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**Breast milk cytokine and IgA composition differ in Estonian and Swedish mothers -  
relationship to microbial pressure and infant allergy**

*Running title:* Milk cytokines & IgA in Estonia & Sweden

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## **Abstract**

The immune system of the neonate is influenced by maternal immunity during pregnancy and lactation. An altered microbial exposure, possibly underlying the increase of allergic diseases in affluent societies, may affect maternal breast milk immune composition. Secretory IgA (SIgA), IL-4, IL-10, IL-13, IFN- $\gamma$ , TGF- $\beta$ 1 and TGF- $\beta$ 2 were analysed with ELISA in colostrum and 1-month mature milk from mothers from Estonia (n=39) and Sweden (n=60), two geographically adjacent countries with different living conditions and allergy incidence. The IL-10 and IFN- $\gamma$  levels were higher in colostrum from Estonian than Swedish mothers, whereas the opposite was true for TGF- $\beta$ 2. In mature milk, higher SIgA and IFN- $\gamma$  levels but lower TGF- $\beta$ 1 and TGF- $\beta$ 2 levels were observed in Estonian than Swedish mothers. Interestingly, **in Sweden but not Estonia, the** TGF- $\beta$ 1 and TGF- $\beta$ 2 levels correlated inversely with environmental endotoxin concentrations, whereas positive correlations to microbial load were observed for IL-4, IL-10 and IFN- $\gamma$ . High colostrum IL-13 levels were associated with allergic sensitisation during infancy in Sweden. In conclusion, Estonian mothers have lower breast milk levels of TGF- $\beta$ , particularly TGF- $\beta$ 2, but higher levels of SIgA, IL-10 and IFN- $\gamma$  than Swedish mothers, possibly due to differences in microbial load.

**Key words:** breast milk; cytokines; IgA; endotoxin; allergy

## **Abbreviations**

SIg            secretory immunoglobulin

SPT           skin prick test

## Background

Mucosal surfaces are the main entry for most environmental antigens, *e g* infectious agents and allergens. An integrated mucosal immune network of lymphoid and non-lymphoid cells and effector molecules, such as secretory IgA (SIgA), cytokines and chemokines, is needed for host protection against pathogens and tolerance to commensal bacteria and innocuous antigens (1). The neonatal period is critical in this respect, as the newborn baby is immediately exposed to numerous micro-organisms and foreign proteins. The mucosal immune system is poorly developed at birth, however, with only traces of SIgA and SIgM in exocrine glands, not reaching adult levels until several years after birth (2). Thus, an adequate supply of breast milk is essential for an optimal mucosal barrier function during the first months of life (3).

Breast milk contains numerous immunological components that compensate for the immature and inexperienced mucosal immune system of the infant (4), including immune cells, antibodies (especially IgA antibodies), pro- and anti-inflammatory cytokines such as TNF, IL-10 and TGF- $\beta$  (5) and factors that may modify immune responses to bacteria, *e g* soluble CD14 (6). Through the integrated mucosal immune system, immune responses induced in the gut and airway mucosal-associated lymphoid tissue are reflected in other secretory tissues, *e g* the mammary gland (3). Thus, primed B- and T-cells are directed from inductive mucosal sites to other glandular tissues through the expression of specific adhesion molecules or “homing receptors” (1). Consequently, the immunological composition of breast milk may vary considerably in mothers from different environments (3). Other factors suggested to affect the composition of breast milk include maternal allergy (5, 7), infections (8), mastitis (9), stress (8), delivery by Caesarean section (8) and supplementation of fish oil (10) and probiotics (11) during pregnancy. The controversial results regarding the role of breast-

feeding for prevention of allergy development have been suggested to be partly due to differences in breast milk composition (12). For instance, high levels of breast milk IgA (13-15) and TGF- $\beta$  (16, 17) have been suggested to affect the allergy development in the child, although this is still controversial (15, 18-20).

We have previously shown that the microbial load differs between Estonian and Swedish populations, both with regard to the commensal gut flora (21) and to environmental endotoxin levels (22). As environmental microbes are the major stimuli of the immune system, the immune composition of breast milk may differ between mothers living in regions with different microbial burden. An increased microbial load is associated with a reduced prevalence of atopic diseases (22, 23). The aim of this study was therefore to investigate the breast milk content of atopy related cytokines, *i e* IL-4, IL-13 and IFN- $\gamma$ , anti-inflammatory cytokines, *i e* IL-10, TGF- $\beta$ 1 and TGF- $\beta$ 2, and SIgA in Estonian and Swedish mothers in relation to environmental endotoxin levels and development of sensitisation and eczema in the breast fed infants.

## **Material and Methods**

### *Subjects*

Samples of colostrum were obtained as soon as milk production started, within the first four days after delivery and mature milk samples were obtained one month after delivery from 39 Estonian and 60 Swedish mothers. The mothers participated in a prospective study regarding allergy development in children in relation to life style factors, as described in detail previously (24). The allergic history of the mothers was recorded by a research nurse during an initial interview. Fourteen (36%) of the Estonian and 29 (48%) of the Swedish mothers reported a history of allergic symptoms, *i e* allergic rhinoconjunctivitis, asthma and/or eczema (Table 1). The high number of allergic mothers was due to the increased interest among these mothers to participate in studies focusing on allergy. Except for allergy, none of the mothers had any autoimmune or other chronic disease. All mothers delivered their babies at term and the perinatal period was normal. None of the Estonian and three of the Swedish mothers were delivered by Caesarean section. The mean duration of exclusive breast-feeding was 2.8 months (range 0.3-6.3 months) in the Estonian mothers and 3.9 months (range 0.3-7.0 months) in the Swedish mothers. For partial breast-feeding, the mean duration was 7.3 months (range 1.0-24 months) in the Estonian mothers and 9.4 months (range 2.0-24 months) in the Swedish mothers. The milk samples were collected in sterile, plastic tubes, using a manual breast-pump. The samples were immediately frozen and stored at -20°C until analysis.

Skin prick tests (SPT:s) were performed at all follow-ups in duplicate at the volar aspects of the forearms with fresh skimmed cow's milk and thawed egg white at 3, 6, 12 and 24 months. At 12 months, the children were also tested with cat allergen extract and in Estonia also with mite allergen extract (*Dermatophagoides pteronyssinus*). At 24 months, birch and timothy



allergen extract was added to the panel, and in Estonia also cockroach (*Blatella germanica*) allergen. All extracts were standardised allergen extracts from ALK (Soluprick®, ALK, Hørsholm, Denmark), except the cockroach extract, which was from Bayer (Spokane, WA, USA). Histamine hydrochloride, 10 mg/ml, was used as positive control and glycerol as negative. The test was regarded as positive if the mean diameter of one of the wheals was at least 3mm. The cumulative incidence of positive SPT up to 24 months of age was 8% (3/39) among the Estonian infants and 25% (13/53) among the Swedish infants. Seven of the Swedish infants did not complete the study protocol up to 24 months of age, while all the Estonian infants did (Table 1).

The presence of IgE antibodies to egg white and  $\beta$ -lactoglobulin were determined in serum samples collected at all follow-ups up to 24 months, and IgE antibodies to cat and birch allergens were analysed at 12 and 24 months by a commercial chemiluminescence method, according to the recommendations of the manufacturer (Magic Lite™, ALK). The test was regarded as positive at >1.43 SU/ml. The Swedish children were regarded as sensitised if they had a positive SPT and/or detectable circulating allergen specific IgE antibodies, and the cumulative incidence of sensitisation up to 24 months of age was 34% (18/53). This definition was not applicable in the Estonian children, since low levels of circulating IgE antibodies is commonly detected and are poorly related to allergy and SPT positivity in these children (24). Consequently, the three SPT positive Estonian children were defined as sensitised.

A clinical examination regarding development of eczema was done by a pediatric allergologist when the infants were 24 months old. Eczema was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution, as

described previously (24). The cumulative incidence of eczema up to 24 months of age was 8% (n=3) among the Estonian infants and 19% (10/53) among the Swedish infants (Table 1).

#### *Cytokine, SIgA and endotoxin analysis*

Before analysis, the breast milk fatty layer and cellular elements were removed as previously described (5). Interleukin-4, IL-10, IL-13, IFN- $\gamma$ , TGF- $\beta$ 1, TGF- $\beta$ 2 and SIgA were analysed as previously described (5). The detection limits were 5.6 pg/ml for IL-4, 4.8 pg/ml for IL-10, 3.2 pg/ml for IL-13, 15.6 pg/ml for IFN- $\gamma$ , 62 pg/ml for TGF- $\beta$ 1 and TGF- $\beta$ 2 and 30  $\mu$ g/ml for SIgA.

Dust samples were collected from a carpet in each household with a dust collector device, containing a 6- $\mu$ m pore size filter connected to a vacuum cleaner (22). Two square meters of the carpet was vacuum cleaned for four minutes. The filters were stored in sterile plastic bags in -20°C until analysis. The endotoxin concentration was determined by a chromogenic Limulus assay (QCL-1000®, Bio Whittaker, Walkersville, ND, USA) as described earlier (22). The lowest detection limit was 0.50 EU/mg dust.

#### *Statistical analysis*

The Mann Whitney *U*-test was employed for unpaired analyses, and Spearman's rank order correlation coefficient test for correlation analyses. The chi-square test was employed for categorical variables, and the Fisher's exact test was used when the expected frequency for any cell was less than five. Differences combined with *p* values less than 0.05 were considered statistically significant. The calculations were performed with the statistical packages StatView version 5.9 for PC (SAS Institute Inc, NC, USA).

*Ethical aspects*

The study was approved by the Ethics Review Committee on Human Research of the University of Tartu and the Human Research Ethics Committee at the Faculty of Health Sciences, Linköping University. The parents of all children gave their written informed consent.

## Results

The TGF- $\beta$ 2 levels were lower in colostrum and mature milk from Estonian than Swedish mothers (fig 1), while TGF- $\beta$ 1 and levels were lower only in mature milk (fig 1). In contrast, the SIgA levels were higher in Estonian than Swedish mothers (fig 2). As the frequency of samples with detectable levels of IL-4, IL-10, IL-13 and IFN- $\gamma$  was less than 35%, the number of samples with detectable levels of these cytokines, rather than the concentrations, is reported (table 2). Interleukin-10 and IFN- $\gamma$  were more often detected in colostrum from Estonian than Swedish mothers, whereas the opposite tended to be true for IL-4. Furthermore, IL-4 was detected more often in allergic than non-allergic mothers in Sweden (44% vs 16%,  $p=0.02$ ), but not in Estonia (14% vs 11%).

The endotoxin levels in carpet dust were higher in Estonian (median 19.4, range 0.5-97.0 EU/mg dust) than Swedish dust samples (median 11.7, range 1.3-68.2 EU/mg dust,  $p=0.007$ ). In the Swedish mothers, TGF- $\beta$ 1 ( $\rho=-0.34$ ,  $p=0.04$ , fig 3) and - $\beta$ 2 ( $\rho=-0.30$ ,  $p=0.07$ ) in colostrum correlated inversely with the endotoxin levels. In contrast, there were positive correlations between colostrum IL-4, IL-10 and IFN- $\gamma$  levels and house dust endotoxin levels in Sweden ( $\rho=0.34-0.35$ ,  $p=0.03$ ). No significant correlations were observed between breast milk cytokine levels and house dust endotoxin levels in Estonia.

High levels of IL-13 in breast milk were associated with early sensitisation in Swedish breast fed infants. This could not be evaluated in the Estonian infants, as only three of them were sensitised. All three Swedish infants who were sensitised at three months had received colostrum with detectable levels of IL-13, whereas the corresponding figure for non-sensitised infants was 30% (7 of 23,  $p=0.046$ ). For the infants who were sensitised at six months, 67% (4 of 6) had received colostrum with detectable IL-13, as compared to 15% of the non-

sensitised infants (4 of 27,  $p=0.02$ ). High colostral TGF- $\beta$ 2 levels also tended to be associated with sensitisation in Sweden, especially during later infancy (median 486, range 240-1400 pg/ml in non-sensitised vs median 586, range 365-1156 pg/ml in sensitised children,  $p=0.11$ ). The IL-13 and TGF- $\beta$ 2 levels were not significantly related to atopic dermatitis development. None of the other analysed breast milk parameters were associated with sensitisation, nor with atopic eczema (data not shown). Worth noticing, however, is that 50% of the infants who were sensitised within the first six months of life and then lost their SPT reactivity had received breast milk with detectable levels of IL-10, as compared to none of the infants with sustained sensitisation ( $p=0.04$ ).

## Discussion

The immune composition differs in breast milk from mothers of two geographically adjacent populations with marked differences in life style, environment and allergy prevalence. Thus, Estonian mothers had lower levels of TGF- $\beta$ , particularly TGF- $\beta$ 2, but higher levels of SIgA, IL-10 and IFN- $\gamma$  in their breast milk than Swedish mothers. The low levels of the anti-inflammatory and IgA-inducing cytokine TGF- $\beta$  in breast-milk from mothers of a country with high microbial pressure and low prevalence of allergic disease may seem counter-intuitive. This finding is in accordance with our recent observations that probiotic *Lactobacillus reuteri* supplementation reduces breast milk TGF- $\beta$ 2 levels, however (11), and possibly related to improved epithelial barrier function. A certain microbial pressure may be mandatory for preservation of an intact mucosal barrier function (25). In response to epithelial damage, TGF- $\beta$  drives tissue remodelling, inducing fibrosis and airway smooth muscle proliferation in asthma (26, 27). Thus, it is tempting to speculate that breast milk TGF- $\beta$ 2 levels may increase after mammary epithelial damage. Also, IL-13 induces epithelial cells to produce TGF- $\beta$ , particularly TGF- $\beta$ 2 (28, 29), a process inhibited by IFN- $\gamma$  (30). In contrast to epithelial cells where TGF- $\beta$ 2 dominates, immune cells including T regulatory cells preferentially express the TGF- $\beta$ 1 isoform (31, 32). Adding to the complexity in TGF- $\beta$ -mediated effects, this pleiotropic cytokine may, in concert with proinflammatory mediators such as IL-6 or the Th2 cytokine IL-4, induce potentially airway inflammation-aggravating T helper cells producing IL-17 (32-34) and IL-9 (27, 35, 36), respectively. These proinflammatory properties may be related to our findings of low TGF- $\beta$  levels in Estonian, as compared to Swedish, mothers.

The integrated mucosal immune system entails that immune responses induced in the gut or airways can be reflected in the mammary gland (3, 37). Since microbes and their products are

major factors regulating mucosal immunity, it is tempting to speculate that the microbial environment would influence the breast milk composition. Supporting this hypothesis, the house dust endotoxin levels correlated inversely to the colostral levels of TGF- $\beta$ , but positively to IL-4, IL-10 and IFN- $\gamma$  in colostrum of Swedish mothers. Taken together, these findings indicate that a high external microbial load is associated with low TGF- $\beta$ , but high IL-4, IL-10 and IFN- $\gamma$  responses at the mammary epithelium. The lack of direct association between endotoxin and breast milk cytokines in the Estonian mothers is possibly due to the generally high endotoxin levels in Estonia (22), already providing a maximal effect.

Many bacteria and microbial-derived products induce the production of IFN- $\gamma$  and IL-10, and occasionally also IL-4, from T-cells and antigen presenting cells located beneath the mucosal epithelium (38, 39). These cells will then specifically migrate to other mucosal sites, and their cytokine production is mirrored in secretions such as the breast milk. This may explain the higher levels of IL-4, IL-10 and IFN- $\gamma$  in individuals living in an environment with high microbial load. We have previously observed higher colostral IL-10 levels in mothers treated with *Lactobacillus reuteri* as compared with placebo (11). Most IgA antibodies in mucosal secretions are directed against microbial antigens (3). Thus, the higher SIgA levels in mature milk of Estonian, as compared to Swedish, mothers are possibly due to the more divergent microbial environment of the former population, although no correlation between SIgA levels and endotoxin was found.

Speculatively, the association between high colostral IL-13 levels and early sensitisation may be related to the IgE-inducing capacity of IL-13, which may dominate the effects of IL-4 when this more tightly regulated cytokine is absent or present at low levels, e.g. during the initiation of allergic responses (40). Furthermore, IL-13 induces epithelial cells to produce

TGF- $\beta$ , particularly TGF- $\beta$ 2 (28, 29). We observed a tendency towards increased colostrals TGF- $\beta$ 2 levels among sensitised infants, in accordance with our findings in the *Lactobacillus reuteri*-treated cohort (11). Possibly, the relation between breast milk TGF- $\beta$ 2 levels and childhood sensitisation is explained by epithelial barrier function. Thus, high TGF- $\beta$ 2 levels in milk mirror increased epithelial permeability, as determined by the milk Na/K ratio (11). An increased epithelial permeability has previously been associated with development of sensitisation and allergic symptoms up to 18 months of age also in another independent cohort (41). In partial contrast to our findings, infants with post-weaning onset allergic disease received colostrum with higher TGF- $\beta$ 2 levels than infants with pre-weaning onset of disease (16). This observation is still controversial, however (15, 18, 20). That children with transient as compared with persistent sensitisation during infancy more often received colostrum with detectable IL-10 may be related to the IgE inhibitory effects of this cytokine (26).

In conclusion, Estonian mothers have lower levels of TGF- $\beta$ , particularly TGF- $\beta$ 2, but higher levels of SIgA, IL-10 and IFN- $\gamma$  in their breast milk than Swedish mothers, possibly due to differences in microbial load. High IL-13 levels may be associated with increased risk of sensitisation during infancy.

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### Figure legends

**Figure 1.** Lower breast milk levels of TGF- $\beta$ , particularly TGF- $\beta$ 2, in Estonian than Swedish mothers. Colostral and 1-month mature milk TGF- $\beta$ 1 and TGF- $\beta$ 2 levels (pg/mL) in Estonian (white) and Swedish (grey) mothers. The 10<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> and 90<sup>th</sup> percentiles and outliers are indicated. \*\*p<0.0001

**Figure 2.** Higher levels of secretory IgA in mature milk from Estonian than Swedish mothers. Colostral and 1-month mature milk secretory IgA levels ( $\mu$ g/mL) in Estonian (white) and Swedish (grey) mothers. The 10<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> and 90<sup>th</sup> percentiles and outliers are indicated. \*\*p<0.0001

**Figure 3.** Inverse correlation between carpet dust endotoxin levels and colostral TGF- $\beta$ 1 levels in Swedish mothers. Colostral TGF- $\beta$ 1 levels (pg/mL) in relation to carpet dust endotoxin levels (EU/mg dust),  $\rho$ =-0.34, p=0.04.

**Table 1. Characteristics of the Estonian and Swedish mothers and children, followed to 24 months of age.**

|                                       | Estonian  | Swedish     |
|---------------------------------------|-----------|-------------|
| N of mothers                          | 39        | 60          |
| Mothers with allergic history         | 14 (36%)  | 29 (48%)    |
| N of born babies                      | 39        | 60          |
| N of babies dropping out of the study | 0         | 7           |
| Cumulative incidence of SPT+          | 3/39 (8%) | 13/53 (25%) |
| Cumulative incidence of eczema        | 3/39 (8%) | 10/53 (19%) |

**Table 2. Number (n) and percentage (%) of colostrum and mature milk samples from Estonian (Est) and Swedish (Swe) mothers with detectable levels (pos) of IL-4, IL-10, IL-13 and IFN- $\gamma$ .**

Statistically significant differences are marked in bold.

|               | Colostrum                    |                             |              | Mature milk                 |                            |             |
|---------------|------------------------------|-----------------------------|--------------|-----------------------------|----------------------------|-------------|
|               | Est<br>n pos<br>(%)          | Swe<br>n pos<br>(%)         | p value      | Est<br>n pos<br>(%)         | Swe<br>n pos<br>(%)        | p value     |
| IL-4          | 4/32<br>(12%)                | 18/60<br>(30%)              | 0.08         | 9/37<br>(24%)               | 11/49<br>(22%)             | ns          |
| IL-10         | <b>14/39</b><br><b>(36%)</b> | <b>6/60</b><br><b>(10%)</b> | <b>0.004</b> | 4/39<br>(10%)               | 3/48<br>(6%)               | ns          |
| IL-13         | 8/23<br>(35%)                | 21/59<br>(36%)              | ns           | 11/32<br>(34%)              | 12/47<br>(26%)             | ns          |
| IFN- $\gamma$ | <b>9/38</b><br><b>(24%)</b>  | <b>4/60</b><br><b>(7%)</b>  | <b>0.03</b>  | <b>6/38</b><br><b>(16%)</b> | <b>1/49</b><br><b>(2%)</b> | <b>0.04</b> |

Fig 1

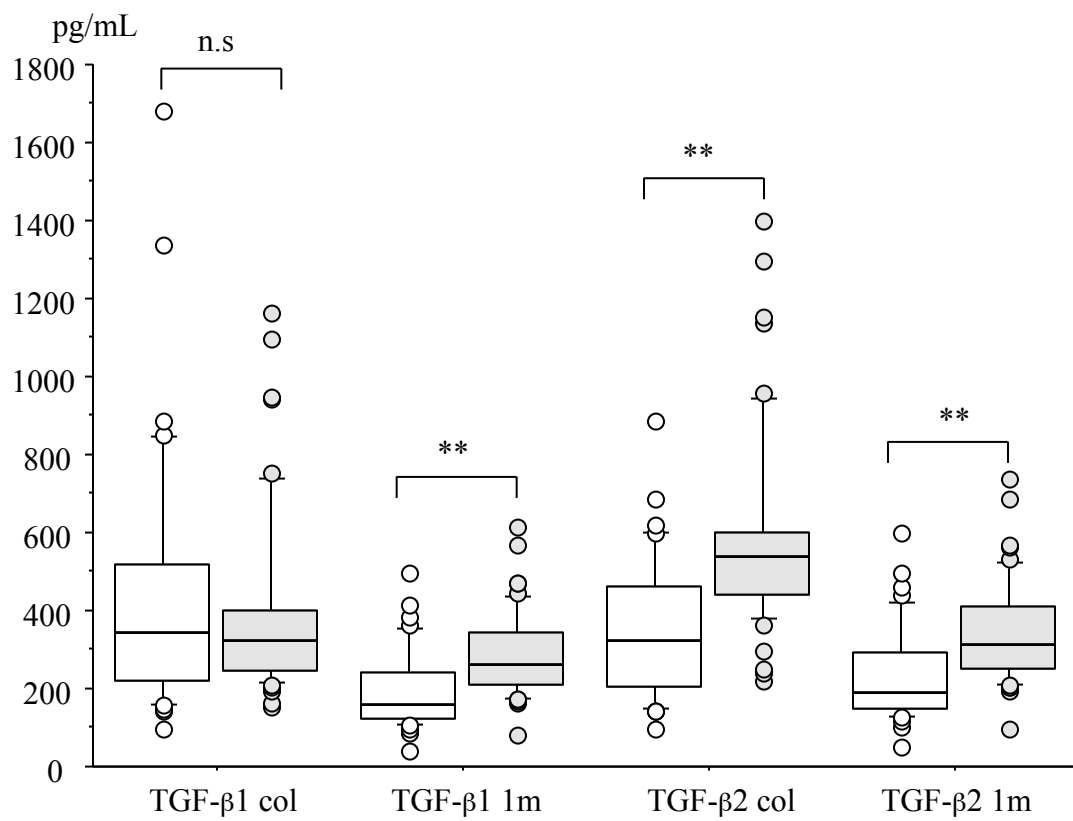




Fig 2

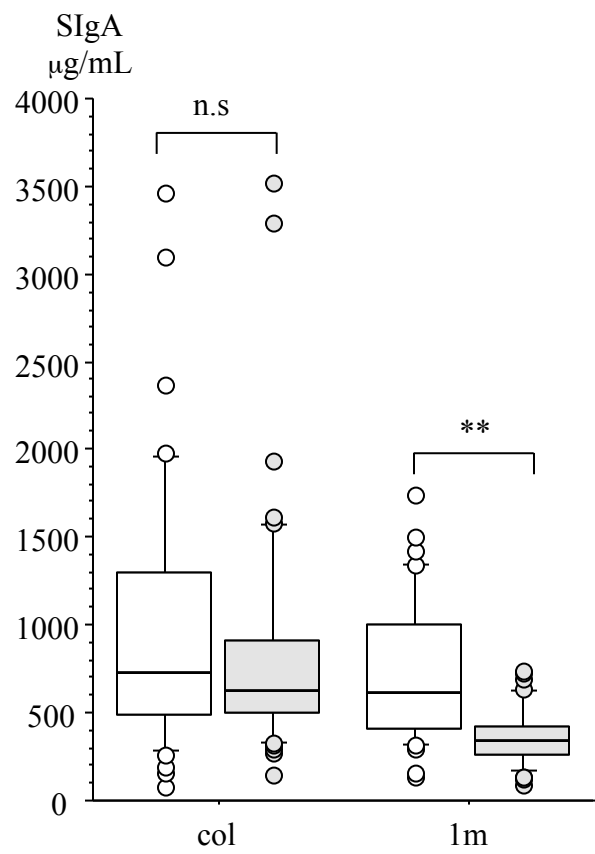


Fig 3

