE (Bos d 8 β)		1.7 $\pm$ 4.8	0.7 $\pm$ 1.6	1.5 $\pm$ 3.4	0.2 $\pm$ 0.4
κ -Casein-IgE (Bos d 8 κ)		2.9 $\pm$ 7.1	1.1 $\pm$ 2.6	1.5 $\pm$ 3.1	0.7 $\pm$ 1.4
β -Lactoglobulin-IgE (Bos d 5.0102)		0.8 $\pm$ 1.7	0.4 $\pm$ 0.8	1.3 $\pm$ 3.0	0.1 $\pm$ 0.2
β -Lactoglobulin-IgE (Bos d 5.0101)		0.8 $\pm$ 2.2	0.4 $\pm$ 0.9	1.5 $\pm$ 3.8	0.2 $\pm$ 0.4
IgG	mg/l				
α -Lactalbumin-IgG		4.6 $\pm$ 4.4	4.9 $\pm$ 5.9	4.2 $\pm$ 2.3	2.8 $\pm$ 0.8
β -Lactoglobulin-IgG		5.3 $\pm$ 6.2	4.3 $\pm$ 5.0	3.1 $\pm$ 3.2	2.1 $\pm$ 0.5
Casein-IgG		11.4 $\pm$ 18.4	9.5 $\pm$ 12.8	12.8 $\pm$ 24.0	7.0 $\pm$ 5.3
IgG4	mg/l				
α -Lactalbumin-IgG4		0.9 $\pm$ 2.5	1.8 $\pm$ 3.2	1.3 $\pm$ 1.9	0.5 $\pm$ 0.7
β -Lactoglobulin-IgG4		1.2 $\pm$ 3.5	1.6 $\pm$ 3.9	0.3 $\pm$ 0.5	0.3 $\pm$ 0.5
Casein-IgG4		2.6 $\pm$ 7.8	3.0 $\pm$ 6.5	2.1 $\pm$ 4.0	1.2 $\pm$ 1.4

New Table 3: Selected sIgE levels (COX-Regression)

	Hazard ratio	95% Confidence interval	p-value
CM-IgE	0.25	0.12-0.56	0.0006
✓Lactalbumin IgE	0.14	0.05-0.35	3.1e-5
⊗Lactalbumin IgE	0.37	0.16-0.85	0.0197
✱Casein IgE	0.26	0.08-0.84	0.025
α_{s1} -casein IgE	0.36	0.15-0.88	0.025

Fig.: 1

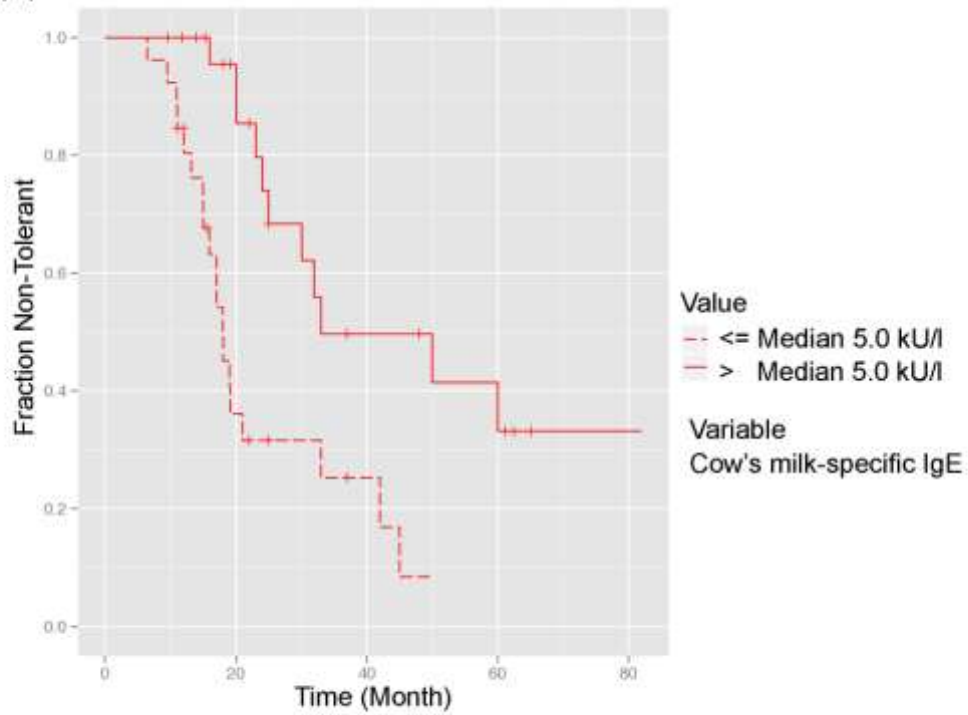


Fig.: 2

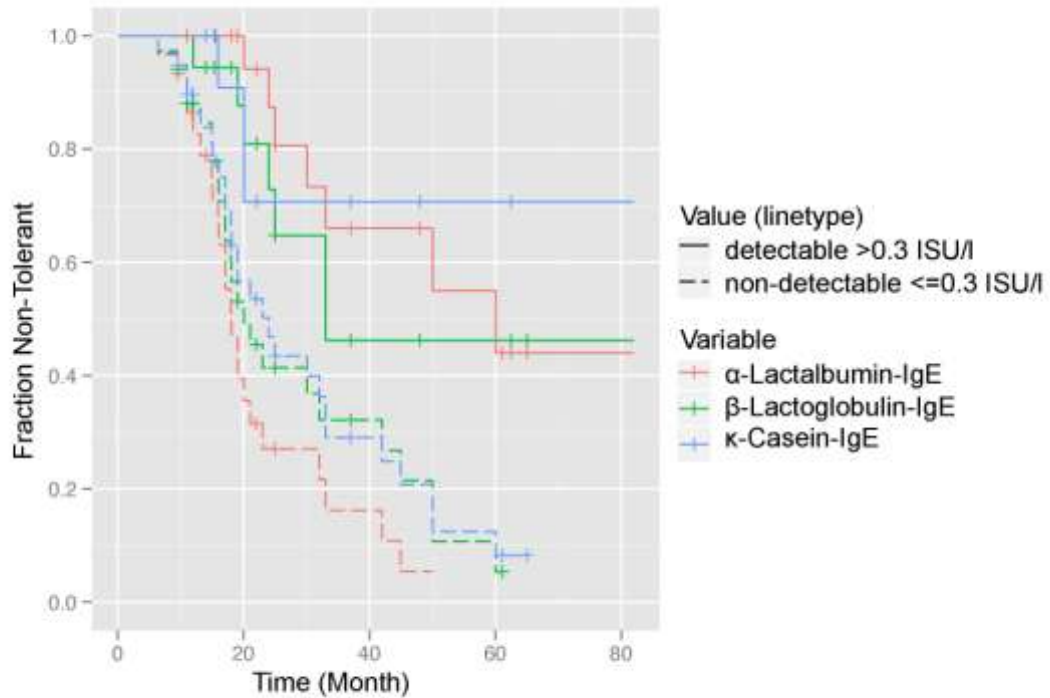


Fig.: 3

