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Maternal milk cytokines: potential immuno-modulatory role in infant *Giardia* infection.

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ABSTRACT

Little is known about constitution of cytokines in breast milk. The current study was designed to explore the transferable immune mediators (IFN-γ, TNF-α, IL-6 and IL-10) from mothers to their fetuses via breast milk and its effect on *Giardia* infection. ELISA assessed infant serum samples for levels of these cytokines in 25 breast-fed (group I) versus 25 non breast-fed infants (group II). The same was done on milk specimens from nursing mothers. Statistically significant higher mean levels of 4 cytokines were detected in infants' sera of group I (12.26±4.37, 15.97±4.38, 8.83±4.41 & 89.77±33.18 pg/ml), when compared to group II (7.49±6.63, 8.78±6.58, 2.87±2.62 & 28.42±19.87 pg/ml) for IFN-γ, TNF-α, IL-6 and IL-10 respectively. Furthermore in group II 24% (6/25), 20% (5/25) & 20% (5/25) infants' sera failed to show any detectable levels of IFN-γ, TNF-α, IL-6 respectively. Significant positive correlation noticed between nursing mothers' milk cytokines and their infants' serum levels in group I, confirming transferred passive immunity. Group I displayed lower parasitic burden, and ameliorated clinical manifestations versus group II. Significant negative correlation of IL-10 with the pro-inflammatory cytokine IFN-γ shown in group I. This study strongly denoted the role of IL-10 in limitation of clinical manifestations or successful elimination of *Giardia* parasite. In conclusions; the results of this study may provide scientific data to improve the supplementation of infant formula to mimic breast milk as closely as possible.

INTRODUCTION

Giardia lamblia is among the major parasites that infect humans and is the most common intestinal parasite diagnosed as a cause of childhood morbidity related to infantile diarrheal diseases (Addiss *et al*. 1992; Nkrumah and Nguah 2011). Although the greater part of infections with *Giardia lamblia* is asymptomatic, it also causes acute and chronic diarrheal illness (Sullivan *et al*.1988; Rauch *et al*. 1990).The relevant host- or parasite-related factors that determine whether an infection remains asymptomatic or results in diarrhea are unknown. In Egypt that was reported as hyper-endemic in *Giardia* infection (Rauch *et al*. 1990), the relation between breast-feeding and *Giardia* infections remains an important issue. Many studies demonstrated that breast-feeding is protective against infection in infants from 0-18 months old (Paramasivam *et al*. 2006; Mihrshahi *et al*. 2007; Edmond *et al*. 2007; Duijts *et al*. 2009; Walker 2010). Yet the mechanisms of protection, as well as effectiveness of such protection have not been recognized, and investigators in this field gave only contradictory results. Literature data indicated that parasitic invasions induce inflammatory response, with the involvement of T cells and release

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of inflammatory cytokines as IL-5, IL-6, IL-8, IL-10, TNF-α and IFN-γ (Matowicka-Karna *et al*. 2009).Cytokines or Interleukins are secreted by lymphocytes, monocytes or macrophages to act on other cells of the immune system and to regulate their function (Solaymani-Mohammadi and Singer 2010; Zeinab *et al*. 2014).

Cytokines are one of the important immunological mediators under constant study. They are multifunctional glycoproteins involved in cell communication and immune system activation and they are present in variable concentrations in human milk (Berdat *et al*. 2003; Schack-Nielsen and Michaelsen 2007).Unfortunately little is documented concerning important immunological aspects as cytokine constitutions of breast milk in different categories. Investigations on the interplay between mother and child after birth, including studies of the content of breast milk, are needed not only to fulfill our academic curiosity but will also lead to new targets and therapeutics to prevent or minimize infectious diseases. Exploring different immunological aspects in milk provides scientific support for the supplementation of infant formula for improvement to mimic breast milk and its functional effects as closely as possible. The aim of the current study was to assess the level of important immuno-mediators (interleukins) particularly, IFN-γ, TNF-α, IL-6 and IL-10 secreted in mothers' colostrum/milk in comparison to their babies' serum levels. It addition, to compare patterns and correlations of these cytokines in breast-fed versus non breast-fed babies.

Patients and Methods:

A hospital-based cross-sectional study was conducted on attendants at Breast feeding counseling clinic, Prophylactic Medicine Center, Abo Elreesh Japanese Hospital, Kasr Al-ainy hospitals for neonatal care services patients, Pediatric Department, Cairo University as well as outpatient clinic attendants at Pediatric Department, Faculty of Medicine, Al-Fayoum University. The study included infant /mother as a pair which randomly selected. The study population was categorized into; group I (gp I) that involved 25 breast-fed infants and group II (gp II) that included 25 non breast-fed infants. Stool and serum samples were collected from both mothers and infants. In addition milk/colostrum, samples were collected from lactating mothers. Cooperative nursing and non-nursing mothers with infants less than one year were included in this study. Immunocompromized, dehydrated infants, cases with circulatory insufficiency, cases who needed emergency or cases whose specimens proved to be positive for bacterial pathogens by routine microbiologic techniques, or for rotavirus or any other causes of infantile diarrhea other than *Giardia* were excluded from this study. Parents of all infants, included in the study, were informed about the purpose of the study, and the collection of samples was performed after obtaining written consent.

Data collection:

Structured questionnaire was used to collect data that involved demographic data; age, sex, occupation, nutritional status, lactation status, treatment status either current or previous. Also, clinical assessment was done. Relevant clinical manifestations were recorded. Severity of symptoms was ranked as described previously (Paniagua *et al*. 1997) grade I: mild, lasting for no longer than 3 days, no fever, no vomiting and good response to rehydration therapy; grade II, moderate, lasting for more than 3 days, with fever and/or vomiting and good response to rehydration therapy; a grade III, severe, diarrhea associated with fever, vomiting, severe dehydration and need for hospitalization. And considered cases of giardiasis apply with the following definition: "patients passing cysts in their feces and/or presenting with one or more of the clinical symptoms of giardiasis" in addition to cases with proven positive fecal sample to *Giardia* copro-antigen.

Sample collection and examination:

Fresh fecal specimens were collected from mothers and infants, not contaminated with water or urine in dry, clean, wide-mouth plastic containers with tight-fitting lids. The containers are transferred as soon as possible to the Lab to be microscopically examined by direct wet preparation and formal ether concentration as been described by (Garcia 2007) Part of the faecal sample was preserved in -20°C to be tested by an immuno-chromatographic (ICT) rapid assay (IVD RIDA®QUICK *Cryptosporidium*/ *Giardia* / *Entamoeba* Test) for the qualitative detection, and for exclusion of other parasitic infection. Furthermore determination of *Giardia lamblia* copro- antigens in stool samples was also performed by fecal direct fluorescent antigen (DFA) detection Kit. The Test procedure was done according to the manufacturer instructions. Briefly, 10% formalin-fixed specimens were tested for presence of copro-antigens of *Giardia* cysts by human fecal direct fluorescent antigen (DFA) detection Kit (IVD Research Inc. Carlsbad, CA 92010 USA) that utilizes mixture of Fluorescein isothiocyanate (FITC) -labeled monoclonal antibodies directed against *Giardia* cysts.

As regard milk specimens, all milk samples were collected from all nursing mothers using a clinically proved manual breast pump. Milk specimens were transported in a cooler on ice to the laboratory, where they were frozen and stored at - 70 °C till investigated. Also, approximately 2 ml of blood was collected from all participants in this study (both mother and infant) by venipuncture into centrifuge tubes. Obtained serum was divided into 4 eppendorf tubes, and preserved in – 20°C until serologically tested.

For detection of cytokine levels; mothers milk specimens and infants serum were all assessed by ELISA

assay, (Assay pro LLC -30 Triad South Drive, St. Charles, MO 63304, Missouri). This assay utilized a quantitative sandwich enzyme immunoassay technique that measured different human cytokines; IFN- γ, TNF-α, IL-6 and IL-10) using a polyclonal antibody specific for each cytokine that had been pre-coated onto a 96-well micro plate. The test was performed according to the manufacturer instructions. The intensity of the color was measured and OD values were calculated. The results were expressed as pg/ml, based on standard curves (Baqai 1996).

Results:

The current study conducted on 2 groups of infants aged less than one year, group I included 25 breast-fed infants, And group II included 25 non breast-fed infants. Their mothers were housewives of low socioeconomic and poor educational level and their age ranged from 22 to 34 years old.

Giardia infection was confirmed by different techniques, applying direct microscopic examination on concentrated faecal samples, and immunological assays detecting copro-antigen (ICT and DFA). Noticeable less number of *Giardia* infection in group I was detected, when compared to group II, 2 cases (8%), versus 6 cases (24%) in group I and group II respectively table 1. In addition, in group I: *Giardia* cysts or trophozoites were not observed microscopically in any of samples, however *Giardia* copro-antigen was detected by ICT & DFA in only 2 fecal samples, which were clinically asymptomatic denoting low parasitic burden. While in in group II: in (4/25) 16.0% *Giardia* infection was directly evidenced by microscopic examination in addition to additional 2 samples that reported positive results by immunoassays ICT & DFA. These 2 additional positive samples were related to infants, their ages more than 6 months table 1. Within the 6 *Giardia* positive cases detected in group II (4/6) were suffering from severe manifestation grade III, and the other (2/6) were manifested with moderate form grade II.

Table 1: shows results of detection of *Giardia* infections in infants of study groups either by direct microscopy after faecal concentration or by detection of *Giardia* copro-antigen using ICT and DFA

Infants study groups	Direct microscopy	Copro-Ag by Immunoassays (ICT & DFA)
Group I (Breast-fed)	0 (0.0 %)	2 (8.0%)
Group II (Non Breast-fed)	4 (16%)	6 (24.0%)

Concerning infantile serum levels of cytokines, detectable levels were obtained in serum of all infants related to group I. The mean values ± SD of different cytokines are identified in (table 2 and figure 1). The levels of all cytokines were significantly higher in breast-fed group I than non breast-fed group II. Group II showed variable levels in different cytokines, even undetectable levels in some samples in all cytokines except IL-10. For IFN- γ, no measureable levels were reported in 6 samples (25%), also for TNF-α, and IL-6, 5 samples (20%) for each cytokine obtained negative results (0=minimum). The maximum values of different cytokines were 18.9, 24.6, 27.10 and 212.00pg/ml for IFN- γ, TNF-α, IL-6 and IL-10 respectively, reflecting a wide variation range table 2.

Concerning level of cytokines in mothers' milk in group I in comparison to the serum of their babies, not only typical patterns in all cytokines were observed (figure 2a), but also positive significant correlations between infants sera & mothers milk cytokines levels were recorded (figure 2b, c, d & e), reflecting the passive transferred immunity from others to babies. On the other hand, no other significant correlation was noticed between different cytokines in both groups except for the IL-10, which recorded significant negative correlation between IL10 and IFN **γ** in breast fed infants (group I), and not in non-breast fed infants (group II) (figure 3).

Table 2: shows calculated mean values and standard deviations and P values for all cytokines in infantile serum, in both studied infants groups breast-fed group as well as non-breast-fed group

	IFNγ		TNFα		IL6		IL10	
	Breast Fed Infants (pg/ml)	Non Breast Fed infants (pg/ml)	Breast Fed Infants (pg/ml)	Non Breast Fed infants (pg/ml)	Breast Fed Infants (pg/ml)	Non Breast Fed infants (pg/ml)	Breast Fed Infants (pg/ml)	Non Breast Fed infants (pg/ml)
Mean	12.35	6.45	16.40	8.19	8.83	2.88	89.77	28.42
SD	4.20±	5.50±	4.30±	5.55±	4.41±	2.63±	33.18±	19.87±
Minimum level	4.70	0.00	9.09	0.00	4.80	0.00	53.70	8.23
Maximum level	18.90	16.90	24.6	15.30	27.10	8.52	212.00	78.00
P value	0.0004		< 0.0001		< 0.0001		< 0.0001	

Discussion:

There is an increasing concern in the effects of breast-feeding on the immunological status of the offspring and in exploring the mechanisms behind these effects (Schack-Nielsen and Michaelsen 2007).Pathophysiological effects of cytokines are well described, yet reference values for infants are indefinite (Berdat *et al*. 2003; Schack-Nielsen and Michaelsen 2007). Broadly, data concerning such issue is very

restricted and variable. A study of (Wallace *et al*. 1997) who measured levels of 2 cytokines granulocyte-colony stimulating factor and IL-6 in 30 milk samples of healthy nursing mothers. They recorded an extremely wide range from 14 pg/mL to 197 > 2500 pg/mL for the first cytokine, whilst in IL-6, milk contained levels from 0.81 pgmL to 306 pg/ mL. The current study was designed in trial to explore the transferable immune mediators from mothers to their fetuses via breast milk and its effect on *Giardia* infection. Therefore, the level of some important immune mediators (interleukines) as IFN-γ, TNF-α, IL-6 and IL-10 in mothers' colostrum/milk in comparison to their babies' serum levels was measured using ELISA technique. In addition, the patterns and correlations of these cytokines in breast fed versus non breast-fed infants were analyzed.

The results of the present study not only reflected the significant higher levels in all cytokines in breast-fed group I than non breast-fed group II. But also showed the failure of immune system in some infants of non breast-fed group II to demonstrate any detectable level of some cytokines in their sera. Unfortunately and as mentioned before, there are no reference levels of cytokines in infants. However, there was similar pattern and significant positive correlation noticed between nursing mothers' milk cytokines and their infants serum levels in group I, which may strengthen the documented scientific fact concerning transferred passive immunity. Particularly in breast-fed infants studied, who displayed lower parasitic burden, and ameliorated clinical manifestations versus non breast fed infants in group II, in addition they showed higher number of cases (6/26, 24%), and were severe to moderate clinical forms of giardiasis. This is more or less in accordance to what recorded by (Wallace *et al*. 1997) who concluded that, cytokines play a role not only in neonatal growth and development, but may also protect the infant against subsequent infection. Transferred passive immunity via milk as well studied by (Schack-Nielsen and Michaelsen 2007), who emphasized the importance of short-term immunological benefit of breast-feeding on the protection against infectious diseases, suggesting that breast-feeding influences the development of the infant's own immune system. This also explained the measurable levels of all cytokines in breast-fed groups in our work, confirming the potentiation of transferred immune mediators for infants' immune system. While about one quarter of non breast-fed infants failed to demonstrate any detectable levels in three cytokines (IFN-γ , TNF-α and IL-6).

In the present study, in spite that *Giardia* cysts or trophozoites were not observed microscopically in any of the 25 faecal samples of breast-fed infants (group I), *giardia* antigen was detected by ICT in 2 asymptomatic cases out of 25 faecal samples. While in non breast-fed infants (group II), 6 out of 25 cases were positive to *Giardia* infection, 4 of them were directly evidenced by microscopic examination of faecal samples, and another 2 samples reported positive by ICT. These 6 cases suffered severe (grade III) to moderate (grade II) forms of clinical manifestations. This observation confirmed what was mentioned above concerning transferred parasitic and clinical partial immunity. This was in accordance with (Mahmud *et al*. 2001) who followed a total of 152 infants from birth to 1 year of age in a rural community of Egypt. The authors recorded lower risk for both asymptomatic and symptomatic infections in addition to milder clinical manifestations among exclusively breast-fed infants compared with infants who were not exclusively breast-fed. Generally, there is reported controversy concerning role of Th1 & Th2 immune responses in *Giardia* infection (Jiménez *et al*. 2014) reported the importance of mixed response Th1 and Th2 in *Giardia* infection, while (Zeinab *et al*. 2014)denied completely the value of both Th1 & Th2 in clearance of *Giardia* parasite. Nevertheless (Singer and Nash 2000) recorded delay in *Giardia* clearance in interferon-gamma deficient animals, when compared to controls, in accordance to our results that showed a significant higher IFN-γ level in serum of infants in breast fed infants versus non breast fed infants, which suggesting that Th1 response may be more substantial in controlling *giardia* infection.

Moreover, the significant negative correlation of IL-10 with the pro-inflammatory cytokine IFN-γ shown in breast fed infants in this study strongly denoted immune modulatory role of IL-10 evident by limitation of clinical manifestations or successful elimination of *Giardia* parasite (detection of parasitic copro-antigen in 2 asymptomatic infants table 1). Likewise, (Walker 2010) recorded that IL-10 among several components of breast milk that can reduce the inflammatory response to stimuli in the newborn intestine. These components of breast milk can act individually or in harmony with others to control the neonatal immature inflammatory response. Furthermore (Belderbos *et al*. 2012) reported a significant high level of IL-10 in serum of breast-fed infants and concluded that, the protective effect of breastfeeding against subsequent infections may be explained by its innate immune modulatory effects in the first month of life.

In conclusion, breast-feeding has been shown to enhance not only the development of the immune system of the newborn but also develop immuomodulatory mechanisms as well as provide protection against *Giardia* infections. Besides, implementation of breast-feeding programs has the potential to save hundreds of thousands of lives worldwide. Our understanding of the importance of immune components in breast milk in immune development and regulation is still in its' initial phases. This will be a fertile area of additional research for nutritionists for many years.

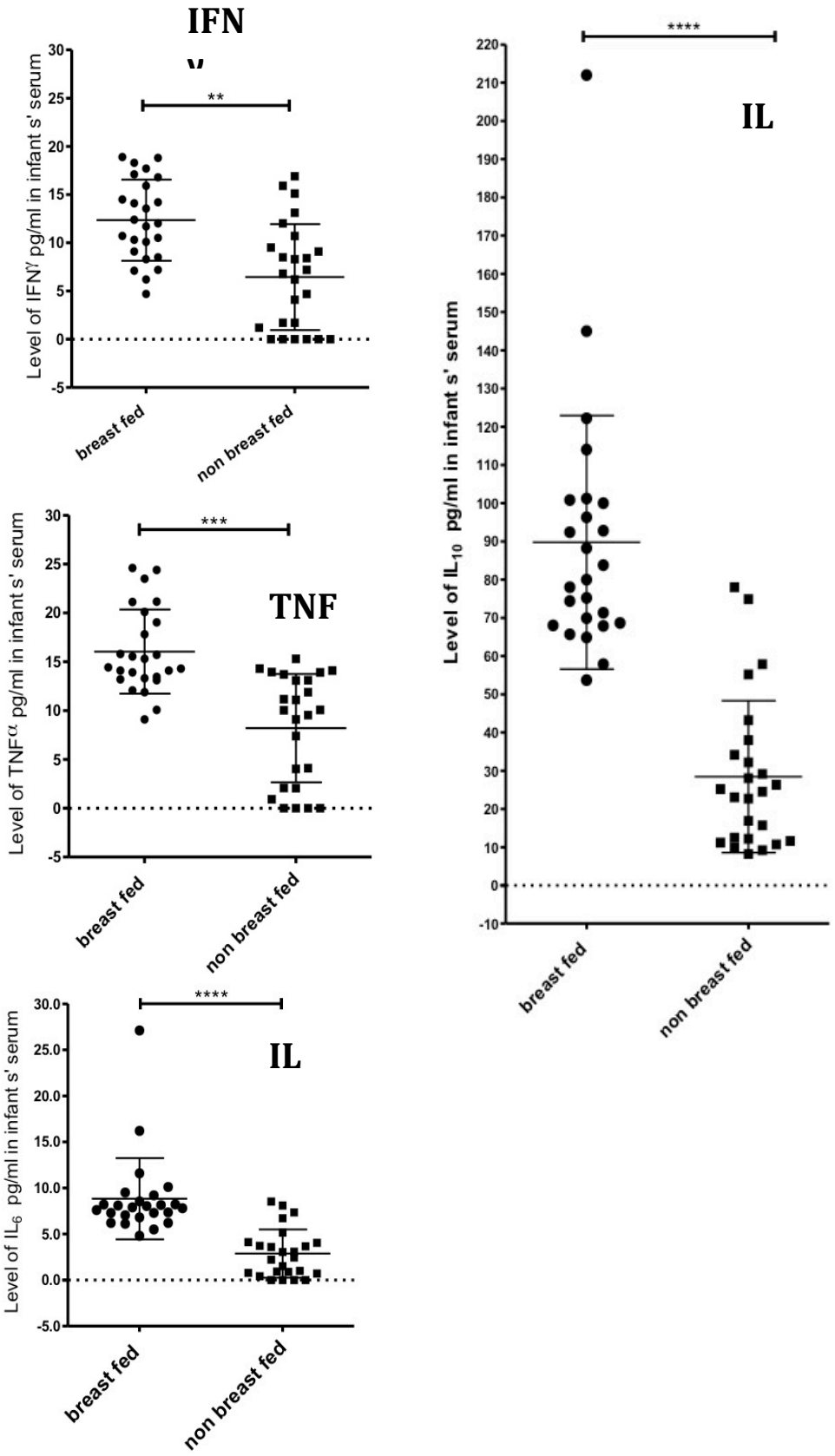
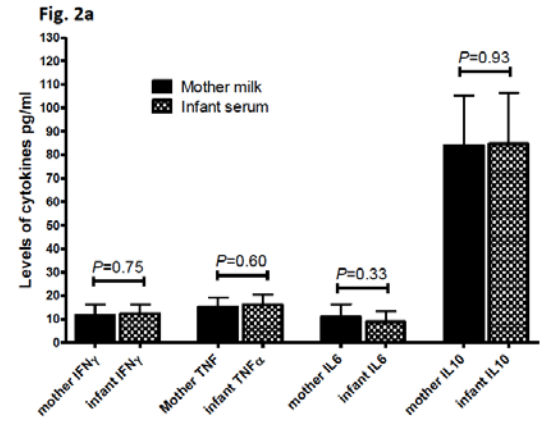
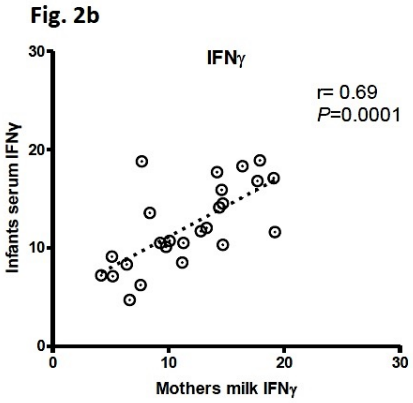
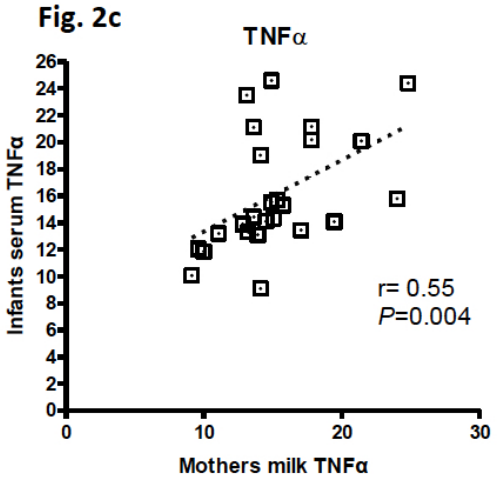
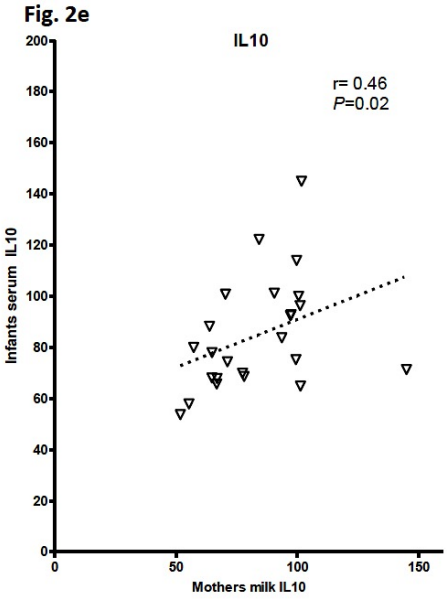


Fig. 1: shows infantile serum levels of all cytokines (A: IFNγ, B: TNFα, C: IL6 & D: IL10) in both breast-fed group & non breast-fed infants group, with strongly statistical significant higher cytokines levels in breast-fed group compared to non breast-fed group. N.B. significance strength shown *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001









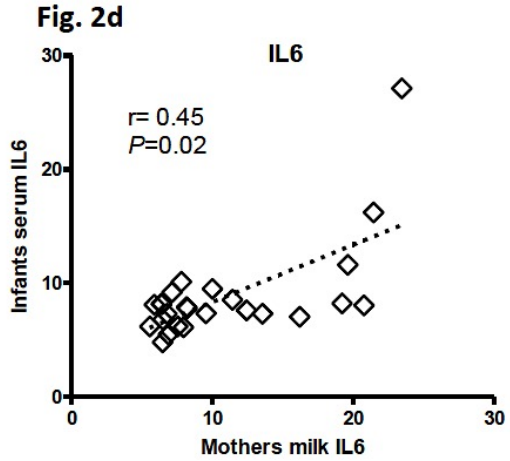


Fig. 2a: shows levels of different cytokines in mothers' milk compared to their infants' serum levels, revealing the same pattern in both, and with no significant difference.

Fig. 2b, c, d: & e show positive significant correlations in studied cytokines between mother's milk & serum infant's levels. Shown *P values* were calculated by Mann-Whitney t test. Correlations calculated by spearman r test.

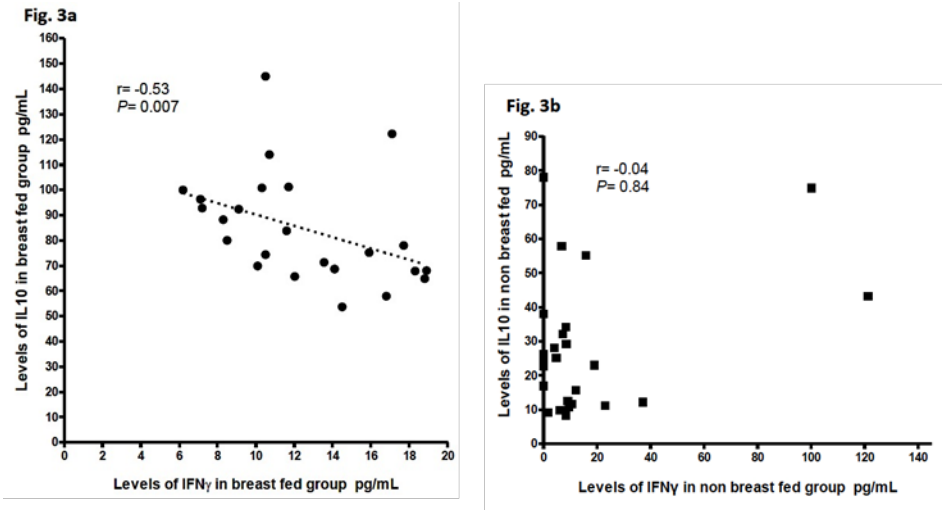


Fig. 3: shows correlation between infantile serums IFN γ & IL10 in both study groups, revealing strong statistically significant negative correlation in breast-fed group, not in non-breast-fed group. Correlation *P values* calculated by Spearman r test.

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