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Immunobiology

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## Breastfeeding and IL-10 levels in children affected by cow's milk protein allergy: A retrospective study

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### ARTICLE INFO

#### Article history:

Received 5 August 2016

Received in revised form 30 August 2016

Accepted 7 September 2016

Available online xxx

#### Keywords:

Breast-feeding

Cow's milk protein allergy

Interleukin-10

Immune tolerance

### ABSTRACT

**Aim:** To assess the protective role of breast-feeding in infants with CMPA-related AEDS as well as IL-10 utility as marker of disease evolution.

**Methods:** 64 breast-feeding children with CMPA-related AEDS (31 males and 33 females; mean age  $5.56 \pm 2.41$  months; 21 mild AEDS; 25 moderate AEDS; 18 severe AEDS) and 60 artificial feeding babies (33 males and 27 females; mean age  $6.01 \pm 2.08$  months; 26 mild AEDS; 19 moderate AEDS; 15 severe AEDS) were evaluated. In all patients serum IL-10 levels were detected.

**Results:** Significant Score Atopic Dermatitis (SCORAD) index point differences between breastfed and not breastfed children ( $p < 0.001$ ) have been detected. The serum IL-10 levels were lower in children with CMPA-related AEDS as compared to the healthy control group ( $p < 0.001$ ). Moreover, a significant inverse correlation between serum IL-10 levels and SCORAD in both enrolled groups has been also noted. In particular, IL-10 levels, in both groups, were significantly lower in children with severe symptoms. Conversely, serum IL-10 levels were significantly increased in children with mild-severe symptoms in both groups. Furthermore, breastfed children, with lower severe symptoms, had higher serum IL-10 levels. Finally, serum total IgE levels were negatively correlated with serum IL-10 levels in both breastfed and non-breastfed children with CMPA-related AEDS ( $p < 0.001$ ).

**Conclusions:** We reported that exclusive breast-feeding induces hyposensitization in children with CMPA-related AEDS and it is associated with minor disease severity and higher serum IL-10 levels, resulting as useful disease-monitor marker.

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### 1. Introduction

Cow's milk protein allergy (CMPA) is the leading cause of food allergy in infants and young children younger than 3 years (Fiocchi et al., 2010; Koletzko et al., 2012), with pleiotropic clinical presentation (e.g., cutaneous, gastrointestinal, and respiratory) of variable intensity in infants (Fiocchi et al., 2010; Koletzko et al., 2012; Katz et al., 2010). Although its prevalence is approximately 2%–7.5% in the infant population (Fiocchi et al., 2010), the incidence falls to <1%

in children 6 years of age and older (Yavuz et al., 2013). CMPA results from an immunological reaction to one or more milk proteins and it may be both immunoglobulin (IgE) mediated and non-IgE mediated, as well as mixed (Fiocchi et al., 2010; Koletzko et al., 2012; Katz et al., 2010).

The aetiology of CMPA is still unclear. Positive family history for atopy, genetic predisposition, time of introduction of cow's milk, the permeability of gastrointestinal tract, and environmental factors have been suggested as possible cumulative causes contributing to disease development (Fiocchi et al., 2010; Koletzko et al., 2012; Katz et al., 2010).

It is becoming evident that multiple factors, through modulation of immune response, can influence the development of CMPA (Yavuz et al., 2013). On this regard, several studies have examined

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the possible benefits of breast-feeding in comparison to cow's milk formula-fed on food allergy in children (Greer et al., 2008). Breast milk is known to present the following characteristics: (i) contains a minor amount of CMP (Fiocchi et al., 2010), (ii) inhibits food antigen absorption, (iii) and finally acts as immune-modulator of gut flora (Greer et al., 2008; Kuitunen et al., 2012; Castellazzi et al., 2013).

In fact, breast milk contains a multitude of immunologically active components, including interleukin (IL)-10, a major regulatory cytokine of inflammatory responses, which, by promoting the induction of Th-1 and the suppression of Th-2 function, helps infants in the essential immune adaptations (Fiocchi et al., 2010; Katz et al., 2010). In particular, IL-10 even influences the regulation of tolerogenic microenvironment, inducing the maintenance of immune tolerance to food antigens (Berni Canani et al., 2015; Leonardi et al., 2015; Manti et al., 2015).

In this retrospective study, we evaluated if breast-feeding can represent a protective factor in CMPA-related atopic eczema/dermatitis syndrome (AEDS) children. Moreover, we measured serum IL-10 levels from breastfed and not breastfed infants with CMPA, during symptomatic and asymptomatic periods, to evaluate its utility as marker of disease evolution.

## 2. Materials and methods

### 2.1. Subjects and experimental design

A total of 124 children, born full-term and aged 2–36 months old, who had been referred to the Section of Immuno-Allergology, Genetics and Pediatric Immunology Unit, Department of Pediatrics, University of Messina, between September 2013 and December 2013, were enrolled in the study.

Subjects were eligible if they were affected by CMPA without alarm symptoms, assessed according to the NICE (Baker, 2013), positive cow's milk challenge test and skin prick tests (SPT) (whole cow's milk or at one cow's milk protein such as alpha-lactalbumin, casein, beta-lactoglobulin) or determination of serum total IgE levels. The severity of AEDS was evaluated according to Score Atopic Dermatitis (SCORAD) index (Anon, 1993).

Exclusion criteria included: associated diseases (hepatic, infectious, or endocrine diseases, genetic syndromes, immunodeficiencies, neurological and psychiatric problems), any infection within one month before the start of the study, other skin diseases, positive SPT for additional food allergens, gastrointestinal (chronic diarrhoea and/or vomiting, gastroesophageal reflux disease, constipation, colic, failure to thrive, enterocolitis syndrome) and respiratory (coughing and wheezing) comorbidities, and use of any treatment, capable of interfering with the results (corticosteroid) in the previous month.

Participants with CMPA-related AEDS were subdivided in two groups: breastfed or not. In particular, 64 (31 males and 33 females; mean age  $5.56 \pm 2.41$  months; 21 mild AEDS; 25 moderate AEDS; 18 severe AEDS) were enrolled in the breastfed with CMPA group; 60 (33 males and 27 females; mean age  $6.01 \pm 2.08$  months; 26 mild AEDS; 19 moderate AEDS; 15 severe AEDS) were enrolled as non-breastfed group with CMPA.

73 healthy children (39 males and 34 females; mean age was  $5.51 \pm 2.93$  months) were enrolled as healthy controls (control group). Apart from CMPA-related AEDS, inclusion/exclusion criteria were identical to those used for the CMPA-related AEDS group.

Written informed consent was obtained from the parents and informed assent from the children and adolescents.

A physician performed the physical examination of each child. Demographic data and other clinical characteristics of the study subjects were collected using questionnaires. It has been also measured of each child's length/weight.

### 2.2. Skin prick test and IgE measurement

Skin Prick Test (SPT) was performed on the volar aspect of the lower arms by using a panel of aero- (including: house dust mite, mixed grass, Parietaria, birch, olive tree, Alternaria, cat, and dog) and food-allergens (casein,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin) (Allergopharma, Reinbek, Germany). Normal saline and histamine were used as negative and positive controls, respectively. Skin wheal diameter was recorded at 15 min as the mean of 2 perpendicular measurements. A positive response was defined as a skin wheal diameter of 3 mm or more compared to negative control (Heinzerling et al., 2013).

Total serum IgE levels were also determined in all subjects by ImmunoCAP100 system (Phadia, Uppsala, Sweden).

### 2.3. Oral challenge test

The children were well and systemic drugs were discontinued at least 15 days previously. Patients were admitted to the hospital for 8 days (Vázquez-Ortiz et al., 2013; Meglio et al., 2004; Vandenplas et al., 2007). The starting dose was a drop of the milk formula on the lips. If no reaction was noted after 15 min, the formula was orally give and the dose was increased as follows: 0.5, 1, 3, 10, 30, 50, 100 ml every 30 min. Afterwards, children received 250 ml of cow's milk-based formula each day for 7 days. The challenge was considered negative or positive with objective symptoms. If the challenge resulted positive, children underwent elimination diet. Patients that did not develop symptoms during the challenge test, resumed normal diet, although they remained under observation. Not all subjects completed the study.

When resumed CMP in their diet, although in presence of negative oral challenge test, 14 patients (5 breastfed children and 9 not breastfed children) reported skin symptoms. They were enrolled in the A sub-group (patients with negative oral challenge test and relapse).

Finally, after the elimination diet, 16 patients (7 breastfed children and 9 non breastfed children) did not show symptoms improvement, thus, they did not undergo oral challenge test and maintained the CMPA elimination diet. They were enrolled in the B sub-group (patients did not undergo oral challenge test).

### 2.4. Assay of serum cytokines

IL-10 Enzyme Linked Immunosorbent Assay (ELISA) kits was used by a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) method on microtiter plate. The assays used monoclonal antibodies directed against epitope of IL-10. Standards (78–5000 pg/ml) and samples reacted with the capture monoclonal antibody coated on microtiter well, a monoclonal antibody labeled with biotin and with a monoclonal antibody labeled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated-human IL-10 biotin-HRP, the microtiter plate was washed to remove unbound enzyme labeled antibody. Bound enzyme-labeled antibody was measured through a chromogenic reaction. Chromogenic solution (TMB) was added and incubated for 15'. The reaction was stopped with the addition of Stop Solution and the micro-titerplate was successively read at the 450 nm wavelength. The amount of substrate turnover was determined colourimetrically by measuring the absorbance, which was proportional to the IL-s concentration. A calibration curve was plotted and IL-s concentration in samples was determined by interpolation from the calibration curve. The use of EASIA reader (linearity up to 3 O.D. units) and sophisticated data reduction method (polychromatic data reduction) resulted in a high sensitivity at the low range and in an extended calibration range. Blood samples were then col-

**Table 1**  
The demographic, clinical and laboratoristic characteristics of the study groups.

| N              | Breastfed Infants with CMPA<br>64 | Non Breastfed Infants with CMPA<br>60 | Healthy Control Group<br>73 | P value |
|----------------|-----------------------------------|---------------------------------------|-----------------------------|---------|
| Gender M:F     | 31:33                             | 33:27                                 | 34:39                       |         |
| Age (months)   | 5.56 ± 2.41                       | 6.01 ± 2.08                           | 5.51 ± 2.93                 |         |
| SCORAD         | 52.19 ± 2.76                      | 54.91 ± 3.98                          |                             | <0.001  |
| Total IgE U/ml | 377.53 ± 9.76                     | 385.58 ± 13.02                        | 28.4 ± 5.38                 | <0.001  |
| IL-10 pg/ml    | 2.63 ± 0.28                       |                                       | 4.95 ± 0.5                  | <0.001  |

Plus–minus values are means ± SD.

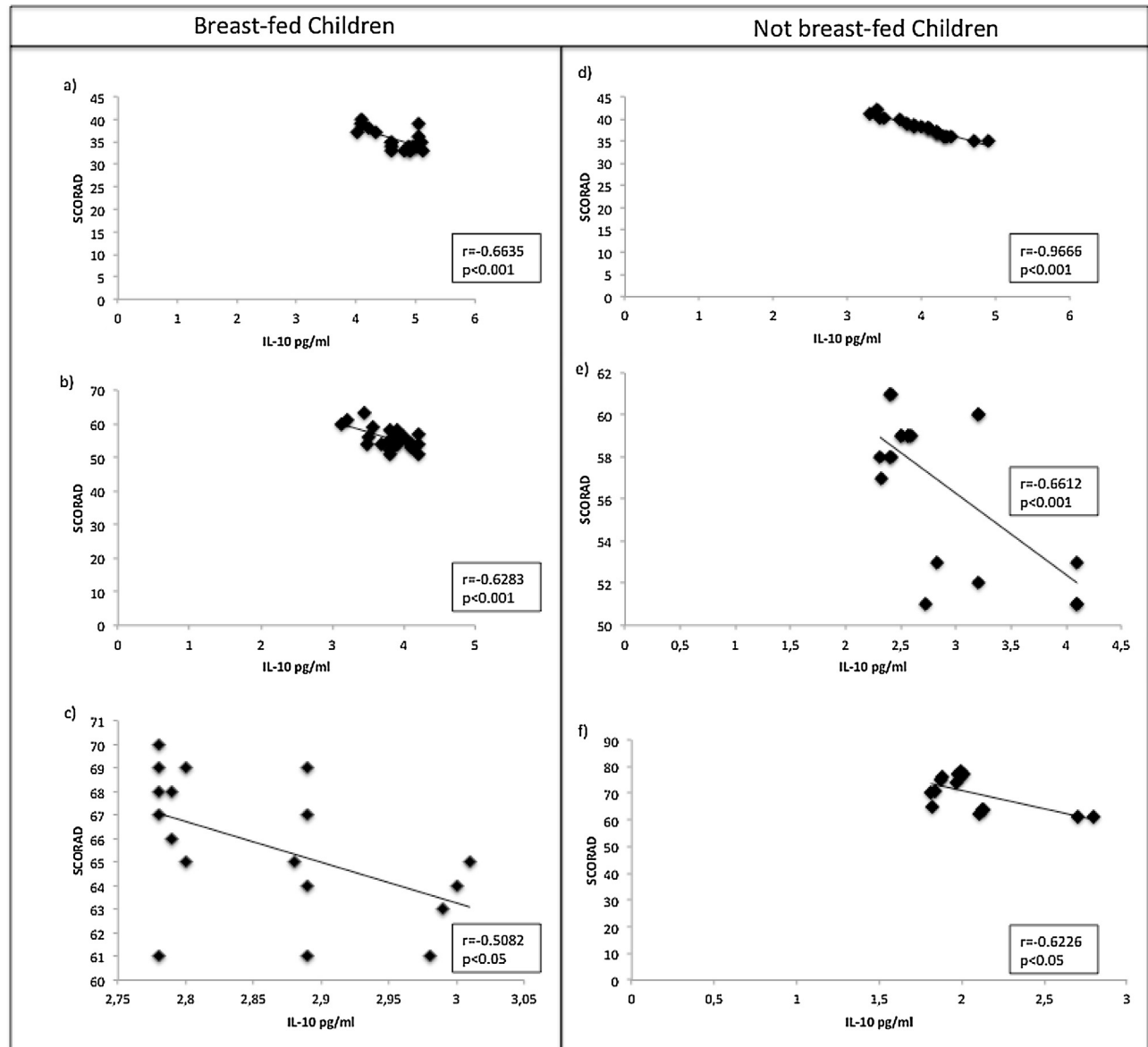


**Fig. 1.** Serum IL-10 levels and SCORAD index value relationship in mild (a), moderate (b) and, severe (c) AD CMPA-related infants with breastfeeding administration or not.

lected in 3 periods: at the time of CMPA diagnosis, 3 month after avoiding cow's milk (pre-challenge), and post-challenge, in order to measure in vitro cytokines response (IL-10) to cow's milk protein.

## 2.5. Data analysis

Data were tabulated using Excel 2003 (Microsoft Corp., Redmond, WA, USA). The data collected were statistically analyzed

by the statistical computer software SPSS, version 15.0. A p value of less than 0.05 was considered to be statistically significant. Mean and standard deviation were calculated for the variables and *t*-test was employed for the comparisons. For the attributes, percentages were calculated first and then  $\chi^2$  test was used for comparisons. Correlations between interleukin levels, disease severity and serum IgE levels were calculated using Pearson's test.

**Table 2**  
Serum IL-10 trend in breastfed and in non-breastfed children with CMPA at time of diagnosis, at pre-challenge time, and at post-challenge time.

|                                                     | Time of diagnosis | At pre-challenge time | At post-challenge time | Pvalue    |
|-----------------------------------------------------|-------------------|-----------------------|------------------------|-----------|
| Breastfed Infants with CMPA                         |                   |                       |                        |           |
| Number of patients remaining in the study over time | 64                | 57 <sup>a</sup>       | 52 <sup>b</sup>        | p < 0.001 |
| IL-10 pg/ml                                         | 3.04 ± 0.27       | 3.62 ± 0.32           | 4.77 ± 0.42            |           |
| Non Breastfed Infants with CMPA                     |                   |                       |                        |           |
| Number of patients remaining in the study over time | 60                | 51 <sup>a</sup>       | 42 <sup>b</sup>        | p < 0.001 |
| IL-10 pg/ml                                         | 2.23 ± 0.30       | 2.58 ± 0.41           | 3.17 ± 0.52            |           |

Plus-minus values are means ± SD.

<sup>a</sup> Nevertheless a negative oral challenge test, 14 patients (5 breastfed children and 9 non breastfed children) reported skin symptoms when resumed CMP in their diet (A sub-group).

<sup>b</sup> Nevertheless elimination diet, 16 patients (7 breastfed children and 9 non breastfed children) did not show symptoms improvement, thus, they did not undergo to oral challenge test and maintain CMPA elimination diet (B sub-group).

### 3. Results

The demographic, clinical and laboratoristic findings of infants with CMPA are summarized in Table 1. There were no significant differences between patients and subjects in accordance to gender and age (Table 1).

With reference to the characterization of disease severity in children with CMPA-related AEDS, we found significant SCORAD index point differences between breastfed and not breastfed children (52.19 ± 2.76 vs 54.91 ± 3.98 respectively,  $p < 0.001$ ).

Serum total IgE levels were significantly increased in both breastfed and non-breastfed groups (377.53 ± 9.76 U/mL and 385.58 ± 13.02 U/mL  $p < 0.001$ , respectively).

The serum levels of IL-10 were lower in children with CMPA-related AEDS as compared to healthy control group (2.63 ± 0.28 pg/ml respectively versus 4.95 ± 0.5 pg/ml;  $p < 0.001$ ) (Table 1).

Moreover, serum total IgE levels were negatively correlated with serum IL-10 levels in both breastfed children with CMPA-related AEDS (at the time of diagnosis:  $r = -0.4016$ ,  $p < 0.001$ ; at pre-challenge time:  $r = -0.4301$ ,  $p < 0.001$ ; at post-challenge time:  $r = -0.4042$ ,  $p < 0.001$ ) and non-breastfed children with CMPA (at the time of diagnosis:  $r = -0.53$ ,  $p < 0.001$ ; at pre-challenge time:  $r = -0.3866$ ,  $p < 0.001$ ; at post-challenge time:  $r = -0.5344$ ,  $p < 0.001$ ).

Moreover, we also detected a significant inverse correlation between serum IL-10 levels and SCORAD index (breastfed group: severe AEDS  $r = -0.5082$ ,  $p < 0.05$ ; moderate AEDS  $r = -0.6283$ ,  $p < 0.001$ ; mild AEDS  $r = -0.6635$ ,  $p < 0.001$ . Non-breastfed group: severe AEDS  $r = -0.6226$ ,  $p < 0.05$ ; moderate AEDS  $r = -0.6612$ ,  $p < 0.001$ ; mild AEDS  $r = -0.9666$ ,  $p < 0.001$ ) (Fig. 1). Precisely, IL-10 levels, in both groups, were significantly lower in children with severe symptoms. Conversely, serum IL-10 levels were significantly increased in children with mild-severe symptoms in both groups (Fig. 1).

In particular, at the time of diagnosis, comparing the two sub-groups, we even found that serum IL-10 levels were significantly lower in non-breastfed than in breastfed children with CMPA-related AEDS ( $p < 0.001$ ) (Table 2).

3 months after elimination diet (pre-challenge time), serum IL-10 levels were significantly increased in both groups.

Precisely, in breastfed children with CMPA-related AEDS, the serum IL-10 levels were significantly higher at 3 months as compared to the levels at the time of diagnosis (3.04 ± 0.27 pg/ml versus 3.62 ± 0.32 pg/ml, respectively;  $p < 0.001$ ) (Table 2).

At post-challenge time, the serum IL-10 levels were significantly higher as compared to the levels at 3 months (4.77 ± 0.42 pg/ml versus 3.62 ± 0.32 pg/ml, respectively;  $p < 0.001$ ) (Table 2).

In not breastfed children with CMPA-related AEDS, the serum IL-10 levels were significantly higher at 3 months as compared to the levels at the time of diagnosis (2.58 ± 0.41 pg/ml versus 2.23 ± 0.3 pg/ml, respectively;  $p < 0.001$ ) (Table 2).

At post-challenge time, the serum IL-10 levels were significantly higher as compared to the levels at 3 months (3.17 ± 0.52 pg/ml versus 2.58 ± 0.41 pg/ml, respectively;  $p < 0.001$ ) (Table 2).

On the contrary, in A e B subgroups, low or not increased serum IL-10 levels (2.47 ± 0.35 pg/ml versus 2.53 ± 0.58 pg/ml, respectively) were detected.

### 4. Discussion

In the present study, we compared a group of breastfed patients with not-breastfed children in order to evaluate the role of breast-feeding on CMPA-related AEDS.

Although several authors investigated the role of breastfeeding in the development of atopic diseases, the available data do not reach clear conclusions (Helm, 2014; Liao et al., 2014; MiceliSopo et al., 2014; Atanaskovic-Markovic, 2014; Järvinen et al., 2014; Flohr et al., 2014).

In our study, we have shown that breast-feeding is associated to a minor severity disease in CMPA-related AEDS children and it also promotes the systemic release of IL-10, reaching similar values to those found in control group.

These data are also in agreement with a recent observation that IL-10 has a central role in down-regulating inflammatory cascades, by modulating Th2 and allergic responses (Tiemessen et al., 2004; Ma and Yin, 2013). In accordance with these findings, herein, we reported lower serum IL-10 levels in artificial-feeding infants with more severe clinical symptoms. Conversely, higher serum IL-10 levels were recorded in breastfed children with lower severe symptoms.

These data also confirm the hypothesis that AEDS severity, in subjects affected by CMPA, might be associated with an impaired regulation of IL-10. During follow-up, we observed a statistically significant increase of IL-10 serum levels in breastfed children that underwent a positive open oral challenge test. Our results suggest that breast-feeding, with the participation of suppressor cytokines, such as IL-10, may induce hyposensitization in children affected by CMPA. These findings indicate that exclusive breast-feeding, conversely to artificial-feeding, promotes an anti-inflammatory cytokine, which is maintained during infancy.

Finally, our data indicate that IL-10 seems to play a key role in the immune balance of CMPA-related AEDS and it may become a useful marker of disease improvement monitoring. Additional studies with more numerous cohorts are warranted in order to validate these preliminary results.

### Informed consent statement

All study participants, or their legal guardians, provided informed written consent prior to study enrollment.

## Conflict-of-interest statement

The authors have no conflicts of interest related to the manuscript.

## Financial disclosure

The authors have declared no conflicts of interest.

## Funding/support

The Authors declare that the study was not supported by any grant support or funding sources.

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