The Association Between Transforming Growth Factor Beta (TGF-β) Isotypes and Thymic Stromal Lymphopoietin (TSLP) in Breast Milk and Infant Gut Permeability in Term Infants: ProPACT Study

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Abstract

The association between transforming growth factor beta (TGF-β) isotypes and thymic stromal lymphopoietin (TSLP) in breast milk and infant gut permeability in term infants: ProPACT study

Background: There is little known about the role of breast milk cytokines on the influence of allergy disease development through modifying gut permeability. We aimed to analyze the association between two cytokines in breast milk: TGF-β isotypes and TSLP, and term infant gut permeability plasma markers: LBP and FABP-2.

Methods: Samples were collected during a randomized-controlled trial cohort within the context of maternal probiotic supplementation's effect on childhood atopic dermatitis and other allergy diseases (the ProPACT Study), consisting of 415 mother-infant pairs. In this sub-study, we analyzed breast milk cytokines from 255 mothers and infant gut permeability plasma samples from 115 children. The association between TGF-β isotypes and TSLP in breast milk and term-infant gut permeability plasma LBP and FABP-2 markers were investigated using multivariate analyses at two breastfeeding timepoints, 10 days and 3 months.

Results: Both at 10 days and at 3 months, TGF-β1 had the greatest number of varied concentrations, TGF-β2 had the highest concentration and TGF-β3 had the lowest concentration. Most of the detectable breast milk TSLP (55.4%, n = 238) at 10 days were in low concentration (32.3%). The median concentration of term infant plasma LBP at 10 days and 3 months were low (1 and 1.4 µg/mL), while term infant plasma FABP-2 concentrations were lower than 2 000 pg/mL (2 ng/mL) at both timepoints and still within the normal range of the assay. There was no statistically significant association between breast milk TGF-β isotypes and TSLP and term infant plasma LBP and FABP-2, although there was an inverse tendency between the concentration of breast milk TSLP and infant plasma LBP at 10 days.

Conclusions: Breast milk TGF-β isotypes and TSLP do not appear to be associated with the term infant gut permeability markers, plasma LBP and FABP-2 both at 10 days and 3 months after birth in a Norwegian population. Further studies are required to investigate and confirm whether increased gut permeability in term infants has a role in the development of childhood allergy diseases.

Keywords: breast milk, TGF-beta isotypes, TSLP, LBP, FABP-2, term infant gut permeability

Introduction

Breast milk nutrition has been highlighted as an important factor in shaping infant's gut health for a prevention of many chronic diseases such as allergy. A review published in 2019 elaborated on how breastfeeding shapes the early life gut microbiota in relation to allergy prevention, through several immunological mechanisms (1). Maternal factors such as maternal allergy diseases (asthma, eczema, rhinitis), smoking, urogenital infections during pregnancy, age, body mass, child sex and season of birth contributed to the variation of breast milk cytokines, chemokines, and Immunoglobulin (Ig) A concentrations during pre and perinatal period, although their associations were somewhat small (2). To preview, breast milk contains factors that can affect key players in allergy development in infants, however, the biological mechanisms behind these effects is less well understood.

1. The PACT Study and The ProPACT Study

In September 2000, a cohort study named Prevention of Allergy among children in Trondheim (PACT) (3) was conducted and mainly aimed to decrease childhood atopic diseases such as atopic dermatitis (AD), asthma, and allergic airway disease (AAD) through several structured interventions to reduce environmental tobacco exposure and housing dampness, and increase intake of omega-3-fatty acid through fish consumption intake during pregnancy and early infancy. The PACT study invited families during pregnancy, and at routine child health checks at 6-weeks, 1-year, 2-years, and 6-years of age. Recruitment occurred at 28 general practices (initially there were 32 agreed but then four withdrew), all seven community-based midwives and all 20 maternity health centers in Trondheim, Norway.

A municipality-wide lifestyle advice intervention started in June 2002, and pregnant women recruited after this date were included in the intervention cohort. The infants of these women and any other infants born after January 2003 were considered part of the intervention cohort. Parallel to the PACT study, a randomized controlled trial investigating the role of probiotics in the prevention of childhood eczema and other allergic diseases during the first two years of life was conducted.

This sub-study was a double-blind randomized controlled trial and named as Probiotics in the PACT study (ProPACT) (3, 4).

The ProPACT study recruited pregnant women in the PACT study who came for check-ups to all seven midwives in Trondheim. All pregnant women were eligible if they understood Norwegian, signed the consent form, were planning to breastfeed during the first three months after birth, were in week ≤ 36 of pregnancy, liked and tolerated fermented milk. Women were excluded if they had been taking probiotic supplements during the last 4 weeks, were planning to move away from Trondheim within 25 months of randomization, or were at risk of developing pregnancy complications such as pre-eclampsia. The ProPACT study allocated eligible pregnant women to receive and drink fermented milk either containing selected probiotics or a placebo from 36th week of pregnancy until 3 months after birth. Compliance to study milk consumption was recorded in the diaries along the randomization period, and acknowledged as compliant if they consumed at least 250 mL of the trial milk on at least 50% of days during the allocated intervention period, they did not consume other products containing probiotics, and the child was at least partially breastfed until 3 months of age. The ProPACT study collected breast milk sample at 10 days and 3 months after birth, and infant blood plasma samples at 10 days and 3 months and also at 1 and 2 years of age (4-6).

The ProPACT study showed a reduction in the cumulative incidence of AD in the children of almost 40% during the first 2 years of life after maternal supplementation with three selected probiotic strains (4). Based on intention-to-treat (ITT) analysis, this significant communal effect needed at least 8 subjects to receive the probiotic supplementation during pregnancy and this effect was stronger for non-Immunoglobulin E (IgE)-associated AD. According to the Nottingham Eczema Severity Score, the children with AD in the probiotic group had a significantly reduced risk of having moderate AD compared with the placebo group (4). Furthermore, the primary preventative effect was the best shown among children

without a family history of atopy in which more than 90% of them were free of AD in the 2 years of life (4).

In addition to the results of the ProPACT study, further analysis was done to understand the mechanism behind those preventative effects of probiotic supplementation in pregnant women against the developing of AD for the first 2 years of life of the child. My co-supervisor, Dr. Melanie Rae Simpson, in one of her papers (5) from her published doctoral thesis in 2018 (6), analyzed the levels of several cytokines, transforming growth factor beta (TGF-β) isotypes and thymic stromal lymphopoietin (TSLP) in the breast milk which was collected in the ProPACT study. She discussed the changes and implications of TGF-β isotypes and TSLP in prevention of atopic dermatitis in the children within the context of maternal probiotic supplementation. She concluded that maternal probiotic supplementation may have increased breast milk levels of TSLP at 10 days postnatal, however, it did not appear as a part of probiotic's mechanism to prevent AD in children (5).

2. Breast milk cytokines: TGF-β isotypes and TSLP

Over the past two decades, multiple cytokines and immunomodulatory factors have been identified in breast milk and listed in a mini review by Dawod and Marshall, 2019 (7). The same review suggested that breastfeeding has impact on the development of allergic diseases, despite the fact that existing extensive studies provided conflicting results.

TGF-β was one of the cytokines in breast milk which has been suggested as a protective factor against allergy development in infants when its level in colostrum is high (8). However, conflicting result from interventional studies are described in a review by Boix (8), which may be explained by heterogeneity in sampling, processing, and outcome assessment. Further, the decline of TGF-β level throughout the lactation period may be responsible for the differences in associations with the allergy development (8) or even the maternal origin before childbirth may also affect the pleiotropic nature of TGF-β together with IL-6 and IL-8 (9). TGF-β was found

abundant in breast milk and it has different concentrations depends on the isotypes (5, 6, 8). In a mini review by Dawod and Marshall, 2019, TGF-β1 and TGF-β2 had similar concentration levels in human colostrum (earliest four days postpartum), around 100 – 3 300 pg/mL (7). TGF-β1 had a slightly higher lowest detectable level compared to that of TGF-β2, however, throughout 6 months lactation periods, TGF-β2 increased sharply in human milk up to 10-fold TGF-β1 (800 – 5 300 pg/mL vs. 80 – 600 pg/mL) (7).

TGF-β together with interleukin (IL)-10 could improve infant gut permeability and block the damaging effect of pathogenic *Escherichia coli* infection on intestinal epithelial cells (10). Several studies from 1997 until 2016 (11-19) had shown protective mechanism of TGF-β against increasing gut permeability on infants. Donnet-Hugghes et al (11) concluded that preterm neonates in particular were suffering due to increased intestinal permeability, however, the event could be blocked by TGF-β2. It can be explained that TGF-β works as an antagonist for the inflammation-mediating cytokines, such as interferon gamma (IFN-γ), tumor necrosis factor alfa (TNF-α), or IL-1β (12).

Further, production of IL-8 either in vitro or in vivo was shown to be inhibited as well by TGF-β2 (13). Rautava (14) also wrote about the anti-inflammatory effect of TGF-β2 in breast milk which significantly attenuated IL-1β-production of immature human gut epithelial cell through ERK signaling, which was necessary for the cytokine response, and this mechanism was completely SMAD-6 protein dependent. Other researchers also submitted proven anti-inflammatory effect of TGF-β2 in which it attenuated LPS-induced releases of IL-6, IL-1β, and TNF-α by reducing early ERK activation, IL-1β-mediated NF-κBp65 (Nuclear Factor-Kappa B p65) release and its nuclear translocation (15, 16).

As TGF-β2 is normally expressed in breast milk in high concentration and TGF-β isotypes' receptors are widely expressed in human fetal as well as murine intestine, enterally administered recombinant TGF-β2 was also found to be protective against inflammatory mucosal injury in infant models' gut epithelial cells and it can suppress

the expression of gut resident macrophage's inflammatory cytokines (13). TGF-β2 was proven to promote and increase gut production of TGF-β (18) which might give an idea of the continuity of child growth in case there is no more breast milk consumed.

Hennet (19) narrated from Laiho, K. et al (20) that breast milk TGF-β and IL-10 work as two of "the umbrella components of breast milk" which means that TGF-β and IL-10 work for the maturation of infant mucosal immunity during the first weeks of life. TGF-β in this earliest period of life is produced by leucocytes present in the breast milk and remain active after passage through stomach due to high gastric pH in infants (approximately 3-5) and remain stable due to maintenance by α1-antitrypsin in early milk which protects cytokines from gastric proteolysis (19).

In contrast, TSLP was mentioned as pro-inflammatory cytokine which promote the developing of AD at the dendritic cell (DC) level (21) ever since the commentary by Liu Yong-Jun in 2006 (22) that TSLP, an IL-7-like cytokine, gained the terms of master switch for allergic inflammation by triggering DC-mediated T helper 2 (Th2) inflammatory responses. Liu Yong-Jun discussed the establishment of a direct link between TSLP expression and the pathogenesis of AD and asthma in vivo due to the facts of the expression of TSLP is high by skin keratinocytes and airway epithelial cells during allergic inflammation, although the mechanism on how TSLP was highly expressed in these cells remained unclear. Aberrant expression of TSLP was also seen in allergy related diseases of the gut, for example food allergy-related disorder eosinophilic esophagitis (EoE) as discussed by Ziegler et al, 2013 (23).TSLP genetic variants and its dysregulated expression have been linked to atopic dermatitis, asthma, allergic rhinitis and EoE as discussed by Cianferoni and Spergel, 2014 (24). However, it was explained that TSLP expression is not absolutely needed to promote Th2-type inflammation since there were different study results (23).

That contrasting idea was partly nulled by Bjerkan et al, 2015 (25) which found that TSLP was associated with protective and tolerogenic roles in the gut system and thymus gland. Some studies reviewed by Ziegler et al (23) suggested that TSLP also

maintains gut homeostasis and modulation of Th1/Th17 inflammation and thus low TSLP expression was seen in non-inflamed colonic tissue in Crohn's disease and ulcerative colitis (UC) (23). They suggested that TSLP positive effect against inflammation was attenuating Th1/Th17 responses and directly acting on the gut epithelium to support wound healing in any colon inflammation (23).

In addition, since TSLP is highly expressed in skin keratinocytes, Macfarlane et al (26) found that TSLP level in the breast milk which was collected at 3-5 days post-delivery significantly higher than those collected at 11-26 days post-delivery (median level of 90 pg/mL and 40 pg/mL respectively). They discussed that there might be a possibility of gut-immune interplay which might be affected by breast milk TGF-β1 and TSLP activities through triggering a tolerogenic DC phenotype within the gut. Nevertheless, the exact mechanism for breast milk TSLP modulating infant gut permeability still remains questionable.

3. Infant gut permeability: plasma LBP and FABP-2

Allergy diseases such as atopic dermatitis might arise from poor gut permeability in infants as well as imbalance in gut immune system (10). The phrase "poor gut permeability" refers to an increase in the rate of flux of molecules across the gut epithelium without proper chemical selection or physical restriction, which can be referred to as gut barrier. Gut barrier is defined as highly specialized mucosa lining where digestion and absorption take places, along with its ability to maintain symbiotic relationships with normal gut stromal milieu and intestinal immune system against invaders (10). Immune system mechanism has always been mediated through various cytokines.

To further explore the biologic plausibility of breast milk cytokines and infant gut permeability, this master thesis explores the association between two cytokines in breast milk, TGF-β isotypes and TSLP, and term-infant's gut permeability which can be measured in two plasma biomarkers, lipopolysaccharides-binding protein (LBP) and fatty-acid-binding protein 2 (FABP-2).

In this current study, the outcome of interest are infant gut permeability markers in term infants which were measured by conducting enzyme-linked immunosorbent assay (ELISA) on the infant's plasma levels of lipopolysaccharides-binding protein (LBP) and fatty-acid binding protein-2 (FABP-2) at 10 days and 3 months after birth to match with their corresponding mother's breast milk TGF-β isotypes and/or TSLP.

LBP is part of the innate immune response that opsonizes lipopolysaccharides (LPS or endotoxin, a cell wall constituent of Gram-negative bacteria) when LPS crosses the gut barrier and LBP serves thus as a marker of metabolic endotoxemia (27). The physiological concentration of LBP in serum of healthy human is 5 – 10 µg/mL (microgram per milliliter, ug/mL) (28-31). A study assessing serum LBP continuously (0-, 12-, 24-, and 72-hours postnatal age) in infants with neonatal bacterial infection (NBI) found that all infants (preterm and term) who were NBI negative had median LBP from 2.9 µg/mL at 0-hour of age., then went steady at 6 µg/mL within 12 to 24 hours and ended up to 4.2 µg/mL at 72 hours postnatal age (32). While the infants with NBI positive had 2 up to 5 times higher levels of serum LBP (17.6 to 20 µg/mL) than those who were NBI negative, and the differences were all statistically significant ($p<0.0001$) (32). Specifically, term infants with NBI negative had median serum LBP 3.9 µg/mL, about five times lower than term infants with NBI positive (17.5 µg/mL) (32).

Two linked studies in 1992 and 1994, explained that LBP in plasma reacts rapidly but transiently with LPS, and monocyte activation resulting in tumor necrosis factor (TNF) release was limited when LBP level was depleted from serum either by dilution of the serum or when anti-LBP antibodies were added (30, 31). Other study by Le Roy (2001) speculated that subnormal LBP is important to increase phagocytosis (LPS-induced neutrophil activation), and clearance of LPS from blood (33). According summary by Sakr, et al (2008), the effects of LBP were concentration dependent: low concentration of LBP, which speculated occur in extravascular fluids, enhanced LPS-induced TNF-α secretion of activated monocyte and may promote

inflammation at local sites of infection (28, 30, 34). Whereas higher concentration of LBP was a protective mechanism by neutralizing cell activation and may prevent LPS-induced systemic inflammation (28, 29). LBP level can increase 2 to 50-fold within 24 hours in sepsis (29, 30, 35), and LBP at this high concentration will attenuate the release of pro-inflammatory cytokines (29). The elevating level of LBP in the earliest days of the newborns were observed in neonatal enterocolitis (NEC) (36).

NEC is known to be one of the most common forms of gut barrier dysfunction and damage in premature newborn which may be modulated or even improved with breastfeeding either own milk or donated milk, but not with formula milk (37). Meng et al (2021) summarized that LBP level can also increase in anyone who consumes high fat and carbohydrates and is therefore also related to obesity, metabolic disorders, diabetes, as those diseases are considered as chronic low-grade inflammation. This mechanism is induced by the alterations of the intestinal flora together with an increase of gut permeability to LPS (38). LBP is known to be transient molecule which has low sensitivity due to short half-life (36). Nevertheless, LBP is thought as better acute-phase marker for early-onset sepsis or metabolic endotoxemia than procalcitonin (PCT), C-reactive protein (CRP) and soluble CD-14 (36), which reacts against LPS/endotoxin from Gram-negative bacteria which may be present as normal flora in mother's birth canal and/or abdomen cavity.

Molés et al, 2018 (39) reviewed that human infant has greater, but temporary, permissiveness of gut mucosa. And the process of infant "gut closure", to have a normal functioning gut barrier, is closely relates to infant gut permeability, which have two physiological mechanisms. The first physiological mechanism of infant gut permeability is mostly associated with the concept of gut closure, which consists of the passage of macromolecules from the lumen of the infant's gut via paracellular space to blood (39). In humans, gut permeability to macromolecules is very high shortly after birth and then drops dramatically within the first 7 days of life, as Molés et al (2018) has reviewed. Molés et al (2018) also mentioned that the infant gut permeability temporarily increased again at the introduction of a weaning food, or in

the case of gut inflammation for any reason. One surrogate marker to estimate infant gut permeability according review by Molés et al (2018) is intestinal FABP (I-FABP, also called FABP-2) (39), which can be present both in urine and blood. FABP-2 is present mainly in villi of mature enterocytes from duodenum to caecum (40), with the highest content of FABP-2 was in jejunum and the lowest was in distal colon (41), and it constitutes 2% of enterocyte protein (42). FABP-2 is filtered in urine after being released in blood when enterocytes detached from gut villi (39) representing enterocyte turnover or damage (40). It has been discussed that there was possibility of increasing gut maturation from the observed post-natal rise in urinary FABP-2 concentration according to gestational age (39, 43). Based on a meta-analysis report, FABP-2 has also been stated as valid serological biomarker for early diagnosis of NEC in premature newborns leading to severe stage III with moderate accuracy, suggesting a possible application in determining intestinal injury in full-term newborns (44).

FABP-2 plasma level in normal healthy adult individual is usually low, even below the detection level of the assay kit (< 0.1 ng/mL), and elevated in patients suffering from intestinal diseases along with liver type FABP (L-FABP, also often described as FABP-1) (41). A study by Guthmann et al (2002) found that FABP-2 level in plasma of healthy preterm neonates was 2.52 ng/mL (median), within the range of 0.46-4.51 ng/mL (45). FABP-2 is noted as sensitively related to acute intestinal injury less than 2 hours (42), and rapidly eliminated through renal clearance within 22 minutes by 50% for every instantaneous release of the contents of 1 gram damaged tissue into plasma (41, 46, 47) and the fact that is due to its villous-only expression in mature enterocyte of mature newborns, the crypt cells have rapid automatic repair of the function despite of damaged villi (41, 42, 47).

Rationale for the study

There is unknown association between breast milk TGF-β isotypes and TSLP and term infant gut permeability plasma LBP and FABP-2 markers. Assuming that breast milk is delivered to the infants and it contains TGF-β isotypes which known to be gut protective molecules, a TGF-β level be might be correlated with a decrease in LBP and FABP-2 level in healthy growing term infants. Whilst TSLP level in breast milk might serve as an inverse relationship with plasma LBP and FABP-2 level in term infants given theirs conflicting natures, both as pro-inflammatory cytokine and in the promoting of infant gut wound healing.

Presumably, mothers' allergy status might induce infant gut sensitization through breast milk cytokines and may result in changing level of infant gut permeability markers. A previous study on food allergy article (48) speculated that serum LBP could also present as a surrogate marker in several non-communicable diseases in infants which shows systemic inflammation, such as allergic diseases (48). However, after adjustment for potential confounding factors, LBP level was not clearly related to allergic diseases, yet may be related to gut-derived low-grade inflammation (48). A sub-study of the ProPACT study that has been published in 2021 showed that markers of intestinal permeability (LBP and FABP-2) in children were not consistently associated with atopic eczema, fungal or bacterial abundance, or bacterial diversity from birth to 6 years follow up (49). A larger sample size and longitudinal investigations are needed to verify these evidences.

Among numerous studies cited above, there were no exact analysis or statements on whether TGF-β or TSLP are really protective for term infant's gut permeability. Moreover, research works which analyzed the role and function of breast milk TSLP, especially on infant gut permeability and allergy diseases are limited. Therefore, this master thesis is written to fill this gap in knowledge and would be one good supportive explanation to fill in the missing links between infant gut permeability and childhood allergy diseases.

In this analysis, we used subset data from the ProPACT study to assess the association between two breast milk cytokines: TGF-β isotypes and TSLP, and term infant gut permeability plasma biomarkers: LBP and FABP-2.

Research questions

Is there any association between specific maternal breast milk cytokines and infant gut permeability in term infants?

Objectives

1. Main aim: to investigate the association between specific maternal breast milk cytokines and infant gut permeability in term infants.
2. Specific aim:

i. To investigate the association between Transforming Growth Factor beta (TGF-β) isotypes (TGF-β1, TGF-β2, TGF-β3) in breast milk and infant gut permeability in term infants measured by plasma lipopolysaccharide binding protein (LBP) and fatty acid binding protein-2 (FABP-2).

ii. To investigate the association between thymic stromal lymphopoietin (TSLP) in breast milk and infant gut permeability in term infants measured by plasma LBP and FABP-2.

Ethical approval

This master thesis is approved by the Regional Committee for Medical and Health Research Ethics (Ref. 13944) and had been decided to go further without a need of NSD (Norsk senter for forskningsdata) approval due to Norwegian University of Science and Technology (NTNU) and NSD agreement on any health research conducted by the Faculty of Medicine and Health Sciences at NTNU.

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Material and Methods

1. Breast milk cytokine quantification

Breast milk cytokine quantification has been clearly described in two previous studies (5, 6). In brief, breast milk samples were collected at 10 days and 3 months postpartum and frozen at home before being transported, then stored at -80 degrees until analysis. When the samples were to be analyzed, they were thawed and centrifuged (16,100*g*, 10 min, 4°C) to separate fatty, solid and aqueous portions. The aqueous portion was extracted without disturbing the pellet and was used for subsequent cytokine quantification.

The concentrations of all TGF-β isotypes were measured using a multiplex assay (Bio-Plex Pro TGF-β assay, Bio-Rad Laboratories, Oslo, Norway). Following the manufacturer's instructions, 20 µL 1 N HCl was added to 100 µL of the aqueous portion of breast milk sample for 10 minutes at room temperature, to serve as an activation step for TGF-β assay, and it was followed by neutralization protocol using 20 µL 1 N NaOH with 0.5 M HEPES. The standard and two internal control samples (low and high) were conducted in duplicate. Standard curves were used to calculate the concentrations and the value was multiplied by 1.4 to account for the dilution in the activation-neutralization process. The intra-assay coefficients of variation (CV) were <13.6, <13.4 and <14.6% and the inter-assay CVs were <9.9, <5.9, and <14.3% for TGF-β1, TGF-β2, and TGF-β, respectively.

The working range of the assay was 1.7 – 27 616 pg/mL for TGF-β1, 14.7 – 30 080 pg/mL for TGF-β2, and 2.8 – 15 031 pg/mL for TGF-β3.

TSLP concentrations were measured using a human TSLP DuoSet ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The assay had range of detection from 31.25 to 2 000 pg/mL. The standards and an internal control were conducted in triplicate which had average value of 266.6 pg/mL (SD 61.1 pg/mL). The intra-assay CV was <13.5% and inter-assay CV was 22%. Due to a large proportion of samples with TSLP concentrations either above or below the range of detection of the assay, we categorized the TSLP concentrations into these following 4 categories: below detection, low detectable, high detectable and above detection. The internal control sample was chosen as the cut-off between the low and high detectable categories, because it was approximately the median concentration (183 pg/mL, IQR: 91 – 576 pg/mL) and maximized the inter-assay comparability.

2. Infant gut permeability measurements

In brief, plasma concentration of LBP was measured by ELISA (Human LBP, Merck, own positive controls) and FABP-2 by ELISA (Human FABP-2/I-FABP Quantikine ELISA Kit, R&D Systems, producer's positive controls) and followed the producers' protocols including negative controls on all plates as previously described (49). Based on R&D Systems website and catalog DFBP20 (50) FABP-2 ELISA assay kit has its lowest detection level of 6.21 pg/mL within range of 15.6 – 1 000 pg/mL, by measuring 10 µL plasma sample.

3. Data management

The candidate and supervisors used personal computers and the data management was done within the server of The University of Oslo.

We utilized data from ProPACT study which had randomized 415 subjects as our initial study population. After data cleaning and sample analysis, there were differing numbers of breast milk TGF-β isotypes and TSLP samples available for each analysis: 255 TGF-β isotypes' concentrations at 10 days, 247 TGF-β isotypes' concentrations at 3 months, 238 TSLP concentrations at 10 days, 225 TSLP concentrations at 3 months were available (Table 2a and 2b). There were also differing numbers of term infant gut permeability plasma LBP and FABP-2 markers available for each analysis (Table 3): 96 LBP concentrations at 10 days, 115 LBP concentrations at 3 months, 96 FABP-2 concentrations at 10 days, 114 FABP-2 concentrations at 3 months. Each cytokine and each infant gut permeability marker were analyzed independently to each other following each timepoint. Since there was less than 10% missing in the current study cohort, any missing data were converted into a new missing category to portray the characteristics of whole samples.

4. Statistical analysis

4.1. Exposure

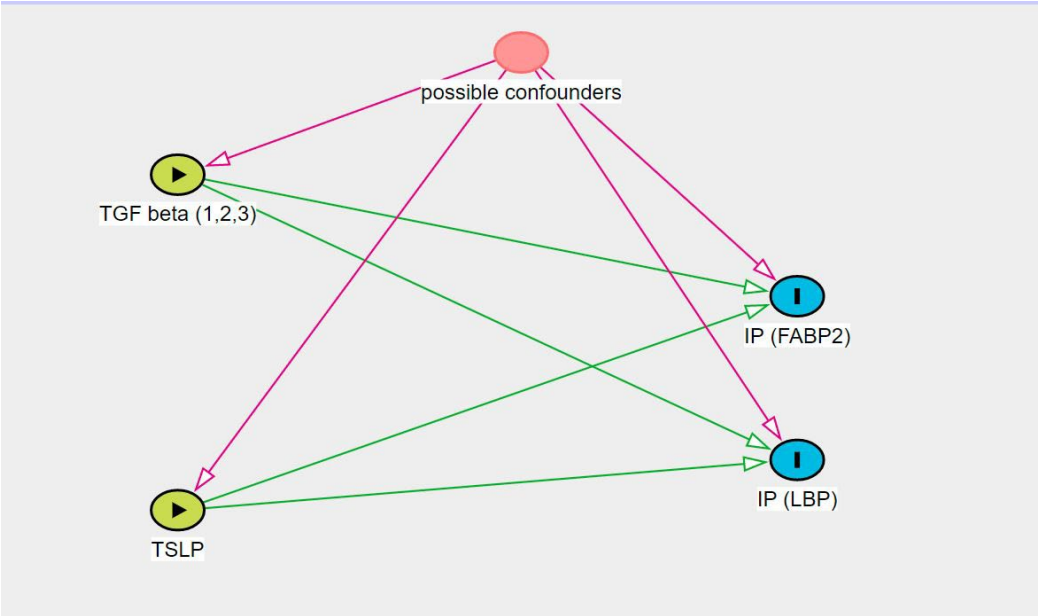
Two breast milk cytokines in this current study, TGF-β isotypes (TGF-β1, TGF-β2, TGF-β3) and TSLP concentrations were recorded as continuous data. TGF-β isotypes and TSLP are presented in median, 25^{th}-75^{th} percentiles range, minimum and maximum values due to right-skewed distribution. TSLP, however, due to a large number of out-ranged detection values, was grouped into four categories based on the level of detection: below detection, low, high, and above detection. Thus, for descriptive purposes, we also display proportion to each TSLP category.

4.2. Outcome

Infant gut permeability plasma LBP and FABP-2 were also continuous variables and did not have a normal distribution. Thus, it is descriptively presented in median, 25^{th}-75^{th} percentiles range, minimum and maximum values.

4.3. Confounders

After thorough initial descriptive statistics and discussions based on existing literatures, we agreed to include the following confounding factors: maternal age (continuous), child sex (male/female), siblings (yes/no), maternal atopy (yes/no), maternal active smoking status within 6 weeks after birth (yes/no/missing). All confounding factors were categorical variables, except maternal age, was a continuous variable.



Picture 1. Directed acyclic graph (DAG) to portray the association between TGF-β isotypes and TSLP and infant gut permeability (IP) markers, and also with possible confounders. (51)

4.4. Detailed analysis approach

Baseline characteristics of the mother and infant pairs were determined based on two conditions: 1) the availability of breast milk at 10 days, thus it showed a group of 255 mothers; and 2) the availability of at least one of breast milk cytokines (TGF-β isotypes or TSLP) concentration and at least one corresponding term infant gut

permeability plasma marker, LBP or FABP-2, thus it showed a group of 147 mothers. To investigate the association between each cytokine and each infant gut permeability marker we ran multiple linear regression analysis, both unadjusted and adjusted, for each breastfeeding timepoint, at 10 days and 3 months.

4.5. Statistical model

A multiple regression model was used to investigate the association between each exposure and each outcome at each timepoint. The association of each cytokine concentration at 10 days was performed toward each outcome marker at both 10 days and 3 months. While the association of each cytokine concentration at 3 months was only performed toward each outcome marker at 3 months. There were 36 models of TGF-β isotypes toward each infant gut permeability marker, 18 unadjusted models and 18 adjusted models; and 12 models of TSLP toward each infant gut permeability models, 6 unadjusted models and 6 adjusted models. In multivariate models for TSLP we only analyzed its categorical data.

For each model of TGF-β isotypes at 10 days, we analyzed data of 90 mother-infant pairs who had complete LBP and FABP-2 samples at 10 days (12 models), while at 3 months there were 109 mother-infant pairs with complete LBP samples (6 models) and 108 mother-infant pairs with complete FABP-2 samples (6 models). For each model of TGF-β isotypes at 3 months, we analyzed data of 107 mother-infant pairs who had complete LBP samples at 3 months (6 models) and of 106 mother-infant pairs who had complete FABP-2 samples at 3 months (6 models).

For each model of TSLP groups at 10 days, there were 88 mother-infant pairs who had complete LBP and FABP-2 samples at 10 days (4 models), while at 3 months there were 102 mother-infant pairs with complete LBP samples (2 models) and 101 mother-infant pairs with complete FABP-2 samples (2 models). For each model of TSLP groups at 3 months, we analyzed data of 97 mother-infant pairs who had complete LBP samples (2 models) and of 96 pairs who had complete FABP-2 samples (2 models).

Adjustment to five confounders: maternal age, child sex, siblings, maternal atopy, maternal active smoking status within 6 weeks after birth were done for each model. We then report beta-coefficients and 95% confidence intervals of unadjusted and adjusted models, and level of statistical significancy set to $p < 0.05$. All statistical analyses were performed using STATA 17.0 SE-Standard Edition (StataCorp, College Station, Texas, USA).

Results

1. Characteristics of Participants

Table 1. Baseline characteristics of the mother-infant pairs

Characteristics of the participants	The available breast milk cytokine samples at 10 days (n1 = 255*)	Pairs of one breast milk cytokine and one infant gut permeability marker (n2 = 147**)
Age of the mothers, years, mean±SD	30.4 ± 4	30.3 ± 4
Sex (male), child, n (%)	120 (47.06)	63 (42.86)
Siblings, n (%)	113 (44.31)	71 (48.3)
Maternal atopy, n (%)		
Yes	124 (48.63)	71 (49.31)
No	130 (50.98)	72 (50)
missing	1 (0.39)	1 (0.69)
Family history of atopy, n (%)	184 (72.16)	106 (72.11)
Smoking		
Maternal active smoking 6 weeks after birth		
Yes	8 (3.14)	5 (3.40)
No	221 (86.67)	126 (85.71)
missing	26 (10.20)	16 (10.88)
Maternal smoking (pregnancy – 1st year of child life)	19 (7.45)	14 (9.52)
Paternal smoking (pregnancy – 1st year of child life)	35 (13.73)	20 (13.61)
Smoking exposure during the 1st year of child life		
Yes (either one or both parents smoke)	31 (12.16)	20 (13.61)
No (neither smoke)	209 (81.96)	121 (82.31)
missing	15 (5.88)	6 (4.08)

*n1 number is among 255 mothers who have breast milk samples at 10 days from the total population of ProPACT study (415 subjects).

**n2 number is among 147 mothers who have at least one breast milk cytokine sample and their corresponding child has at least one plasma gut permeability sample.

Among 415 subjects of ProPACT study we included 255 mother-infant pairs based on only the availability of mothers' breast milk TGF-β isotypes samples at 10 days. Analyses considering the association between breast milk cytokines and intestinal permeability markers included the 147 pairs whose mothers have at least one breast milk sample and their child has at least one gut permeability sample. Mothers in both subgroups were aged around 30 years old. Both subgroups have similar proportion of male child (47.06% and 42.86%) and birth of older siblings (44.31% and 48.3%) of the current child. Family history of atopy was reported high for this study population and of similar proportion for both subgroups (72.16% and 72.11%). Maternal active smoking 6 weeks after birth was also similar in both subgroups (3.14 and 3.4%) although maternal smoking during pregnancy until the first year of child life was slightly higher in the current paired study population (n=147) compared to the n=255 mothers with available breast milk cytokines at 10 days (9.52% vs. 7.45%). Generally, the number of smoking exposure during the first year of child life was 12.16% in n1 group and 13.61% in n2 group. It was almost equal to paternal smoking proportion only (13.73% in n1 group and 13.61% in n2 group). Overall, the two subgroups are similar. We wanted to check that the subgroup used in this study was not a highly selected group.

2. TGF-β isotypes concentration in breast milk

Table 2a. Maternal breast milk Transforming Growth Factor-βs (TGF-βs) concentration

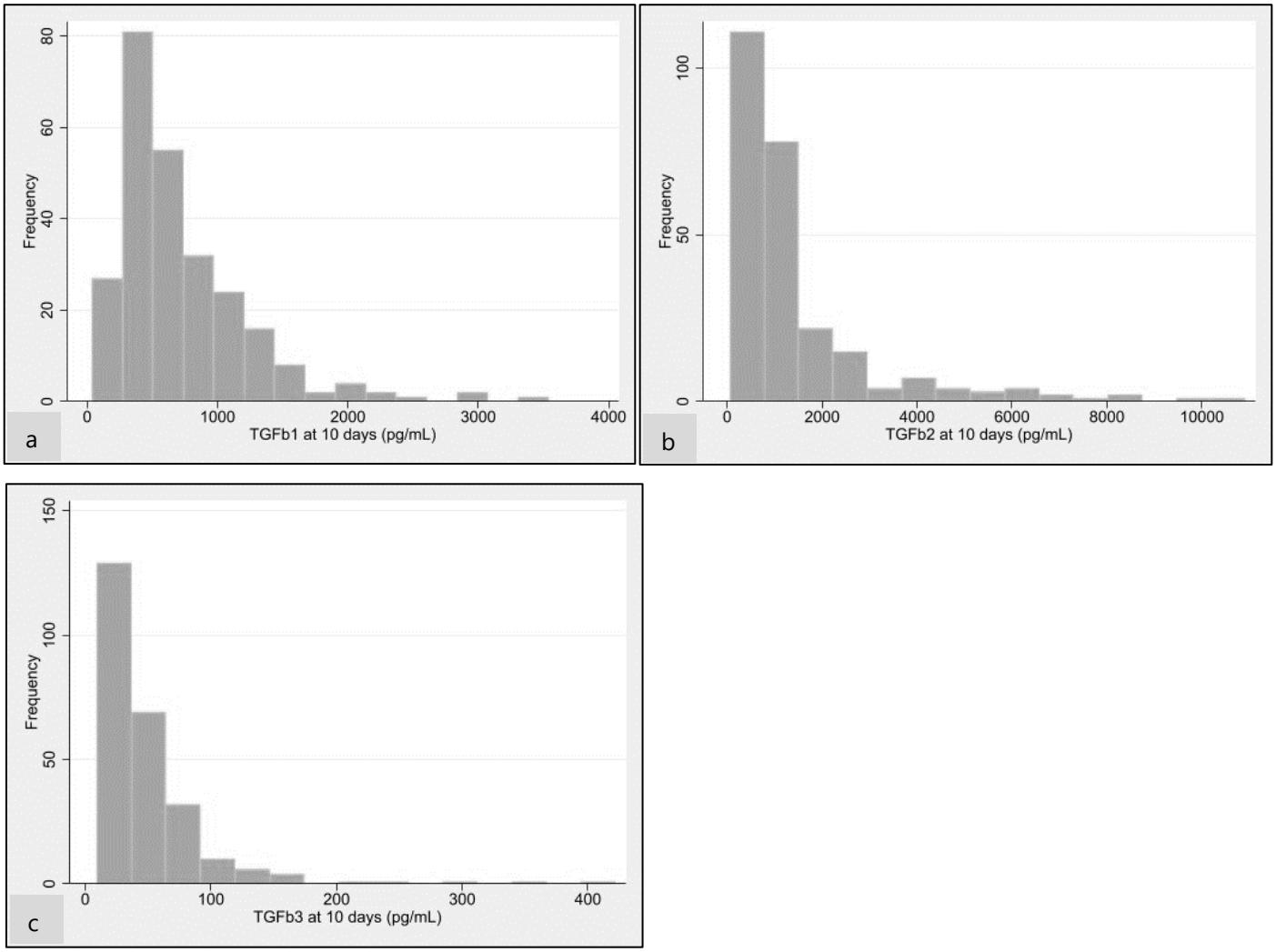
Breast milk TGF-β isotypes	The available breast milk cytokine samples at 10 days			Pairs of one breast milk cytokine and one infant gut permeability marker		
	n1*	Median (25^{th} – 75^{th} percentile); pg/mL	Min, Max; pg/mL	n2**	Median (25^{th} – 75^{th} percentile); pg/mL	Min, Max; pg/mL
at 10 days						
TGF β1	255	617 (395 – 896)	37; 3538	144	619 (384 – 824)	37; 3537
TGF β2	255	971 (482 – 1540)	56; 10914	144	973 (482 – 1417)	56; 10914
TGF β3	255	36 (25 – 57)	9; 422	144	36 (25 – 54)	9; 361
at 3 months						
TGF β1	247	427 (286 – 646)	71.9; 2427.7	143	393 (278 – 605)	115; 2375
TGF β2	247	577 (298 – 1120)	57; 26128	143	536 (276 – 1022)	56; 26128
TGF β3	246	30 (19 – 45)	7; 553	143	29 (19 – 41)	7; 150

*n1 number is among 255 mothers who have TGF-β isotypes samples at 10 days from the total population of ProPACT study (415 subjects). **n2 number is among 147 mothers who have at least one breast milk cytokine sample and their corresponding child has at least one plasma gut permeability sample.

Among 415 mothers in the ProPACT study, there were 255 mothers at 10 days who collected breast milk samples and all of them had detectable TGF-β isotypes, and so did 247 mothers at 3 months. Among those three isotypes in the group of n=255 at 10 days and in the group of n=247 mothers at 3 months, TGF-β2 had the highest median concentration (971 pg/mL and 577 pg/mL), followed by TGF-β1 (617 pg/mL and 427 pg/mL) and TGF-β3 had the lowest median concentration (36 pg/mL and 30 pg/mL) (Table 2a). It should be noted that n2 group was a group of mother-infant pairs in which mother and infant had at least one available cytokine and plasma gut permeability marker sample. This explains three missing data of TGF-β

isotypes at 10 days and four missing data at 3 months. This applies to for the table of breast milk TSLP concentration and term-infant plasma gut permeability markers.

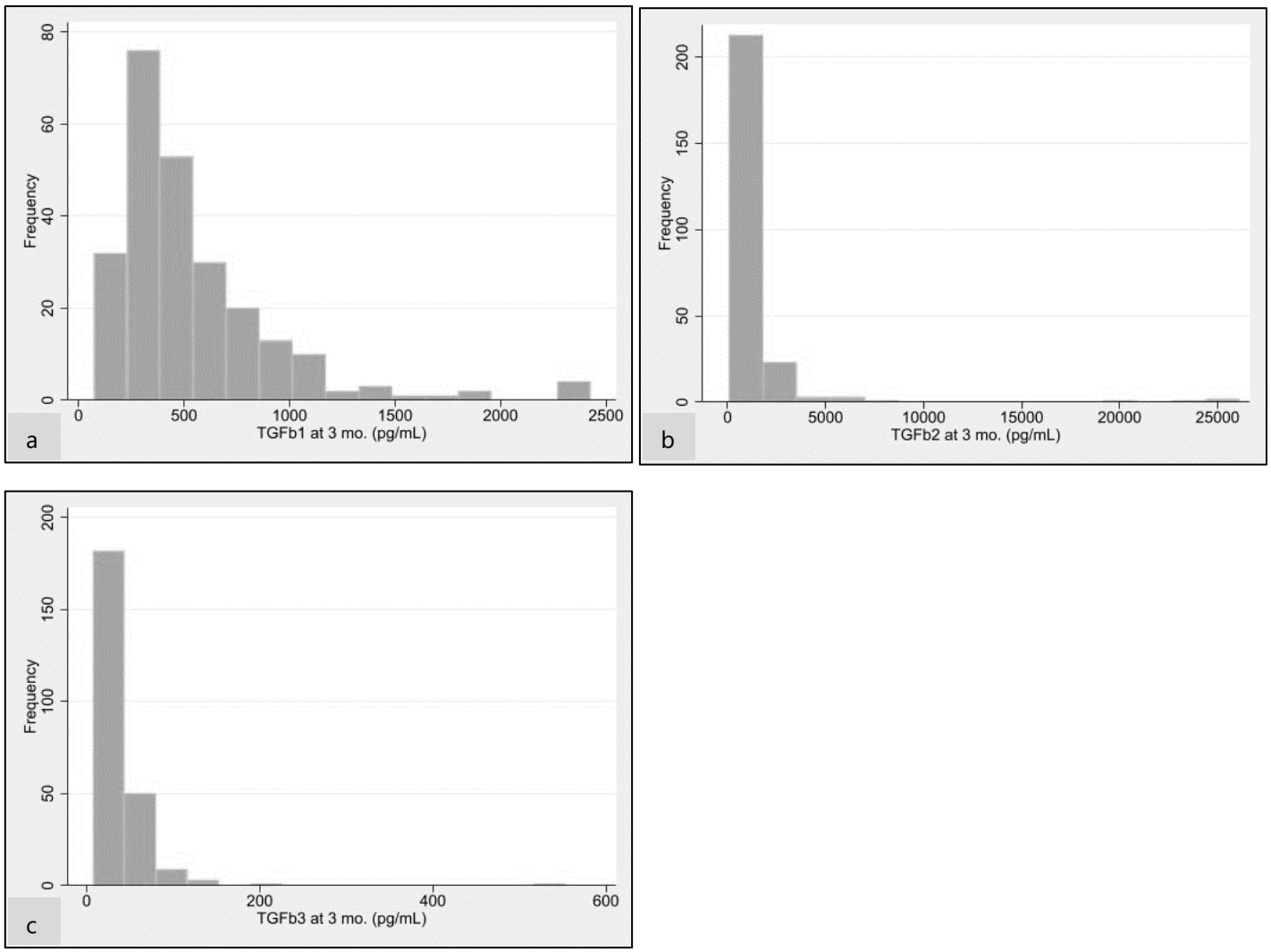
Figure 1 (a-c): TGF-β isotypes at 10 days, TGF-β1 (a), TGF-β2 (b), TGF-β3 (c)



The TGF-β median concentration for each isotype in the other subgroup (n=144 at 10 days and n=143 at 3 months) were similar in the order of the concentration values (TGF-β2, TGF-β1, TGF-β3). At 3 months, there was one particular subject mother, who was in both groups, had a very high concentration of TGF-β2 (26 128.2 pg/mL) compared to other mothers. There were also some variations of TGF-β concentration values between two subgroups at 3 months: among minimum

values of TGF-β1 (71.9 pg/mL to n=247 vs. 115 pg/mL to n=143) and maximum values of TGF-β3 (553 pg/mL to n=247 vs. 150 pg/mL to n=143).

Figure 2 (a-c): TGF-β isotypes at 3 months, TGF-β1 (a), TGF-β2 (b), TGF-β3 (c)



The histograms portray wider varieties of TGF-β1 concentration compared to the other two isotypes (Figure 1a and 2a), and both of TGF-β2 and TGF-β3 concentrations at 3 months become less varied (Figure 2b-c). The TGF-β isotypes' concentrations decreased along with the breastfeeding timepoints after birth, specifically a drastic concentration change occurred in both TGF-β1 and TGF-β2 at 3 months (Figure 2a-b) compared to those at 10 days (Figure 1a-b), while TGF-β3 median concentration seemed steady with a decreasing trend and less variation at 3

months (Figure 2c) than at 10 days (Figure 1c). TGF-β2 had also much fewer variation of concentration values at 3 months (Figure 2b) compared to that at 10 days (Figure 1b).

All TGF-β isotypes had right-skewed distributions as many breast milk cytokine studies revealed previously (5, 7, 52, 53), and median values for each isotype, at each timepoint were likely similar for both subgroups in this study population.

3. TSLP concentration in breast milk

Among 415 mothers in the ProPACT study, there were only 238 mothers who have detectable TSLP samples at 10 days and at 3 months follow-up there were only 225 mothers (Table 2b).

TSLP concentration categories had similar general proportion of detectable samples (low and high concentration) both at 10 days and 3 months (54.4% and 56%, in n1 group). While the proportion of detectable samples (low and high values) in n2 group were slightly higher than in n1 groups (58.3% and 60%, respectively to at 10 days and at 3 months). Most of detectable TSLP concentrations were in low value in both subgroups, 32.3% (n=238) and 34.6% (n=136) at 10 days, and the proportion for the low concentration at 3 months was even higher, 36% (n=225) and 39% (n=125) (Table 2b).

Samples with TSLP concentration both below and above detection were in steady proportion for both at 10 days and 3 months, around 44% (n1) and 40% (n2). Along with the breastfeeding timepoint, more than 60% of the mothers in both n1 and n2 subgroups (62.7% vs. 65.6%) have either below detection or low concentration of TSLP at 3 months. And it is portrayed in Table 2b that the TSLP median concentration in this study population was shown to be decreasing following breastfeeding timepoint, from 224.4 pg/mL (n1=238 at 10 days) to 144.2 pg/mL

(n1=225 at 3 months), and from 221.2 pg/mL (n2=136 at 10 days) to 142 pg/mL (n2=125 at 3 months). In table 2b, we can see that the subgroup n2 had fewer available breast milk TSLP samples (n2 = 136) than TGF-β isotypes (n = 144, table 2a). This did not affect the description of whole TSLP sample (n1).

Table 2b. Maternal breast milk Thymic Stromal Lymphopoietin (TSLP) concentration

TSLP level of detection at each timepoint	The available breast milk cytokine samples at 10 days	Pairs of one breast milk cytokine and one infant gut permeability marker
	n1* (%)	n2** (%)
At 10 days	238 (100)	136 (100)
Median (25th-75th percentile), pg/mL	224.4 (57 – 2105)	221.2 (66 – 1267)
Min, max, pg/mL	-11.6, 12702.4	1.2, 12702.4
TSLP categories:		
below detection	45 (19)	24 (17.6)
low	77 (32.3)	47 (34.6)
high	55 (23.1)	35 (25.7)
above detection	61 (25.6)	30 (22.1)
At 3 months	225 (100)	125 (100)
Median (25th-75th percentile), pg/mL	144.2 (46 – 1086)	142.0 (46 – 690)
Min, max, pg/mL	-46.155 – 7216.73	-46.16 – 7216.73
TSLP categories:		
below detection	60 (26.7)	33 (26.4)
low	81 (36)	49 (39.2)
high	45 (20)	26 (20.8)
above detection	39 (17.3)	17 (13.6)

*n1 number is among 255 mothers who have TGF-β isotypes samples at 10 days from the total population of ProPACT study (415 subjects). **n2 number is among 147 mothers who have at least one breast milk cytokine sample and their corresponding child has at least one plasma gut permeability sample.

4. Term infant plasma gut permeability markers: LBP and FABP-2

Table 3. Term-infant plasma gut permeability concentration at each timepoint

	The available breast milk cytokine samples at 10 days			Pairs of one breast milk cytokine and one infant gut permeability marker		
	n1*	Median (25^{th} – 75^{th} percentile)	Min; Max	n2**	Median (25^{th} – 75^{th} percentile)	Min; Max
at 10 days						
LBP[a], µg/mL	96	1.0 (0.7 – 1.6)	0.3, 4.0	93	1.0 (0.7 – 1.6)	0.3; 4.0
FABP-2[b], pg/mL	96	431 (291 –590)	36.2, 1343	93	431 (290 – 578)	36.2; 1105
at 3 months						
LBP, µg/mL	115	1.4 (1 – 2.7)	0.2; 11.8	110	1.4 (1 – 2.4)	0.2; 11.8
FABP-2, pg/mL	114	362 (243 – 586)	102.7; 1703.7	109	362 (252 – 579)	102.7; 1703.7

*n1 number is among 255 mothers who have TGF-β isotypes samples at 10 days.
**n2 number is among 147 mothers who have at least one breast milk cytokine sample and their corresponding child has at least one plasma gut permeability sample.
[a] LBP: Lipopolysaccharides-binding protein
[b]FABP-2: Fatty-acid-binding protein-2

Term infant plasma LBP and FABP-2 median concentration were not different between two subgroups, n1 and n2, for each timepoint. LBP concentration seemed to increase slightly from 10 days to 3 months (median level 1 µg/mL at 10 days to 1.4 µg/mL at 3 months), while FABP-2 concentration decreased, from median level 431 pg/mL at 10 days to 362 pg/mL at 3 months (Table 3).

The number of samples were nearly similar for both subgroups for each timepoint: 96 and 93 samples at 10 days, 115 and 110 samples at 3 months. We found a high concentration of LBP at 3 months 11.8 µg/mL, and higher minimum and maximum concentration values of FABP-2 at 3 months (102.7 pg/mL and 1703.7 pg/mL) compared to those at 10 days.

Figure 3. Lipopolysaccharide binding protein (LBP) in µg/mL (ug/mL), at 10 days (a), at 3 months (b)

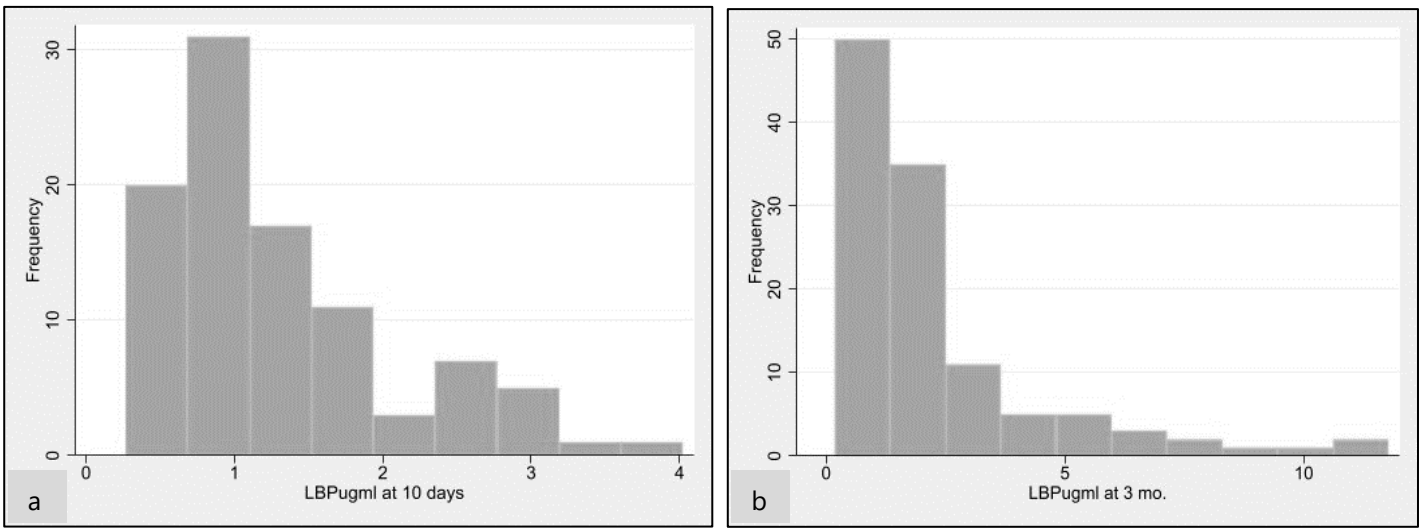


Figure 4. Fatty acid binding protein-2 (FABP-2) in pg/mL, at 10 days (a), at 3 months (b)

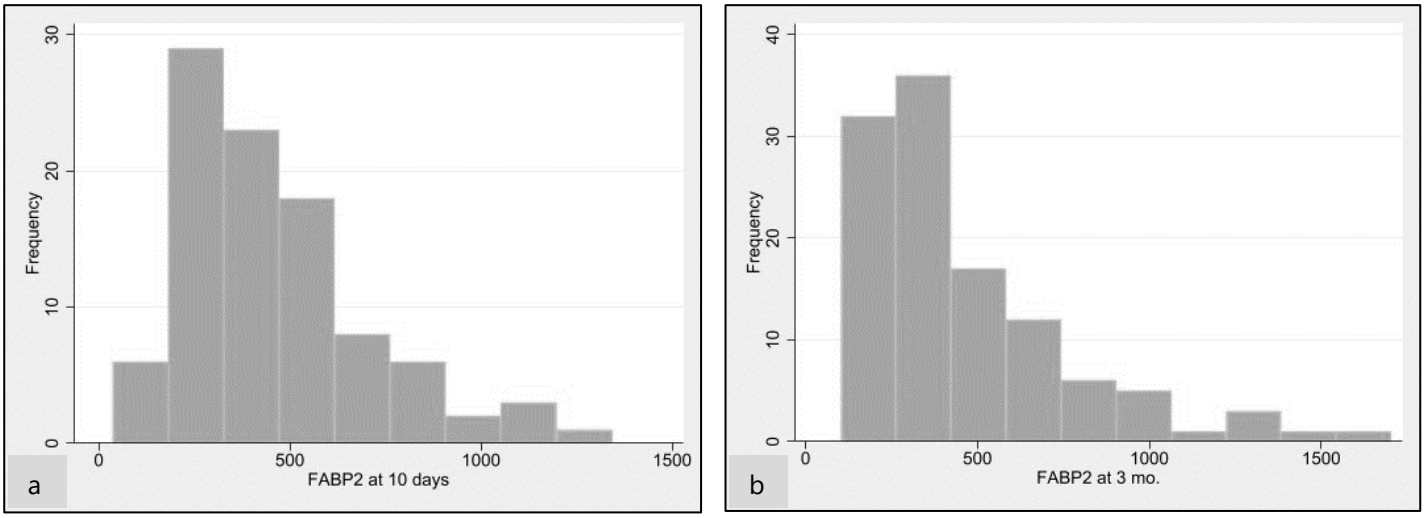


Figure 3a shows a distinct majority of term infants had LBP concentration lower than 5 µg/mL at 10 days. Most term infants at 10 days had LBP concentration about 1 µ g/mL (figure 3a). However, figure 3b portray a wider variation of higher LBP concentration values at 3 months (up to more than 10 µg/mL) compared to that

at 10 days (figure 3a), and more mothers had the lowest value of LBP concentration at 3 months (closer to 0 µg/mL) compared to that at 10 days.

Meanwhile, FABP-2 concentration of the term infants in this current study had slightly similar variation in concentration for both breastfeeding timepoints (figure 4a and 4b). However, mostly term infants at 10 days had FABP-2 around 300-550 pg/mL (figure 4a), while at 3 months they had mostly lower FABP-2 concentration (figure 4b) than that at 10 days. We can see at 3 months, there were few infants who had FABP-2 concentration more than 1 500 pg/mL. We can see also at 3 months more infants had the lowest FABP-2 concentration than that at 10 days, although the lowest FABP-2 concentration at 3 months was higher than that at 10 days (which closer to 0 pg/mL) (figure 4a-b).

5. Association between maternal breast milk TGF-β isotypes and TSLP and term infant gut permeability plasma LBP and FABP-2 markers

In multivariate models, most of the gut permeability marker concentration had an increase trend when the concentration of TGF-β isotypes and TSLP increases (Table 4a and 4b). Generally, there were no statistically significant association between breast milk TGF-β isotypes and TSLP and infant gut permeability markers at 10 days and 3 months after birth, except for two (Table 4a).

Firstly, the LBP infant gut permeability marker concentration at 10 days tended to be lower when TSLP concentration in breast milk was above the level of detection, compared to below the level of detection. Although it was only among those participants with high detectable levels of TSLP in breast milk which had a statistically significant decrease in LBP infant gut permeability markers compared to those with below detectable levels of TSLP in breast milk [beta coefficient = -0.64, 95% CI (-1.2) to (-0.12), *p* value = 0.02]. After adjustment for confounders, the decreasing trend of LBP concentration was not statistically significant. Nevertheless, overall difference of LBP infant gut permeability marker concentration at 10 days among four categories

of breast milk TSLP detection value at 10 days was not statistically significant (p = 0.13), and adjustment of the confounding factors did not alter this conclusion (p = 0.21).

Secondly, the FABP-2 infant gut permeability marker concentration at 3 months seemed to be lower only when TSLP concentration in the breast milk at 3 months were detected low, with a borderline statistical significance, p = 0.059. After adjustment for the confounders, the association between maternal TSLP concentration in breast milk and plasma FABP-2 infant gut permeability marker concentration was not statistically significant (p = 0.438). Overall, the association between breast milk TSLP and FABP-2 infant gut permeability marker at 3 months was not statistically significant, even after adjustment.

Table 4a. Multivariate models of association between maternal breast milk TGF-β isotypes and plasma gut permeability parameters in term infants.

	Unadjusted			Adjusted		
	n	**Beta Coefficient (95 % CI)**	***p-value***	**n**	**Beta Coefficient (95 % CI)**	***p-value***
TGFβ1 at 10 days						
LBP at 10 days	90	0.00017 (-0.00013 – 0.0005)	0.264	90	0.00009 (-0.0002 – 0.0004)	0.550
LBP at 3 months	109	0.00003 (-0.0008 – 0.0009)	0.953	109	0.0001 (-0.0008 – 0.001)	0.750
FABP-2 at 10 days	90	0.03 (-0.06 – 0.12)	0.525	90	0.03 (-0.07 – 0.13)	0.528
FABP-2 at 3 months	108	-0.007 (-0.12 – 0.11)	0.905	108	0.04 (-0.07 – 0.2)	0.459
TGFβ2 at 10 days						
LBP at 10 days	90	0.0000023 (-0.00008 – 0.00009)	0.957	90	-0.000025 (-0.0001 – 0.0001)	0.572
LBP at 3 months	109	0.00002 (-0.00025 – 0.0003)	0.9	109	0.00007 (-0.0002 – 0.0003)	0.602
FABP-2 at 10 days	90	-0.008 (-0.03 – 0.02)	0.543	90	-0.0077 (-0.04 – 0.02)	0.570
FABP-2 at 3 months	108	-0.0034 (-0.04 – 0.03)	0.841	108	0.005 (-0.03 – 0.04)	0.774
TGFβ3 at 10 days						
LBP at 10 days	90	0.00002 (-0.004 – 0.004)	0.992	90	-0.001 (-0.01 – 0.003)	0.478
LBP at 3 months	109	-0.006 (-0.014 – 0.003)	0.215	109	-0.005 (-0.015 – 0.004)	0.287
FABP-2 at 10 days	90	-0.21 (-1.4 – 1)	0.722	90	-0.17 (-1.4 – 1.1)	0.795

FABP-2 at 3 months	108	-0.69 (-1.9 – 0.5)	0.249	108	-0.2 (-1.5 – 1.1)	0.728
TGFβ1 at 3 months						
LBP at 3 months	107	0.0002 (-0.0009 – 0.001)	0.77	107	0.0002 (-0.001 – 0.001)	0.666
FABP-2 at 3 months	106	0.009 (-0.13 – 0.14)	0.889	106	0.036 (-0.1 – 0.2)	0.590
TGFβ2 at 3 months						
LBP at 3 months	107	-0.00003 (-0.0001 – 0.0001)	0.599	107	-0.00002 (-0.0001 – 0.0001)	0.670
FABP-2 at 3 months	106	-0.007 (-0.02 – 0.01)	0.258	106	-0.005 (-0.02 – 0.01)	0.416
TGFβ3 at 3 months						
LBP at 3 months	107	-0.01 (-0.03 – 0.002)	0.101	107	-0.013 (-0.03 – 0.003)	0.107
FABP-2 at 3 months	106	-0.56 (-2.6 – 1.5)	0.580	106	-0.53 (-2.5 – 1.5)	0.602

CI: Confidence Interval of beta coefficient

*Adjusted model is accounted for five possible confounders: maternal age, maternal atopy, sex of the child, siblings, and maternal active smoking status within 6 weeks after birth.

Table 4b. Multivariate models of association between maternal breast milk TSLP and plasma gut permeability parameters in term infants

TSLP categories	**Unadjusted**				**Adjusted**		
	Mean ± SD of outcome at each timepoint	**n**	**Beta Coefficient (95% CI)**	***p* value**	**n**	**Beta coefficient (95% CI)**	***p* value**
TSLP at 10 days	**LBP (µg/mL) at 10 days**	**88**		**0.13**	**88**		**0.21**
below detection	1.7 ± 0.9	15	0			0	
low	1.3 ± 0.7	33	-0.41 (-0.9 – 0.07)	0.092		-0.43 (-0.9 – 0.1)	0.085
high	1.0 ± 0.7	19	-0.64 (-1.2 – (-0.12))	0.02		-0.59 (-1.2 – (-0.03))	0.039
above detection	1.3 ± 0.9	21	-0.36 (-0.9 – 0.2)	0.175		-0.43 (-1 – 0.1)	0.122
TSLP at 10 days	**LBP (µg/mL) at 3 months**	**102**		**0.96**	**102**		**0.95**
below detection	2.4 ± 2.6	18	0			0	
low	2.2 ± 2	37	-0.16 (-1.5 – 1.2)	0.811		-0.16 (-1.6 -1.3)	0.825
high	2.1 ± 2.2	24	-0.25 (-1.7 – 1.2)	0.734		-0.12 (-1.8 – 1.5)	0.881
above detection	2.4 ± 2.8	23	0.07 (-1.4 – 1.5)	0.925		0.1 (-1.4 – 1.7)	0.901

TSLP at 10 days	**FABP-2 at 10 days**	**88**		**0.6**	**88**		**0.91**
below detection	485.3 ± 311.5	15	0			0	
low	451.9 ± 225.2	33	-33.4 (-181.5 – 114.7)	0.655		-43.5 (-197.2 – 110.2)	0.575
high	401.8 ± 209.4	19	-83.5 (-247.8 – 80.8)	0.315		-98.3 (-274.3 – 77.7)	0.270
above detection	498.6 ± 227.5	21	13.3 (-147.5 – 174.1)	0.870		3.9 (-166.2 – 174)	0.964
TSLP at 10 days	**FABP-2 at 3 months**	**101**		**0.75**	**101**		**0.18**
below detection	437.7 ± 279.6	18	0			0	
low	494.2 ± 330.5	37	56.5 (-111.7 -224.7)	0.507		65.4 (-106.8 – 237.6)	0.453
high	423.1 ± 255.3	24	-14.6 (-197.1 – 167.9)	0.874		16.03 (-179.5 – 211.6)	0.871
above detection	425 ± 282.4	22	-12.7 (-198.8 – 173.3)	0.892		-2.8 (-189.6 – 184)	0.976
TSLP at 3 months	**of LBP (µg/mL) at 3 months**	**97**		**0.85**	**97**		**0.91**
below detection	2.5 ± 2.5	28	0			0	
low	2.2 ± 1.8	35	-0.37 (-1.6 – 0.8)	0.542		-0.2 (-1.6 – 1.1)	0.737
high	2.6 ± 2.8	21	0.05 (-1.3 – 1.4)	0.942		0.27 (-1.2 – 1.7)	0.706
above detection	2.0 ± 2.7	13	-0.49 (-2.1 – 1.1)	0.545		-0.34 (-2 – 1.4)	0.691
TSLP at 3 months	**of FABP-2 at 3 months**	**96**		**0.29**	**96**		**0.15**
below detection	529.5 ± 286.8	28	0			0	
low	389.7 ± 313.1	35	-139.8 (-285.3 – 5.6)	0.059		-62.5 (-221.9 – 97)	0.438
high	426.2 ± 191.6	21	-103.3 (-268.9 – 62.3)	0.218		-57.1 (-227 – 112.7)	0.506
above detection	449.1 ± 354.7	12	-80.37 (-278.3 – 117.5)	0.422		-21.5 (-221.6 – 178.5)	0.831

Discussion

Thymic stromal lymphopoietin (TSLP) was the only breast milk cytokine with a possible tendency in decreasing term infant gut permeability markers, plasma LBP and FABP-2, although the association was not statistically significant. Breast milk TSLP acted in different timepoints and different concentrations for different term infant gut permeability plasma markers, LBP and FABP-2. Lower concentration of term infant gut permeability plasma LBP marker was observed at 10 days when breast milk TSLP concentration was high at 10 days, and conversely lower concentration of term infant gut permeability plasma FABP-2 marker at 3 months was observed when breast milk TSLP concentration was low at 3 months. Both unadjusted and adjusted models indicated that TGF-β isotypes and TSLP concentration in breast milk did not influence term infant gut permeability plasma markers either LBP nor FABP-2 concentration for the first 3 months of breastfeeding periods. Thus, TGF-β isotypes and TSLP in breast milk are unlikely to affect term infant gut permeability plasma markers, LBP and FABP-2.

Eczema development in children may be due to increased infant gut permeability, which in turn may be due to variability in TGF-β isotypes concentration in breast milk in different nationalities (7, 54, 55) and lifestyle including nutritional intakes (8, 56). Due to the already-matured gut immunity in our term infants' population, it may be that the pro-inflammatory nature of breast milk TSLP was not properly activated to fight against invaders or react to inflammatory gut mucosal injury. This may be one reason why breast milk TSLP in our study population did not really influence the concentration of plasma LBP and FABP-2 as term infant gut permeability markers.

Breast milk TGF-β1 role might be more visible when we investigate it against allergy disease outcomes such as atopic dermatitis, as previous prospective findings in 2006 (57) showed that in atopic mothers, the colostrum and the mature milk have significant difference in TGF-β1 concentration. But in this master thesis, we try to find

out the biologic mechanism within. Further, TGF-β1 in mature milk of atopic/allergic mothers was significantly lower than that in non-allergic mothers at 1 month (57), and at 6 months follow-up, 46% (6 out of 13) of the babies from allergic mothers presented atopic dermatitis symptoms. Moreover, four of the babies who developed atopic dermatitis had received breast milk with no TGF-β1 level both in colostrum and mature milk (57). The similar findings were reproduced in 2018 in Japan from a study cohort of 500 babies with multiple breast milk sampling for both colostrum and mature milk, and they found that lower levels of TGF-β1 in mature milk compared to that in colostrum was responsible for eczema development in later life (6 month-infants) (58). Our study findings were not able to provide sufficient evidence to support that breast milk TGF-β1 might affect term infant gut permeability markers at 10 days or 3 months, which may be an indication of childhood allergy development. The current study could not investigate allergy disease since that diagnosis in our study population was done on children at 2 years of age.

Whilst breast milk TGF-β2 has been noted as the highest TGF-β isotype in breast milk during 6 months of age after birth (7), its connection to later eczema in infants when the level in breast milk is high was already demonstrated previously (54). Term infants in this study population were assumed to be normal and healthy both at 10 days and at 3 months. Diagnosis of allergy diseases such as atopic dermatitis was only confirmed at 2 years of follow up which was not included in our current analysis. Supposedly, those term infants had a matured growing gut system, including well-functioning gut mucosal immunity. Despite their mothers' breast milk that contained high concentration of TGF-β2, this cytokine would probably contribute to other immunological process, such as promoting more gut epithelial differentiation (11) into maturation and gut immunity cascades with other immune factors, instead of directly suppressing any molecular expression originating from inflammatory gut mucosal injury reactions, such as LBP and FABP-2. We implied that

the changes in plasma LBP and FABP-2 of our term infant population could not be a proper representation of modified gut permeability due to the role of TGF-β2 solely.

In normal gut tissue of mature and term infant, it is proven in vitro that resident intestinal macrophages undergo progressive inflammatory down-regulation and resemble macrophages in the adult intestine (13). While in the preterm infant mouse model, premature bacterial colonization would predispose inflammatory gut mucosal injury due to incomplete process of inflammatory down-regulation of the resident macrophages, for example in necrotizing enterocolitis (NEC). Normally, TGF-β2 is expressed widely in the lamina propria of mature intestine, whether in infant or adult human gut tissue, in the breast milk (in high concentration), and in the lung (but not in the distal airspaces and alveoli). Therefore, premature infants would not have either mature macrophage function or high concentration of gut-origin TGF-β2. Furthermore, infant gut-origin TGF-β2 is usually stimulated and produced when there is exposure and induction by maternal breast milk TGF-β2 (59) in which mothers from preterm infant usually do not have in high levels of, a loop feedback system. Thus, when NEC is occurring, inflammatory gut mucosal injury is inevitable.

Reported by Maheshwari (13) in 2011, enterally administered recombinant TGF-β2 was found to be protective against inflammatory mucosal injury in infant models' gut epithelial cells and it can suppress the expression of macrophage's inflammatory cytokines. Newly updated findings on this can be seen in the schematic diagram of breast milk TGF-β for the infants' gastrointestinal tract and maturation of the immune system by Brenmoehl, 2018 (59). As the authors reviewed that infant with NEC had diminished level of gut mucosal TGF-β2 expression, even lower than the premature or fetal intestine, and reduced breast milk TGF-β bioactivity. Several previous research findings had been reviewed by Brenmoehl, 2018 (59) that breast milk decreases the severity of NEC in preterm infants, which is distinguished by elevated gut weight, mucosa proportion and villus height. Even TGF-β2 through its action phosphorylates its receptor, leading to villus growth of gut epithelium in

newborn pigs, and increased expression of tight junction proteins that regulates gut epithelial permeability. Consequently, breast milk TGF-β2 is protective by decreasing apoptosis of intestinal cells and suppression of macrophage cytokine expression. Thus, genetic/epigenetic variability in TGF-β2 expression is associated with the risk of NEC in premature newborns (59), which we suggest that it cannot actually be detected only by measuring plasma LBP and FABP-2 to represent an increase in term-infant gut permeability.

Meanwhile, TGF-β3 is responsible for mammary gland involution at after milk stasis thus the levels rapidly rise during initial phases of involution (60) which clearly was not happened in our study subjects due to randomized trial requirement to breastfeed the child at least until 25 months after birth. Further, TGF-β3 local expression induces apoptosis (61) thus it is an irreversible commitment in the second phase of involution and therefore, we thought that TGF-β3 role cannot be associated with infant gut immunity and infant gut maturation process. Previous finding by Munblit et al (62) was also supported this suggestion that only TGF-β1 and TGF-β2 which were positively correlated with maternal breast milk IgA in the context of infant gut maturation and infant gut immunity.

Ziegler and Artis (23) found that low TSLP was seen in non-inflamed colonic adult tissue in Crohn's disease and ulcerative colitis. This positive effect was attenuating Th1/Th17 inflammatory response and directly acting on the gut epithelium to support wound healing in any colon inflammation. TSLP has many effector immune cells (23), thus we suggested that TSLP role explained in their study is probably suitable to support our study findings in which lower plasma LBP in term infant at 10 days was observed when breast milk TSLP was high at 10 days. In this case, we implied that infant plasma LBP, in which at low concentration might represent an acute-phase immunologic reaction and involved in monocyte activation (28, 30), might have probably been modulated together by TSLP and TGF-β2 (as explained above) in breast milk.

Therefore, we can see that all term infants in our study population had low LBP concentration level (median 1 µg/mL, 25^{th}-75^{th} percentile 0.7-1.6 µg/mL; median 1.4 µg/mL, 25^{th}-75^{th} percentile 1-2.7 µg/mL, respectively for each subgroup), compared to physiological LBP level in serum or plasma of healthy human (5-10 µg/mL) (28, 30, 31, 38) and to term infants with negative neonatal bacterial infection (3.9 µg/mL) (32). Although it is unsure if our LBP finding is comparable with the reported normal values since the sample population in the first references (28, 30, 31, 38) and the sample collection time in the second reference (32) were different from our plasma LBP characteristics. Other explanation of this finding might due to normal healthy term-infant study population in which they actually had the subnormal level of LBP to increase phagocytosis and clearance of lipopolysaccharides (LPS) antigen from blood (33, 38). Thus, the association between high TSLP in breast milk and low plasma LBP at 10 days in our study population was possibly a coincidence.

Further, lower plasma FABP-2 at 3 months was seen in this current study when breast milk TSLP was low at 3 months which probably indicated that there was no inflammation or intestinal injury occurred in the gut tissue of our term infant population. As mentioned before that classical cell death, apoptosis, will not release cell contents into circulation (41). Breast milk TSLP role against childhood atopic dermatitis might be likely more visible if we also investigate AD outcome at 2 years. As Jepsen et al (63) found that low TSLP in maternal breast milk was significantly protective against eczema during early childhood (0-3 years).

To date, this current study provides new information on plasma FABP-2 concentration in term infant at 10 days and at 3 months after birth. Previous research findings mainly provided FABP-2 concentration in either autopsy samples of the adult intestine (duodenum, jejunum, ileum, colon) (41); preterm infants serum with NEC (44); plasma of healthy preterm infants and preterm infants with NEC (45); early postnatal urine of preterm and term infants (43); autopsy samples of gut tissue obtained from extremely preterm, moderately preterm, and term born infants who

died within 24 hours after birth (43). Guthmann et al (45) found plasma FABP-2 median concentration in healthy preterm neonates 2.52 ng/mL (2 520 pg/mL), within a range of 460 – 4 510 pg/mL.

Meanwhile, term infant plasma FABP-2 gut permeability marker concentration at each timepoint in our current study were mostly in normal range of the assay detection level (15.6 – 1 000 pg/mL), except for few infants who had FABP-2 concentration more than 1 000 pg/mL but less than 2 000 pg/mL.

Reisinger et al (43) concluded that FABP-2 expression is changed along with gut maturation in utero environment, from crypts to intestinal villi, which both displayed on intestine samples of experimental lambs and intestine samples from dead human newborns of three gestational periods: premature, moderately premature, and full-term. According to Pelsers, 2005 (42) that when we evaluating FABPs as clinical tissue injury marker, the time appearance of these markers, we have to take into account for the time course of the disease, molecular size and distribution over extravascular compartments. It is noted that to analyze FABP-2 concentration, the assay must have a good detection property since an early study of FABP-2 in 2003 (41) had difficulties to find FABP-2 concentration more than 100 pg/mL (less than 0.1 µg/L or 0.1 ng/mL), due to its rapid clearance from blood circulation and its low affinity ligand binding sites (50). In fact, under normal physiological conditions, FABP-2 is detectable in serum at low levels (50). So, it is not unexpected that our term infants' plasma FABP-2 concentrations were in a normal range supposed that the infants in our study population were normal and healthy full-term newborn. Results from this paper may there serve as an important reference regarding expected FABP-2 concentrations in term-born infants.

Multivariate analysis in our study did not elucidate any associations between selected breast milk cytokines and term infant gut permeability plasma markers, although maternal atopy and cigarette smoke exposure in late pregnancy (trimester

3) until the first year of child life were thought to have an effect on both breast milk cytokines and term infant gut permeability markers.

Exposure to cigarette smoke in our study population were high, considering the study population lived in Norway in which Tobacco-control initiative is continuously encouraged. A registry study in southern Norway in 2019 (64) reported that there were a decrease trend more than doubled in cigarette smoking within third trimester among pregnant women between 2012 and 2017 (8.9% to 4.1%). However, the current study population includes pregnant women between 2000 and 2009 (the PACT study time period). Results in a registry study from 1999 to 2004 (65) confirmed our smoking status findings by showing the daily smoking prevalence among pregnant women at the end of pregnancy was reduced from 17.3% in 1999-2001 to 13.2% in 2002-2004, although there was an increasing social polarization with regard to education level and smoking habits among multiparous (3+), teenage mothers, single women, and low educated women.

Despite null findings, we have study strength. We used a robust dataset from a sub-study population, the ProPACT Study, which was conducted within a large community study in Trondheim, Norway, the PACT study. As ever mentioned in previous publication of the ProPACT study, this study population was the largest to investigate TSLP concentrations in breast milk and the only one to consider infant outcomes and maternal atopy status (5). Moreover, to detect TSLP in breast milk is quite challenging due to its rapid degradation, and many studies reported low concentration within a relatively narrow range. In 2010 (26) Macfarlane et al reported TSLP concentration, in a study from 44 women who submitted samples at one of two timepoints, was only 90 pg/mL (median level) at 3-5 days after birth and it was significantly higher than those collected at 11-26 days after birth. Our result provides much higher TSLP concentration at 10 days (median 224.4 pg/mL) and even at 3 months (median 144.2 pg/mL). TSLP concentrations in our study at 10 days were quite similar in proportion of its detection level, between below detection to low

(19% to 32.3%) and high to above detection (23.1% to 25.6%). Although the proportion of TSLP concentrations detected were slightly lower at 3 months (62.7% were either below detection or low) compared with those at 10 days. Another thing to note is that our study was able to detect FABP-2 concentrations in term infants within a good detection range, which previous studies had not done in the similar aged study subjects.

Selection bias was inevitable since we only used available samples among 415 subjects of the ProPACT study, of which data on 147 mother-child pairs was available for analysis. If this study was reproduced with a higher sample size, it may alter the conclusion. Reasons for this might due to 1) inaccurate cytokine representatives, 2) the infant gut permeability markers may not have been the correct end-point for our association of interest, 3) selected timepoints was not really precise since increased in infant gut permeability might occurred at the earliest of infants' life (4-7 days) (39), and 4) the immune factors in breast milk may work together in such a way that there may be no single molecule that has a clear association. Subgrouping for n1 and n2 created discrepancies in the number of mothers and cytokine samples but not the infant gut permeability samples. However, this discrepancy did not have any effect on association tests and proved that there was no highly selected group in our study.

Conclusion

Breast milk TGF-β isotypes and TSLP do not appear to be associated with the term infant gut permeability markers, plasma LBP and FABP-2 both at 10 days and 3 months after birth in a Norwegian population. Further studies are required to investigate and confirm whether increased gut permeability in term infant has a role in the development of childhood allergy diseases.

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