Determinants of colostrum and breast milk immune composition and consequences for infant health

Daniel Munblit

Department of Paediatrics

Department of Medicine

Imperial College London

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy in Clinical Medicine

Abstract

Background:

Breast milk is the principal source of nutrition during a critical period of immune programming - maternal and environmental exposures may influence breast milk composition and infant health.

Objectives:

To examine whether environmental and/or maternal factors influence levels of immune active components in colostrum and breast milk and identify associations between these factors and health outcomes. To examine if colostrum/breast milk immune composition can be grouped into specific 'lactotypes'.

Methods:

A prospective cohort study of mother/infant pairs in London, Moscow and Verona. Colostrum samples (days 0-6) and Mature Breast Milk (4-6 weeks) were analysed in duplicate using electrochemiluminescence, and the relationship between levels of immune active factors and maternal/environmental/infant factors was evaluated using mixed models. Lactotypes were identified using Principal Components Analysis.

Results:

Levels of immune active cytokines and growth factors in colostrum declined rapidly over time ($r=-0.39$ to -0.16; $p<0.01$). The effect of time could not be corrected using total protein or sodium as correction factors, due to different kinetics for each mediator measured.

There were significant differences in colostrum and breast milk composition between countries, which could not be explained by the environmental and maternal factors examined.

Using PCA there were two clusters of mediators, suggesting that four human breast milk ‘lactotypes’ exist, based on immune composition. There was some evidence in support of a relationship between human milk mediator levels and/or lactotypes, and infant health outcomes.

Conclusions:

The data support an important role for breast milk cytokines, and especially growth factor, levels as determinants of infant health. Further work is needed to identify improved methods for analysing colostrum and mature milk composition, which account for time of collection and/or stage of lactation.

List of abbreviations

ANPEP: Alanine aminopeptidase

B2M: Beta-2 microglobulin

BM: Breast Milk

CFB: Complement factor B

CoA: Coenzyme A

CST3: inhibitor of cysteine proteinases

CTSS: Cathepsin S

EGF: Epidermal growth factor

ERAP: endoplasmic reticulum aminopeptidase 1

GALT:Gut associated lymphoid tissue

HGF: Hepatocyte growth factor

HLA-DRB5: HLA class II histocompatibility antigen, DRB beta chain

IFN-γ: Interferon-gamma

Ig: Immunoglobulin

IL: Interleukin

MCP-1: monocyte chemotactic protein-1

MFGM: Milk fat globule membrane

MIP-1α: Macrophage Inflammatory Protein

PCA: Principal Component Analysis

PUFA: Polyunsaturated fatty acid

RANTES: regulated and normal T cell expressed and secreted

SERPIN: serine protease inhibitor

SNPs: Single Nucleotide Polymorphisms

SPINT: serine peptidase inhibitor

TGF-β: Transforming growth factor beta

TLR: Toll-like receptor

TSLP: Thymic stromal lymphopoietin

Table of Contents

List of figures

List of key tables

Declaration of originality

The work contained in this thesis is my own, unless otherwise referenced. I led the conception and design of the study, recruited participants, conducted the laboratory work, and the analyses, interpreted the data and wrote all the study texts.

A team of the following researchers made a substantial contribution to the conception and design of the studies, statistical analysis and interpretation of the data and provided critical comments on written texts. Specifically, they made the following contributions:

- *Supervisors:*
  - Prof. John O Warner (first supervisor, Imperial College London) provided guidance and support, and funding for the PhD;
  - Dr. Robert J Boyle (second supervisor, Imperial College London) provided guidance and support;
- *Collaborating researchers at the Institute of Paediatrics in Moscow, Russian Federation:*
  - Prof. Alexander Pampura provided support for the recruitment in Russia;
  - Dr. Marina Treneva provided support for the recruitment in Russia. She recruited participants in Moscow, conducted samples collection, storage and shipment to UK;
- *Collaborating researchers at the University of Verona, Verona, Italy:*
  - Prof. Diego Peroni provided support for the recruitment in Italy. He was responsible for participants recruitment in Verona, conducted samples collection, storage and shipment to UK;
- *Collaborating researchers and medical students at Imperial College London*
  - Shobana Dissanayeke (research technician) participated in laboratory work;

- Gabriela Feketea (Master's student) collected paired breast milk/maternal blood serum samples in Greece and participated in lab work and analysis;
- LiYan Chow, Nisha Patel (BSc students) participated in recruitment, samples collection, participants interviews, laboratory work and data input. Shreya Sheth, Priya Abrol (BSc students) conducted total sodium and total protein analysis; Binta Umar (medical student) participated in data input, Ruby Ramjan (medical student) participated in data input and one year follow-up. Lakmal Mudalige participated in one year follow-up.
- Silvia Colicino (PhD student) provided statistical analysis and expert advice.

Author’s signature: Dr. Daniel Munblit

Date: 11 November 2015

Copyright declaration

Acknowledgements

“In most of mankind, gratitude is merely a secret hope for greater favors”

- Duc de la Rochefoucauld, Maxims (1665)

It is a great pleasure to thank the many people who made this thesis possible.

It is difficult to overstate my gratitude to my PhD supervisor, Professor John Warner. With his enthusiasm, inspiration and his great efforts to explain things clearly and simply, he helped to me to understand a wonderful world of science better. Throughout my thesis-writing period, he provided encouragement, sound advice, good teaching and lots of good ideas. I am also especially grateful for his endless support, even when my initiatives seem to be unrealistic. I anticipate the possibility of working with him in the future with great hope that our breast milk research will never stop.

I would like to express my gratitude to my second supervisor and great friend, Dr. Robert Boyle who was always there to help. He was very helpful and patient throughout my research. His help was priceless. His critical appraisal of my work taught me a lot and provided with a new look at science. Most of my teaching skills that I have developed in the last few years are due to his efforts. I just followed his example.

I am grateful to Dr. Jill Warner who always gave me support and kindness throughout MSc in Allergy course and gave me an opportunity to be involved in teaching and marking during my PhD years. It is hard to imagine a better course director and person.

I am very thankful to Prof. Paul Cullinan, Prof. Neena Modi and Dr. Claudia Gore for critically examining the early and late stage PhD reviews.

I would like to express gratitude to my collaborator and friend Prof. Diego Peroni. I met him at the very beginning of my PhD and his help, support, friendliness and extremely professional approach to work was a fortune for me.

I am more than grateful to my collaborator and friend Dr. Marina Treneva. She is a gem as a scientist and her personal qualities are truly unique. Her kind support, both as a researcher and as a friend was second to none. Her belief in my abilities was a true inspiration to me.

I would like to thank Prof. Alexander Pampura, who always believed in my capacity of doing research and helped me with his advice throughout my “allergy journey”. I am very grateful for his support of my breast milk research.

I am extremely grateful to Silvia Colicino, a fantastic statistician and friend. Without her expertise in statistics I would never be able to complete my data analysis so efficiently. She is a truly unique person, and I am very lucky to meet her.

My special thanks go to Prof. Nikolay Gusev. I am extremely grateful for his kind advice and support throughout all these years. His belief in my capabilities and talks about science provided me with a morale boost. He is an example for me to follow as a scientist and also as a person. It is a fortune that he is a part of my life and my family.

I am indebted to my friends Heather Hannah, Bella Procktor and Katherine Phillips. It would be impossible to finish PhD without your kindness, help, endless support and positive emotions. I am lucky to work with such an amazing people who turned into my friends.

I am grateful to all medical students (Nisha Patel, Shreya Sheth, Priya Abrol, Binta Umar, Ruby Ramjan, Lakmal Mudalige) donating their time to my research. It was not just work but fun to supervise you.

My special thanks goes to LiYan Chow, a medical student, who already turned into a doctor. You joined me at the very beginning of my research and provided me with your support throughout all these years. You are like a family member to me. I am very honoured to be your friend.

I owe my deepest gratitude to Professor Ismail Osmanov, without his wise advice I would not have come to Great Britain. He is always a great support and is one of the most honourable men I have ever met.

Thanks to Lisa Carrier, for all her help and support throughout both, MSc and PhD course, she is a great person and a good friend.

I am indebted to Yvonne Clements and Shobana Dissanayeke for their invaluable help with lab work. I developed my laboratory skills only due to your efforts.

I want to thank all Paediatric Research Unit staff for their help and support during my study.

A very special thanks, goes out to the midwifery staff of Birth Centre, St. Mary's Hospital. Their help in recruitment and friendliness were great.

I would like to thank my friend Charlie Bedford for his undying hopefulness which gave me strength throughout all my research.

Thanks also, goes out to all my friends around the globe, especially my friends from London: Ilya Sheynzon, Alexey Sorokin, Maria Divid, Maria Feld, Lidia Bolotova who supported me throughout this long journey and brought a lot of positive emotions into my life.

A very special thanks, goes out to my best friend Konstantin Tutelman he is like a brother to me, with unwavering support and great friendship.

I am indebted to my friend Alexander Natanzon, for his friendship and optimism.

I am grateful to my medical friends from Moscow (especially Mikhail Volkov, Alan Osmanov, Dmitry Vlodavets, Ilya Korsunskiy and Mikhail Bolotin) for their endless belief in my abilities, support, wise advice and positive emotions they always bring into my life.

I also would like to thank my friend and fitness coach Aryan Kakar, without his trainings I would not have enough energy to finish my PhD.

I would like to thank the many people who introduced me to allergy and medicine and worked with me side by side (especially Tatyana Deeva, Tatyana Solovey and Marina Smirnova), they are always my family.

I reserve special thanks to my mother, without her help and support I would never have completed this course. I am very grateful for her belief in my abilities.

I am very grateful to my father for his never ending optimism and morale support.

I would like to thank my step-father for his support and positive approach.

I would also like to thank all my family, especially my cousin Lev Munblit, for the support they provided me through my entire life and during the whole time I was doing my PhD.

1 Introduction

1.1 History of Infant feeding

1.1.1 Primitive tribes

Some barbarian tribes imposed a delay of several days before a mother could commence feeding her baby. Initiation of breastfeeding varied from tribe to tribe, with the average period for commencement being four days, though in some cases exceeding nine days. This reflected attitudes to the use of colostrum. This taboo is evident from the writings of 17th century English and French authors which had been passed on from Greek and Roman times. The duration of the breastfeeding period varied from one tribe to another but on average, mothers continued lactation for three to four years with the rare exception of King William Land (now known as a King William Island) where children were breast fed for up to 15 years. Mother may have been feeding two or more children of different ages at the same time under such circumstances (Wickes, 1953a).

Other infant feeding practices which have evolved include wet nursing, bottle feeding, and formula use (Stevens et al., 2009).

1.1.2 Wet nursing

Wet nursing has been in use from as early as 2000 BC and has continued into the 21st century (Stevens et al., 2009). In many cultures women of high rank employed wet nurses, indeed it was standard practice for the English royal family. However, in some cultures it was considered that “strangers milk” was harmful to the infant. Eskimos froze the infants of mothers that died in child birth rather than expose them to this kind of

danger. In contrast, some Arab communities passed suckling infants round many lactating women.

In tribes associated with Eastern civilization, such as Java, grandmothers were expected to put the child to the breast whilst the mother worked. There is considerable doubt as to whether persistent sucking ever stimulated true lactation in this way, though there are several reputed instances which suggest that it is possible and there are even reports of fathers successfully suckling infants (Wickes, 1953a).

In Israel, children were regarded as a blessing and breast feeding as a religious obligation. Evidence for the existence of wet nurses is noted in Exodus 2, 7, approximately one thousand years before Christ and clearly demonstrates the hire of wet nurses as a well organized procedure. It seems probable that the average duration for suckling was about three years (2 Maccabees 7, 27), though there is no mention of artificial feeding anywhere in the Talmud (Wickes, 1953a).

In Homeric Greece (950 B.C.) wet nurses were in frequent demand, particularly by women of the higher classes. In these households, wet nurses came to hold positions of great responsibility with authority over the slaves and often with prolonged care of their charges up to adolescence (Wickes, 1953a). From approximately 100 AD through 400 AD, medical authors such as Soranus of Ephesus, Galen of Pergamus, and Oreibasius listed the qualifications for a wet nurse (Radbill, 1981). For example, Soranus of Ephesus (98 AD to 117 AD) composed an obstetrical and gynecological treatise of 23 chapters that provided a model for infant feeding (Osborn, 1979).

In parts of Africa (e.g. Cameroon) it was customary for the women also to suckle, if required, domestic puppies and piglets. The converse of infants suckling animals has also been recognized since the time of Romulus and Remus, and was still practised in France in the 19th century where babies at L'Hopital des Enfants Assistes in Paris were regularly put to the teat of asses that were permanently housed in stalls adjoining the ward (Wickes, 1953a).

1.1.3 Bottle feeding

Despite wet nursing being a suitable option for mothers not able to breastfeed for various reasons other approaches existed in the ancient world (Osborn, 1979). Vessels of different size and shape have been used for feeding over many centuries and have been found by archaeologists in the graves of newborn infants (Weinberg, 1993, Stevens et al., 2009, Wickes, 1953a). These bottles were initially thought to be containers for filling oil lamps but casein found present after chemical analysis, suggested that animal milk had been in use in ancient times as an alternative to breast milk (Wickes, 1953a, Weinberg, 1993, Stevens et al., 2009). Poorly made feeding bottles were in use from the Roman Era to Renaissance time with frequent adverse consequences due to hygiene issues. During the time of the Industrial Revolution, improved feeding bottles were developed that were more hygienic (Wickes, 1953b).

During the Middle Ages, feeding bottles were made from various materials: wood, ceramics, and interestingly, the most common type was a perforated cow's horn (Stevens et al., 2009). In 17th and 18th century, feeding bottles were usually made of leather and wood. By the 1800s, many infant-feeding devices were made from lead and silver (Weinberg, 1993).

Given the lack of knowledge on milk storage, sterilization and infancy nutrition in earlier centuries, it is surprising that infants survived their first year of life. Even as recent as the 19th century, more than one third of all artificially fed babies in 1870 were expected to die during the first year of life (Weinberg, 1993, Stevens et al., 2009).

1.1.4 Formula feeding

The first scientific comparison of human and animal milk was reported in the 18th century by Jean Charles Des-Essartz in his "Treatise of Physical Upbringing of Children in 1760" and provided analysis of human, cow, sheep, ass, mare and goat milk composition. Based on the chemical composition of these milks, Des-Essartz concluded that human milk is the best source of infant nutrition (Stevens et al., 2009). There have been many attempts to create an artificial milk comparable or of 'improved quality' to human milk (Radbill, 1981). In 1865, Liebig's formula made of cow's milk, wheat and malt flour, and potassium bicarbonate was marketed widely and was considered as a perfect infant food (Radbill, 1981). More formulas started to appear after the marketing of Liebig's infant food and the invention of evaporated milk resulted in 27 patented brands of infant feeds by 1883 (Radbill, 1981, Fomon, 2001).

These dietary products for babies were produced in powdered form and consisted of carbohydrates such as sugars, starches, and dextrins added to cow's milk (Stevens et al., 2009). These formulas were lacking important nutrients, such as protein, vitamins, and minerals but their gradual addition over time improved formula nutritional qualities (Radbill, 1981, Fomon, 2001).

Artificial formulas in the 19th century were known to be a cause of many summertime infant deaths (Wickes, 1953b) due to "spoilage of milk left in bottles" (Weinberg, 1993)

and were explained by germ contamination (Stevens et al., 2009). As a consequence of "germ theory" developed in early 20th century, the aim became to purify formula milk of pathogenic bacteria. First attempts to produce non cow's milk formulas to address the needs of children allergic to cow's milk protein began in 1920 when soy based products became available to the public (Stevens et al., 2009).

Very active and aggressive marketing of formula milk caused a global decline in breastfeeding worldwide, especially in developing countries (Stevens et al., 2009). With direct advertising to the public, formula industry companies created a tension between themselves and physicians. The American Academy of Pediatrics released a statement listing reasons for the organization's opposition to advertising infant formulas to the general public. It stated that advertisements had a negative impact on breastfeeding practices, interfered with physicians' advice on infant feeding, led to confusion amongst parents purchasing formula milk and increased the cost of feeding an infant (Greer and Apple, 1991).

The World Health Organisation (WHO) recommends exclusive breastfeeding for at least 6 months in all infants (WHO, 2001). However this is only achieved for 35% of infants worldwide and 19% in Europe (WHO, 2009b). The effect of the WHO recommendations on risk of allergy development has been investigated in several observational studies since a link between milk feeding and eczema was originally made by Grulee and Sanford (Grulee and Sanford, 1936). It is important to highlight that in many studies authors used various definitions of exclusive breastfeeding, and these are not always consistent with the WHO definition. According to WHO "Exclusive breastfeeding means that the infant receives only breast milk. No other liquids or solids are given – not even

water – with the exception of oral rehydration solution, or drops/syrups of vitamins, minerals or medicines"(WHO, 2009a).

1.2 Stages of human lactation

There are three stages in human lactation that are currently identified and explained by changes in specific components of the breast milk (e.g. whey proteins, lactose). These compositional changes seem to match the changing physiological needs of the infant, and can be stratified into colostrum, transitional milk and mature milk. Unfortunately there is no alignment in the precise definition of the terms mentioned above. According to various sources, colostrum is the special milk that is secreted in the first 2–3 days (WHO, 2009a) or 1–5 days (Darragh and Lönnerdal, 2011) after delivery; transitional milk is usually produced 7-14 (WHO, 2009a) 5–21 (Darragh and Lönnerdal, 2011) days postpartum; and mature milk after two weeks (WHO, 2009a) or >21 days (Darragh and Lönnerdal, 2011) post-partum.

It is known that so-called colostrum contains higher levels of protein and lower lactose content in comparison with mature milk. The specific gravity of colostrum is 1.040 compared with 1.060 for mature milk, and the mean energy content of colostrum is also lower at 67 kcal 100 ml^{-1} compared with mature milk, which supplies 75 kcal 100 ml^{-1} of energy. Fat and ash composition of colostrum and mature milk do not differ significantly (Darragh and Lönnerdal, 2011). At the same time colostrum is particularly rich in immunological components such as secretory IgA, lactoferrin, a variety of growth factors and cells (Kobata et al., 2008, Pang and Hartmann, 2007).

Lack of unified definitions of milk during different stages of lactation leads to variability in results in compositional studies. Due to a lack of agreement on current definitions which are empirical rather than evidence based, this highlights the need for a consensus.

1.3 Breastfeeding and GI and allergy outcomes

BM is the optimal source of nutrition for a newborn, and an important factor helping the newborn adapt to the extra-uterine environment. Human milk provides the developing infant with a range of bioactive factors influencing immune system maturation, physical and cognitive development and the infant intestinal microbiome (Borsutzky et al., 2004). At the time of birth, the infant intestinal immune system is relatively mature compared with other parts of the immune system, and therefore able to actively respond to signals from antigens and other immune constituents in BM (Jones et al., 2002a).

We know that the infant's immune system is influenced by maternal immunity via BM (Orlando, 1995), in addition to other routes (Billington, 1992). It has been shown in a number of studies that BM can affect intestinal immunity and may have long-term health consequences (such as influence on blood pressure, type 2 diabetes and intelligence quotient in later life) (Penttila et al., 2003, Ogawa et al., 2004, Nguyen et al., 2007, Horta B, 2007, Horta and Victora, 2013). Moreover, breastfeeding during introduction of gluten into the infant diet may reduce the risk of celiac disease, suggesting important interactions between BM components, dietary antigens and the gut associated lymphoid tissue (GALT) (Akobeng et al., 2006). This protective effect on celiac disease remains unclear as studies have produced conflicting evidence (Vriezinga et al., 2014).

Non-human milk feeds during infancy are known to increase the risk of infectious diarrhoea, due to of the lack of exposure to pathogen-specific sIgA in human milk (Newburg et al., 2004, Morrow et al., 2004, Kramer and Kakuma, 2012) As cow milk derived formulae also increase the risk of respiratory infections (Bachrach et al., 2003), this suggests that human milk has a broader effect on infant immune development than that achieved by sIgA supplementation to the gastrointestinal tract. In the early 20th century Clifford et al. were among the first to show that artificial milk use was associated

with a higher incidence of eczema in comparison with exclusive or partial breastfeeding (Gazzinelli et al., 1994). More recent data suggest that this may be due to the absence of prebiotic in the artificial milk used at that time (Gruber et al., 2010).

WHO and UNICEF proposed the Global Strategy for infant and young child feeding in 2002 (WHO/UNICEF, 2003). The main goal of this document was to focus world attention on the influence of various feeding approaches on infants' development, health, morbidity and mortality.

As mentioned above, the World Health Organisation (WHO) recommends exclusive breast feeding for at least 6 months in all infants (WHO, 2001). To what extent the WHO recommendation delays introduction of non-milk food sources at a critical period where tolerance induction may be important beyond 4 months of age and thereby affects the risk of allergy development is uncertain (Tomicic et al., 2010).

1.3.1 Evidence that mode of infant milk feeding changes allergy risk

One of the first attempts to systematise present evidence on the breast feeding protective effect on the development of allergic diseases was performed by Gdalevich et al. in the beginning of 21-st century. This led to the production of two meta-analyses. One of them was focused on the breast feeding protective effect on bronchial asthma development in children (Gdalevich et al., 2001b) and the second one addressed the protective effect of breast feeding on the development of atopic dermatitis in children (Gdalevich et al., 2001a). In the first meta-analysis they analyzed relevant papers from MEDLINE database which had been published in English between 1966 and 1999. Out of 177 papers (these included 41 studies, which varied in designs) there were completely different outcomes, from a protective effect to even a positive association between breast feeding and atopy. Twelve prospective studies have been included into

meta-analysis (a total of 8183 subjects) to support the preventive effect of exclusive breast feeding on the development of asthma. Systematic review of observational studies has shown a protective effect of exclusive breast feeding (as defined by WHO) for at least 3 months on the development of asthma, at least for children with a positive family history of asthma or atopy [OR = 0.52, 95% CI 0.35 - 0.79] (Gdalevich et al., 2001b). The outcomes of the second meta-analyses done by the same group of authors on the protective effect of breast feeding on atopic dermatitis also supported their theory. Authors included 18 prospective studies into meta-analysis (a total 4158 subjects) and found a protective effect against atopic dermatitis in children with a family history of atopy [OR = 0.58, 95% CI 0.41 - 0.92] (Gdalevich et al., 2001a).

In 2003, van Odjik et al. provided a comprehensive but non-systematic review on the mode of early feeding in infancy and its influence on future atopy development. Of 132 studies reviewed only 56 were regarded as qualitatively acceptable. Studies were divided into one or more of the four following categories: studies of breast feeding and atopic manifestations in cross-sectional populations; studies of breast feeding and atopic manifestations in children with atopic heredity; studies of formula feeding and atopic manifestations in children with atopic heredity and studies of cow's milk exposure and development of atopic manifestations. For the purpose of my thesis I will focus on the data from the first two groups. Results supported the conclusion that breastfeeding protects against development of allergic disease, especially among children with an atopic heredity. Authors found that exclusive breastfeeding reduced the risk of asthma and any breast feeding decreased the risk of recurrent wheezing and development of atopic dermatitis. Furthermore, the protective effects increased with the duration of breast feeding, up to at least 4 months and persisted at least for the first decade of life (van Odijk et al., 2003).

Two of 3 large cohort studies published subsequent to these systematic reviews, showed breastfeeding protective effects on wheeze in general [RR = 0.67, 95% CI 0.48 - 0.96, p=0.021] and in the first 3 years of life [OR = 0.80, 95% CI 0.70 - 0.90] and eczema [OR = 0.64, 95% CI 0.45 - 0.90] but one study, from Denmark (COPSAC birth cohort) on 321 exclusively breastfed babies (eczema was diagnosed in 122 (38%), in contrast found that exclusive breastfeeding increased the risk of eczema development [RR = 2.09, 95% CI 1.15 - 3.80, p=0.016] adjusting for demographics, filaggrin variants, parents eczema, and pets at home. Elliott et al. using data from ALSPAC study highlighted that the protective effect on wheeze did not extend at ages 7-8 years [OR = 0.98, 95% CI 0.79 - 1.22] (Laubereau et al., 2004, Giwercman et al., 2010, Elliott et al., 2008).

Ip and co-authors produced a meta-analyses of 18 prospective studies done in developed countries, assessing atopic diseases development as an outcome, dividing groups into infants breastfed for more than 3 months with a family history of atopy [OR = 0.58, 95% CI 0.41 - 0.92] in comparison to those who were exclusively breastfed for less than 3 months (Ip et al., 2007) and [OR = 0.84, 95% CI 0.59 - 1.19] infants without a family history of atopy respectively (Ip et al., 2007). They reported that in children without a family history of asthma breastfeeding for more than 3 months was associated with reduced risk of asthma compared to infants without 3 months of exclusive breastfeeding [OR = 0.73, 95% CI 0.59 - 0.92].

Yang et al. failed to find a protective effect of exclusive breastfeeding for at least 3 months against eczema development [OR = 0.89, 95% CI 0.76 - 1.04), and for study populations with atopic heredity [OR=0.78, 95% CI 0.58 - 1.05] (Yang et al., 2009). However, there was a protective breastfeeding effect on eczema development when

analysis was restricted to the studies comparing breastfeeding with conventional formula feeding (OR 0.70, 95% CI 0.50–0.99). Authors highlight that their results should be interpreted with caution due to substantial heterogeneity across studies ($\chi 2$ = 83.6, d.f. = 26; $p < 0.001$).

Another meta-analysis did not provide strong evidence that breast feeding is protective against wheezing illness in children aged 5 years and over. The authors stated that any breast feeding slightly lowered the odds of wheeze [OR = 0.92, 0.86 - 0.98] but slightly increased the odds of asthma defined by specific criteria [OR = 1.10, 95% CI 1.00 - 1.22] (Brew et al., 2011).

A recent meta-analysis by Dogaru et al. adds more evidence to support breastfeeding protective effect on wheezing [OR = 0.78, 95% CI 0.74 - 0.84] during the first two years of life (Dogaru et al., 2014).

None of the studies included in these systematic reviews were controlled trials and therefore, they have a significant risk of confounding. The large randomized control trial of a breastfeeding support programme from Kramer et al. did not show a protective effect of prolonged and exclusive breast feeding against asthma and showed an increased risk of inhalant sensitisation at age 7, but did find reduced risk of early eczema [OR = 0.54, 95% CI 0.31 - 0.95] (Kramer et al., 2001).

The Cochrane review done by Kramer did not show any evidence that exclusive breastfeeding for six months reduced risk of allergic disease, compared with lesser durations of exclusive breastfeeding (Kramer and Kakuma, 2012). In addition exclusive breastfeeding for six months did not have any long-term protection against obesity or

allergic disease, cognitive ability or behaviour, compared with exclusive breastfeeding for three to four months with continued partial breastfeeding to six months.

Data on the relationship between breastfeeding and food allergy are not sufficiently robust at the moment to make firm conclusions. A review by Kneepkens and Brand (2010) (Kneepkens and Brand, 2010) does not provide us with any additional data to support or reject the protective effect of breastfeeding against allergic diseases. An overview of existing data from meta-analyses, prospective cohort studies and reports published up to date suggests that current evidence is not sufficient and that it is impossible to conclude that exclusive breastfeeding and/or duration of any breastfeeding influences the risk of atopic diseases, asthma, wheezing, or eczema development (Hornell et al., 2013).

However, there is a suggestion that the effect of breast-feeding on food sensitization is influenced by genetic predisposition. Hong et al. found that the effect of breast-feeding on food sensitization was modified by Single Nucleotide Polymorphisms (SNPs) in the Interleukin (IL)12RB1, TLR9, and TSLP genes in infants both individually and jointly (Hong et al., 2011). This suggests that genetic variability must be considered alongside other factors when analyzing BM composition in relation to effects on atopy development. It is also likely that conflicting data in relation to the protective effects of breast feeding are due to differences in immune modulatory constituents between populations. Thus Tomicic et al. studying BM from Estonian and Swedish mothers found differences in immunological constituents (Tomicic et al., 2010), and Amoudruz et al. showed that levels of IL-6, IL-8 and TGF-β1 were higher in the milk of mothers who migrated to Sweden from developing countries in comparison to local mothers (Amoudruz et al., 2009).

Variability in nutrional constituents may also be important and Plagemann et al found that BM from diabetic mothers can lead to a higher risk of obesity in comparison to donor milk (Scharton-Kersten et al., 1996). As asthma and obesity commonly co-exist the association with maternal diabetes may be relevant to atopic disease.

One of the reasons why breastfeeding may have beneficial effects compared to mixed or exclusive feeding with bovine milk, may be species differences in protein composition. Milk proteins are usually classified into four main groups: caseins, peptones (low molecular weight peptides), whey, and milk fat globule membrane proteins. D'Alessandro and co-authors listed 285 human milk proteins, of which only 106 were "protein core" based on homology in human and bovine milk (D'Alessandro et al., 2010). This highlights the significant difference between human and bovine milk in protein composition.

Randomised controlled trials have shown that variations in formula milk constitution may alter allergy risk, and this suggests that the same may be true for variations in breast milk composition. Data from studies done on large cohorts have not consistently shown a preventative effect of hydrolysed milk formulas on allergy and eczema development (von Berg et al., 2008, Foisy et al., 2011) but oligosaccharides enriched formula was found to have a eczema preventive effect in children with low atopy risk in 2 studies (Gruber et al., 2010, Piemontese et al., 2011).

The majority of published studies, investigating the association between breast feeding and atopic disease, suffer from significant methodological shortcomings. For example there is a lack of data on the precise duration of breast feeding, and degree of

exclusivity (Kramer and Kakuma, 2012). There are problems with accurate measurement and control for potential confounding factors, including family allergic history. Finally, there is a lack of objective or unified classification of allergic outcomes (Kramer, 1988). Research attempting to assess the protective effect of breast feeding usually do not take into account breast milk composition. Accumulated knowledge provides enough data to highlight that breast milk immune composition differs significantly from one woman to another and differences in protective effect may be partially explained by these individual variations. Nevertheless, current evidence taken together supports the concept that variations in the timing and nature of infant milk feeds can impact on immune development and risk of allergic disease.

1.3.2 Infant milk feeding influence on GI outcomes

Gastrointestinal infections are very common in early infancy and childhood. Rates of diarrhoeal disease in US were estimated to be 1.1 episodes per child per year in children less than 5 years of age (Imhoff et al., 2004) and in developing countries 3.2 episodes per child per year (Parashar et al., 2003). A review of diarrhoea morbidity in developing and developed countries published two decades ago stated that the risk of diarrhoea in infants who did not receive breast milk in the first 6 months of life was 3.5 - 4.9 times higher compared to infants who have been breastfed exclusively (Feachem and Koblinsky, 1984). 88% of the studies confirmed an exclusive breast-feeding protective effect compared to no breast-feeding and 83% of studies found that exclusive breast-feeding was protective compared to partial breast-feeding.

One systematic review examined the relationship between breastfeeding and subsequent development of GI infections in children from developed countries aged less than 1 year (Chien and Howie, 2001). Authors stated that “it was not possible to

pool the adjusted relative measures of association" in the cohort studies reviewed. Using a fixed effect model, the summary crude odds ratio of the 14 cohort studies for the development of GI infection was [OR = 0.36, 95% CI 0.32 - 0.41; heterogeneity p<0.01]; and for the two case-control studies [OR = 0.54, 95% CI 0.36 - 0.80; heterogeneity, p=0.35]. Ip and co-authors similarly found that available evidence from three primary studies that controlled for potential confounders suggests that breastfeeding is associated with a reduction in the risk of non-specific GI infection during the first year of life in infants from developed countries, but highlight difficulty of taking into account all potential confounders, and summary adjusted effect estimate could not be determined (Ip et al., 2007).

Breastfeeding protective effect against GI infections may be explained by the presence of oligosaccharides, lactoferrin, secretory IgA, other immunoglobulins and immune active molecules in breast milk which may protect the infant from various infections through passive immunity (American Academy of Pediatrics Committee on Nutrition, 2004). Thus In vitro and in vivo binding studies have demonstrated that α1,2-linked fucosylated glycans, which include oligosaccharides in their free and conjugated forms and play an important role in innate immunologic mechanism by which breast milk protects babies against infections, inhibit binding by campylobacter, stable toxin of enterotoxigenic *Escherichia coli*, and major strains of caliciviruses to their target host cell receptors. Epidemiologic data demonstrate that higher relative concentrations of α1,2-linked fucosylated glycans in human milk are associated with protection of breast-fed infants against diarrhea caused by campylobacter, caliciviruses, and stable toxin of enterotoxigenic *E. coli*, and moderate-to-severe diarrhea of all causes (Morrow et al., 2005, Newburg et al., 2004, Morrow et al., 2004).

1.4 What is BM? Which immune factors may be related to gastrointestinal health and children allergic status?

The human infant is born with a physiological relative immune deficiency, and is dependent on maternally transferred IgG antibody for systemic humoral immune defence. Their T cells have a predominantly naive phenotype, and the capacity of newborn circulating mononuclear cells to generate pro-inflammatory cytokines is low (Ehlers and Smith, 1991). This immune deficiency is partly compensated by immune-active factors in human milk including IgA, anti-microbial peptides, and cytokines, in addition to growth factors and essential nutrients which promote development of the infant GALT (Playford et al., 2000, Lawrence and Pane, 2007). Cytokines from colostrum and BM are not destroyed in the stomach; some are protected by being bound to other molecules such as soluble components of their receptors (Garofalo et al., 1995). A variety of protease inhibitors (e.g.CST3, SERPIN-A1, A3, B1, C1, G1 and SPINT1) which are present in human milk have been proposed to limit the activity of pancreatic enzymes, (Lonnerdal, 2003, Chowanadisai and Lonnerdal, 2002) and in the critical first few weeks of life the gastric pH is high which considerably reduces peptic digestion.

In the past 10 years several cytokines and other immunoreactive substances have been identified in human milk and colostrum (Garofalo, 2010). The actual functional and physiological effects of each of these factors regarding their influence on the infant's evolving immune responses have not been clarified (Garofalo and Goldman, 1998, Lawrence and Pane, 2007). However, we know that certain immune active molecules (e.g. sCD14, TGF-β, HGF) are present in very high concentrations in colostrum (Jones et al., 2002a, Kobata et al., 2008) and levels of some, such as IFN-γ are higher in colostrum in comparison with maternal serum (Prokesova et al., 2006) which suggests

they are actively secreted in BM and may therefore be presumed to have a role in infant immune defence or development. It is known that TGF-β is able to promote IgA production (van Vlasselaer et al., 1992, Kalliomaki et al., 1999, Ogawa et al., 2004) and is actively involved in induction of oral tolerance (Strobel, 2002). CD14 which exists in 2 forms, membrane bound and soluble, is expressed mainly by monocytes or macrophages and plays an important role in innate immunity as a component of the complex with endotoxin (Landmann et al., 2000, Kielian and Blecha, 1995).The levels of CD14 on monocytes and macrophages and sCD14 are very low in the neonate and the high levels in BM may compensate for this relative deficiency. sCD14 is increased in clinical conditions where local or systemic activation of macrophages or monocytes is involved (Maliszewski, 1991).

1.4.1 Cells

A variety of cells are present in human colostrum and breast milk including macrophages, lymphocytes, T cells, and stem cells. It is known that during early lactation the infant consumes about 10^{10} maternal leukocytes a day. Over 80% of the cells present in early breast milk are so-called breast milk macrophages, emerging as peripheral blood monocytes that exit the bloodstream and migrate into milk through the mammary epithelium (Ballard and Morrow, 2013). These monocytes transform into potent cells which possess unique functional features, such as an ability to differentiate into dendritic cells that stimulate infant T-cell activity (Ichikawa et al., 2003, Yagi et al., 2010). Stem cells have also been found in breast milk but their actual function is still unknown (Patki et al., 2010, Indumathi et al., 2013).

A study done a decade ago in Finland suggests that levels of certain cells may influence allergy development as infants subsequently developing cow's milk allergy consumed

breast milk with a much smaller proportion of macrophages, high proportions of neutrophils and eosinophils comprising >1% of milk cells (Jarvinen and Suomalainen, 2002).

1.4.2 Lipids

A triglyceride core surrounded by a thin membrane (about 10–20 nm in cross-section) forming fat globules can be found in milk, and is known as the Milk Fat Globule Membrane (MFGM). This membrane works as an emulsifier and protects the globules from coalescence and enzymatic degradation.

Martin and co-authors identified xanthine oxidase, an enzyme present in the MFGM, has properties as an antimicrobial agent in the gut (Martin et al., 2004). This protein is expressed in gut mucosa cells, and its antimicrobial function is associated with production of reactive oxygen species, superoxide and hydrogen peroxide in the gut. Xanthine oxidase catalyses the reduction of inorganic nitrite to nitric oxide and in the presence of oxygen to peroxynitrite, the latter of which has bactericidal properties.

Sphingomyelin (SM) is one of the components of MFGM lipid fraction and is a major sphingolipid membrane component There is some evidence that SM intake plays an important role in neonatal gut maturation during the suckling period in rats and contributes to the process of myelination of the developing rat central nervous system (Oshida et al., 2003), but relevance to humans remains to be established. Milk phospholipids in the MFGM can have a protective properties in the gut (Kivinen et al., 1995). Phosphatidylcholine may protect the gut mucosa from toxic attack and reduce the rates of necrotizing enterocolitis in preterm infants (Anand et al., 1999, Carlson et

al., 1998).There have been no studies on the levels of BM MFGM lipids or proteins in relation to infant immune function and/or outcomes.

1.4.3 Oligosaccharides

Oligosaccharides are the third largest fraction in BM after lactose and lipids. Their exact physiological role remains unknown. Oligosaccharides are closely related to the Lewis blood group and four different milk groups have been described depending on their specific Lewis blood group-dependent oligosaccharide patterns (Thurl et al., 1997). It is known that colostrum contains more oligosaccharides then mature BM (Coppa et al., 1993) Oligosaccharides have various properties, including being prebiotic, anti-adhesive, glycome-modifying and anti-inflammatory , which can directly or indirectly influence infant immune responses (Donovan, 2009, Bode, 2009). As an illustration of these effects Ward et al. showed that oligosaccharides intensified the growth of *Bifidobacterium infantis* to a much higher degree than inulin or glucose (Ward et al., 2006). Human milk oligosaccharides also inhibit attachment of intestinal bacteria to the surface of intestinal epithelial cells, and direct evidence for the relevance of this come from clinical trial findings that the addition of certain oligosaccharides to cow's milk formula reduces infection risk in infants (Newburg, 1997, Newburg, 1999).

Among the oligosaccharides, the monosaccharide N-acetylglucosamine, a component of several oligosaccharides is important for the growth of Bifidobacterium bifidums (Bezkorovainy, 2001) which has been found in more healthy infants faeces samples in comparison to those who were allergic (Ouwehand et al., 2001).

1.4.4 Polyunsaturated fatty acids (PUFA)

There is some evidence that PUFA may play a role in immune development (Prescott and Dunstan, 2007). Some studies have shown that lower levels of n-3 PUFA and/or higher concentrations of n-6 PUFA in the serum of atopic infants and in their mother's BM (Reichardt et al., 2004, Duchen et al., 2000, Kankaanpaa et al., 2001) were associated with a higher risk of allergic disease while others found a positive correlation between elevated n-3 long-chain-PUFA levels in colostrum and food or inhalant-allergen sensitisation and atopic eczema in infants at 6, 12 and/or 24 months of age (Stoney et al., 2004).

The relationship between dietary linoleic acid and arachidonic acid, the intake of n-3 PUFA and modifications in enzyme expression and activity appear to have a substantial impact on allergy manifestation. However, more research is required to establish the relationship between the availability of n-3 and n-6 LC-PUFA during breast feeding and atopy development in children (Enke et al., 2008).

1.4.5 Exosomes

Exosomes are small, 30–100 nm membrane vesicles, which are released extra-cellularly after fusion of multivesicular endosomes with the cell membrane of a wide range of mammalian cells. The mechanism of action of exosomes within the immune system is not fully understood. They can be secreted by various cells types (dendritic cells, mast cells, epithelial cells, B-cells, T-cells). Those secreted by mast cells, can induce dendritic cell maturation (Skokos et al., 2003). There is also a suggestion that mast cell exosomes can transport functional mRNA to recipient cells (Lotvall and Valadi, 2007). Exosomes from B cells can present Bet v 1 peptides and stimulate Bet v 1-

specific T-cell lines to proliferate and to produce the Th2-like cytokines IL-5 and IL-13 (Admyre et al., 2007a).

Exosomes have been identified in colostrum and mature BM expressing MHC class II, CD86, and the tetraspanin proteins CD63 and CD81. These milk exosome complexes inhibit anti-CD3-induced cytokine production from PBMC and increase the number of $Foxp3^{+}CD4^{+}CD25^{+}$ T regulatory cells. These findings suggest that exosomes in human BM could have a significant influence on immune ontogeny, risks of atopic and other immune mediated diseases (Admyre et al., 2007b).

1.4.6 Immunoglobulins

Immunoglobulins are actively secreted in colostrum and mature breast milk and are identical to those found in blood or secretions. They are a family of bioactive protective proteins divided into several classes including IgM, IgA, IgG, IgE, and IgD (Mix et al., 2006). IgG, IgA and IgM are the major immunoglobulin classes in mammal animal secretions (Huang et al., 2008).

Immunoglobulins are found in both colostrum and BM in significant concentrations (Bottcher et al., 2003, Striker et al., 2004, Bachour et al., 2012). IgA is the major immunoglobulin class (88-90% of total immunoglobulin), whereas IgG is the primary immunoglobulin class found in bovine and porcine colostrum and milk. The content of IgG in human colostrum is of little consequence as it is actively transported across the placenta to the foetus in the last trimester of pregnancy (Hurley and Theil, 2011). Savilahti and co-authors found an association between low levels of IgA antibodies to cow's milk in colostrum and atopy and allergy development (Savilahti et al., 2005). Conversely Kuitunen et al. showed that high OVA IgA antibodies in colostrum were

associated with atopy development by 2 years of age (Kuitunen et al., 2012). Other studies failed to reveal any influence of sIgA on immunological outcomes in children (Bottcher et al., 2008, Saarinen et al., 1999, Bottcher et al., 2003, Boyle et al., 2011). Maternal intestinal antigens shape her GALT before pregnancy, and that GALT migrates to the mammary gland to produce BM immunoglobulins. These BM Igs then act by binding microbial and non-microbial antigens in the infant intestine, and preventing them from breaching infant intestinal epithelium and causing inflammation (Hurley and Theil, 2011). Therefore intestinal exposures in women may be priming both their and their own infants' immune development. This could explain the association between maternal antibiotic administration during pregnancy and a higher risk of asthma in off-spring.

1.4.7 Interleukin-2

Interleukin 2 (IL-2) actively secreted by Th1 cells plays an important role in T cell-mediated immune responses activation. It may increase natural killer (NK) cell cytolytic activity (Sakaguchi et al., 2008), influence Treg cells development and regulate the expansion and apoptosis among activated T cells (D'Souza and Lefrancois, 2003). IL-2 production is known to be reduced in newborn babies (Hassan and Reen, 1996), though additional supply of this cytokine via breast milk may compensate for this deficit.

Few studies have assessed IL-2 levels in colostrum and BM. Ustundag and co-authors found that the IL-2 level in colostrum from mothers of preterm infants was higher compared to the mothers of mature babies (Ustundag et al., 2005). However, this effect was diminished in later stages of lactation and an effect on the infant's immune development was not established. Bryan and co-authors (Bryan et al., 2006), found detectable levels of IL-2 in BM of 42 out of 52 BM samples, and a significant correlation

between milk aqueous IL-2 and maternal serum IL-2, but there was considerable diversity in the timing when BM was collected (15 – 357 days postpartum).

1.4.8 Interleukin-4

Interleukin 4 (IL-4) is one of the key cytokines in the development of allergic inflammation. It is involved in institution of the ε isotype shift and secretion of IgE or IgG4 by B lymphocytes (Coffman et al., 1986, Gascan et al., 1991). In addition, IgE-mediated responses are strengthened by IL-4 as it is able to upregulate IgE receptors on the surface of the cells via the low-affinity IgE receptor (FcεRII;CD23) on B lymphocytes and mononuclear phagocytic cells and high-affinity IgE receptor (FcεRI) on mast cells and basophils (Pawankar et al., 1997). Another known mechanism of IL-4 contribution to airway obstruction in asthma is via induction of mucin gene expression and the hypersecretion of mucus (Dabbagh et al., 1999). IL-4 increases eotaxin expression as well as other inflammatory cytokines from fibroblasts being partly responsible for inflammation and lung remodelling in chronic asthma (Doucet et al., 1998). The cell that initially produces IL-4, thus inducing Th0 differentiation, has not been identified yet, but recent studies suggested that basophils could be an effector cell (Sokol et al., 2008) but the source may be innate lymphocytic cells. They in turn are triggered by TSLP, Il-25 and IL33 from stressed epithelial cells. However, the most prominent effect of IL-4 from Th2 cells is induction of B-cell Ig class switching to IgE production

Levels of IL-4 in colostrum vary from one study to another (<1.6 pg/mL to 172 pg/mL), possibly due to an individual variations, difference in laboratory methods used and time of colostrum collection postpartum, with a tendency to decrease in mature milk (Agarwal et al., 2011).

Studies on IL-4 in colostrum and BM of allergic and non-allergic mothers have produced conflicting results. Bottcher et al. found higher concentrations of IL-4 in colostrum from allergic compared to non-allergic mothers (Bottcher et al., 2000). In contrast median IL-4 levels did not differ significantly between the two groups in a Polish study, nevertheless IL-4 was more often detected in the colostrum of allergic compared to non-allergic mothers (Marek et al., 2009). In both studies, IL-4 was less commonly detected in colostrum and BM, than several other cytokines (Bottcher et al., 2000, Marek et al., 2009). Similarly Rudloff and co-authors detected IL-4 in only 20% of the colostrum samples with no significant difference between allergic and non-allergic mothers (Rudloff et al., 1999). In the milk of allergic mothers the IL-4 rise and peak at 3 months after delivery has been observed with the decline after this time point (Prokesova et al., 2006).

1.4.9 Interleukin-5

Interleukin 5 (IL-5) plays an important role in the induction of B-1 cell proliferation and enhances B-1 and B-2 cells differentiation into antibody secreting cells (Takatsu, 1998). It is produced by T helper-2 cells and mast cells and is considered as a key cytokine in eosinophil production, activation and localization. IL-5 alongside IL-4, IL-9, and IL-13 together with eotaxin, plays critical roles in orchestrating and amplifying allergic inflammation in asthma (Larche et al., 2003, Takatsu and Nakajima, 2008). It is known to be a key mediator in eosinophil activation and life span determination (Takatsu and Nakajima, 2008) leading to eosinophils migration to the lungs in asthmatic patients who are known for distinct eosinophil infiltration of their lungs (Kay, 2001). It has also been shown that IL-5 is involved in IgA production (Schoenbeck et al., 1989).

Concentration of IL-5 in colostrum varies from high (79 pg/mL) (Prokesova et al., 2006) to low (6 pg/mL) (Bottcher et al., 2000) with a similar trend seen in mature breast milk

(Agarwal et al., 2011). These differences in the colostral IL-5 concentration may be explained by individual variations, difference in laboratory methods used and time of colostrum collection postpartum.

There are few studies analysing IL-5 levels in colostrum or BM in general. Prokesova et al. failed to find any difference in IL-5 concentration between mothers with atopic dermatitis and controls, (Prokesova et al., 2006). Two studies (Bottcher et al., 2000, Bottcher et al., 2003) detected IL-5 in less than 10% of the colostrum and 1 month mature milk samples. There was no significant difference between concentrations of IL-5 in colostrum of allergic and non-allergic mothers, but a significant correlation between levels of IL-5 and IL-10 (Bottcher et al., 2000). This is consistent with understanding of cytokine interactions in that IL-10 suppresses IL-12 production and therefore IFN-gamma which will remove one of the regulatory influences on Th-2 activity.

Very few studies aimed to compare the levels of IL-5 in other compartments in parallel: Prokesova and co-authors found similar levels of IL-5 in colostrum and maternal serum (Prokesova et al., 2006) and Ziska et al found that levels of IL-5 in colostrum (73.0 pg/mL) and cord serum (86.5 pg/mL) were higher than in amniotic fluid (9.2pg/mL) (Zizka et al., 2007).

1.4.10 Interleukin-6

Interleukin 6 (IL-6) is a proinflammatory (Kopf et al., 1994) and anti-inflammatory cytokine (Xing et al., 1998) and involved in a wide spectrum of cellular mechanisms, including T-cell activation and immunoglobulin production by B cells, and thus it enhances IL-4-dependent IgE synthesis

IL-6 is detectable in colostrum or BM with no significant difference between the levels of IL-6 in colostrum of allergic and non-allergic mothers (Bottcher et al., 2003, Prokesova et al., 2006) although there was a correlation with IgA in colostrum as well as with IL-10 and TGF-β which are involved in IgA synthesis (Bottcher et al., 2000).

IL-6 levels do not differ much during transitioning from colostrum to mature milk but levels in various studies varied from 7.3 to 81 pg/mL in colostrum and up to 105 pg/mL in 6 month milk, with similar concentrations detected in amniotic fluid, cord serum and maternal serum (Agarwal et al., 2011).

1.4.11 Interleukin-10

Interleukin (IL-10) is considered to be the most important anti-inflammatory cytokine alongside TGF-β and IL-35. IL-10 influences three important functions of monocytes/macrophages: the release of immune mediators, antigen presentation, and phagocytosis. It suppresses all functions of monocytes/macrophages that are responsible for a positive role of these cells in both innate and specific immunity (Sabat et al., 2010). IL-10 has a range of anti-allergic and anti-inflammatory properties. It suppresses the synthesis of a variety of cytokines (IL-1, IL-4, IL-5, IL-6 and TNF-α) and chemokines (IL-8, RANTES, eotaxin and MIP-1α) (Berkman et al., 1995, Berkman et al., 1996, Chung et al., 1999).

A very high concentration of interleukin 10 (IL-10) has been demonstrated in samples of human milk collected during the first 80 hours of lactation (Garofalo et al., 1995). IL-10 is present not only in the aqueous phase of the milk, but also in the lipid layer. Its bioactive properties were confirmed by experiments showing that human milk samples inhibited blood lymphocyte proliferation and that this property was greatly reduced by

the pre-incubation with anti-IL-10 antibody (Garofalo, 2010). In contrast studies have either detected no IL-10 (Snijders et al., 2006), or only rarely in colostrum (12%) and mature milk (6%) (Bottcher et al., 2000) while others have found IL10 in most if not all samples (Garofalo et al., 1995, Marek et al., 2009).This variation may be due to the timing of colostrum collection. In the Garofallo's study, high concentrations of IL-10 were detected in samples collected during the first 24 hours and concentrations were lower in milk collected later (Garofalo et al., 1995). The colostrum samples in the study done by Bottcher et al. were collected 3–4 d postpartum (Bottcher et al., 2000).

IL-10 levels in colostrum and mature milk vary from < 5 pg/mL to 3304 pg/mL (Yilmaz et al., 2007, Garofalo et al., 1995) and 0 to 246 pg/mL, respectively (Prokesova et al., 2006, Rigotti et al., 2006). Data analysed in the review on cytokine concentrations in breast milk suggests that there is no decline in IL-10 levels in milk over time (Agarwal et al., 2011).

1.4.12 Interleukin-12

Interleukin-12 (IL-12) plays central role in TH1 responses (Kobayashi et al., 1989, Hsieh et al., 1993, Manetti et al., 1993). IL-12 is rapidly produced, independently from IFN-γ and signals from T cells (Trinchieri, 2003). It is mainly secreted by such inflammatory cells as macrophages, neutrophils, DCs and monocytes (Gazzinelli et al., 1994, Scharton-Kersten et al., 1996). IL-12 plays an important role in the TH1 response maintaining organ-specific autoimmunity as has been shown in animal models, and is involved in resistance mechanism to infective agents, in particular bacterial and intracellular parasites (Trinchieri, 1998).

Neutrophils, lymphocytes, and macrophages are found in human milk during the first few months of lactation (Smith and Goldman, 1968), and one or more of these cell types can secrete Interleukin 12 (IL-12). The immune environment in utero is regulated predominantly by Th_2- cell predominance (Marzi et al., 1996). Restoration of a balance between Th_2-type and Th_1-type responses postnatally may be orchestrated, by being exposed to IL-12 from the milk of a breast-feeding mother (Bryan et al., 1999).

IL-12 was detected in 62% of BM (Bryan et al., 1999) samples, but there was no association between IL-12 in BM and maternal atopic status or maternal illness/infection. There was no significant difference in the level of IL-12 in colostrum of allergic compared with non-allergic mothers (Snijders et al., 2006).

IL-12 levels in colostrum varies from < 3 pg/mL (Ogawa et al., 2004) to 310 pg/mL (Bryan et al., 1999). Bryan et al. found a gradual reduction of IL-12 concentration in milk over time (Bryan et al., 1999), in contrast Yilmaz and co-authors failed to reveal any difference (Yilmaz et al., 2007).

Comparable concentrations of IL-12 have been found in amniotic fluid, cord serum, and breast milk (Agarwal et al., 2011).

1.4.13 Interleukin-13

Interleukin-13 (IL-13) was originally described as a cytokine that inhibits inflammatory cytokine production (Minty et al., 1993, McKenzie et al., 1993). Similar to IL4 it promotes IgE production but also regulates eosinophilic inflammation, mucus secretion, and airway hyper-responsiveness in asthma patients. At the moment it is still unclear what regulates IL4/IL-13 receptor expression, and how this affects the functional activity of IL-13 (Wynn, 2003).We also do not know what role IL-13 plays in colostrum.

Bottcher et al. (Bottcher et al., 2000) and (Bottcher et al., 2003) detected IL-13 in only 12 out of 48 (25%) colostrum samples but the concentration correlated with the levels of IL-4, IL-5 and IL-10. Prokesova and co-authors, in contrast, found IL-13 in almost every sample tested (Prokesova et al., 2006). This may be due to ethnic and environmental differences in the population groups (from Sweden and Czech Republic).

Colostral levels of IL-13 vary in different studies averaging from 3.2 pg/mL (Bottcher et al., 2000) to 243 pg/mL (Zizka et al., 2007). Interestingly, levels of IL-13 and IL-4 are within the same limits (Agarwal et al., 2011), which may be explained by similarities in their immunological functions, highlighting the importance of multiple cytokine analysis, rather than singly, in breast milk.

1.4.14 Interleukin-15

Interleukin-15 (IL-15) is secreted by various cells but predominantly by mononuclear phagocytes. It has structural similarity with IL-2, and activates Natural Killer cell proliferation, cytotoxicity, and cytokine production and regulates Natural Killer cell/macrophage interaction (Fehniger and Caligiuri, 2001). This cytokine is known to play a key role in anti-HIV responses by stimulating both CD8 T cells and Natural Killer cells (Mastroianni et al., 2004). The single study of IL-15 concentrations in BM showed an association with decreased risk of HIV transmission. It was hypothesised that IL-15–mediated immunity may protect against HIV transmission during breast-feeding (Walter et al., 2009).

1.4.15 Interferon-γ

Interferon-γ (IFN-γ) is a TH-1 cytokine and has extensive and diverse immune-regulatory effects on many cells including an inhibitory effect on Th2-cells. It is produced

by Th1 cells, natural killer cells and dendritic cells (Bogen et al., 1993, Pistoia, 1997). IFN-γ increases antigen presentation of macrophages and suppresses Th2 response (Oriss et al., 1997). Allergen-specific immunotherapy in allergic patients results in increased production of IFN-γ by circulating T-cells (Lack et al., 1997) and in an increase in IFN-γ producing T-cells in nasal biopsies (Durham and Till, 1998).

IFN-γ has been found in colostrum and BM (Bocci et al., 1993, Eglinton et al., 1994) but biological activity in the infants gut remains to be determined. Botcher and co-authors detected IFN-γ in less than 10% of their samples (Bottcher et al., 2000) and Rudloff et al. failed to detect any in 42 samples (Rudloff et al., 1999). In contrast, Prokesova et al. found IFN-γ in almost every sample (Prokesova et al., 2006) which again raises the question why cytokine presence in colostrum varies so much from one study to the other. It may relate to differing environmental influences. It has been suggested that lower levels of IFN-γ in breast milk lead to higher incidence of subsequent eczema development in children (Linnamaa et al., 2013).

Levels of IFN-γ in colostrum vary from <67 pg/mL (Bottcher et al., 2000) to 708 pg/mL (Prokesova et al., 2006). Results of the recent review suggest that IFN-γ concentration does not change over time (Agarwal et al., 2011).

1.4.16 Transforming growth factor-β

Transforming growth factor-β (TGF-β) is a family member of regulatory cytokines that have pleiotropic functions in various cell lineages involved in many physiological and pathological processes such as embryogenesis, carcinogenesis, and the immune response (Cerutti et al., 1998). TGF-β regulates cell differentiation, proliferation, adhesion, apoptosis and migration of various cell types and promotes the production of

extracellular matrix proteins (Dennler et al., 2002). TGF-β plays a crucial role in the maintenance of immune cell homeostasis.

In animal model experiments TGF-β1 null mice died shortly after weaning due to multifocal, inflammatory disease, signs of lymphocyte infiltration into multiple organs (Kulkarni et al., 1993, Shull et al., 1992) and autoimmune manifestations (Christ et al., 1994, Yaswen et al., 1996)[27, 28]. At present three members of the TGF-β family have been found (TGF-β1, TGF-β2, and TGF-β3) TGF-β1 is the prevalent form expressed in the immune system (Eglinton et al., 1994).

A number of studies have shown that high levels of TGF-β in human milk are associated with the prevention of allergic diseases during infancy. A study done in Finland in 1999 found that the concentration of TGF-β was higher in colostrum in comparison to mature milk and infant's serum, in infants with the onset of allergic disease after the weaning period comparing to babies with an onset of allergy prior to weaning. This suggests an allergy delaying effect of TGF-β in colostrum (Kalliomaki et al., 1999).

A systematic review summarises the associations between TGF-β in human milk and immunological outcomes in infants and children (Oddy and Rosales, 2010). Eight of 12 studies quoted in this review reported an association between higher TGF-β1 or 2 levels in colostrum or BM and reduced risk of atopic outcomes in the infant. The authors suggest that TGF-β found in human milk is involved in maintaining homeostasis in the intestine, regulating inflammation, promoting oral tolerance and thereby reducing allergy development (Oddy and Rosales, 2010). A high level of heterogeneity among the published studies makes interpretation difficult with large differences in study populations, design and methodology, TGF- β isoform type assayed, time when milk

samples were collected, age at which outcome was measured and, finally, lack of consistency in the immunological outcomes measured.

Ando and co-authors showed that orally administered TGF-β can mediate its activity in the intestinal mucosa and enhance the induction of oral tolerance to a high-dose dietary antigen (Ando et al., 2007) and Nakao suggests that oral administration of TGF-β simultaneously with an allergen might potentially be a useful approach for the primary prevention of allergic diseases in infants (Nakao, 2010).

1.4.17 Hepatocyte growth factor

Hepatocyte growth factor (HGF) plays an important role in cell growth, cell motility, and morphogenesis regulation by activating a tyrosine kinase signaling cascade binding to the proto-oncogenic c-Met receptor. This makes it a crucial marker of angiogenesis, tumorogenesis, and tissue regeneration. Hepatocyte growth factor is produced by mesenchymal cells and is involved in immunological activity as a multi-functional cytokine on cells of mainly epithelial origin. HGF regulates mitogenesis, cell motility, and matrix invasion (Gene database, 2008). Recent data, from animal models studies, suggests that HGF also may be involved in airway hyper-responsiveness and airway remodelling in allergic asthma (Ito et al., 2005, Okunishi et al., 2009, Nakamura and Mizuno, 2010).

Existing data show that HGF receptor C-met, can be found on the surface of the intestinal crypt epithelial cells as well as muscle layers of the intestine (Kermorgant et al., 1997). HGF and c-met mRNA expression have been detected not only in adult but also in fetal intestine (Kermorgant et al., 1997, Wang et al., 1994). Cummins and Thompson found that the infant mucosa and crypt enterocytes have increased c-met

mRNA and protein expression compared with adult subjects but low HGF expression in the neonatal ileum, suggesting that breast milk HGF interacts with c-met receptor and induces a response (Cummins and Thompson, 2002).

Very few studies assessed HGF levels in colostrum and breast milk. It is evident that levels of this factor in human milk are very high (Kobata et al., 2008). A few studies suggest that these enormous concentrations of HGF in breast milk (20-30 times higher than in maternal serum) may be a part of a physiological mechanism aiding infant GI maturation as well as protection within the first days of life (Srivastava et al., 1999, Kobata et al., 2008).

Levels of HGF in colostrum vary from <0.59 ng/mL (Yamada et al., 1998) to 52.2 (Kobata et al., 2008) ng/ml. Existing evidence does not allow us to make any conclusions in regards to breast milk concentration of HGF and allergic and/or immunological outcomes. However, HGF suppresses the antigen-presenting capacity of dendritic cells in murine models, thus inhibiting sensitisation (Okunishi et al., 2009), suggesting that HGF may be a potential therapeutic option for chronic asthma with airway re-modelling (Okunishi et al., 2005, Ito et al., 2008).

1.4.18 EGF (epidermal growth factor) and VEGF (vascular endothelial growth factor)

EGF is involved in a number of activities, including proliferation, maturation, migration, and apoptosis (Gleave et al., 1993) and VEGF is a key regulator of physiological and pathological angiogenesis and tissue repair. Studies show that VEGF may stimulate endothelial proliferation, motility, and capillary morphogenesis and provide vascular permeability regulation (Veikkola and Alitalo, 1999). Growth factors are present in high

concentrations in breast milk, similar trends can be seen in VEGF. Concentration of both, EGF and VEGF, in breast milk is much higher than in maternal serum suggesting a mammary gland source of these growth factors. (Ballard and Morrow, 2013). Breast milk EGF is believed to increase gut mucosal barrier development (Donovan and Odle, 1994). Data from animal research suggest that EGF may reduce the risk of necrotizing enterocolitis development in infancy (Dvorak et al., 2002).

Loui et al. found higher levels of VEGF in the BM of the mothers delivering term babies when compared with preterm (Loui et al., 2012). This finding in conjunction with the known disregulation of VEGF in retinopathy of prematurity, which causes dysregulated vascularization of the retina (DiBiasie, 2006), lead to a hypothesis that VEGF in BM may reduce the risk of retinopathy of prematurity development (Ballard and Morrow, 2013).

1.4.19 Soluble Cluster of Differentiation 14

Cluster of differentiation 14 (CD14), a pattern-recognition receptor plays an important role in the innate immune response to endotoxin (Pugin et al., 1994). Polymorphisms in the promoter region for the CD14 gene have been associated with susceptibility to atopic sensitization (Vercelli, 2008). The soluble form (sCD14) is secreted by liver and is found in breast milk (Labeta et al., 1993).

sCD14 is found in very high concentrations in human milk. Increased levels of circulating sCD14 correlate with infection and autoimmunity (Filipp et al., 2001). Polymorphisms in the promoter region of the DNA encoding CD14 have been associated with reduced levels of circulating sCD14and in turn inversely correlated with total IgE levels (Feachem and Koblinsky, 1984). Savilahti et al. studied a large cohort of 4676 children in four groups, with either long or short exclusive breast-feeding (3.5 months or 2 weeks) who subsequently were classified by the presence or absence of

atopy. Children with atopic symptoms and IgE sensitization at the age of 4 received colostrum with a significantly lower concentration of sCD14 than those without symptoms and IgE sensitization (Savilahti et al., 2005). Jones et al. (Jones et al., 2002a) and found that infants who subsequently developed eczema received at 3 months of age BM with lower levels of sCD14 than did those without eczema, however another study failed to show sCD14 protective effect against eczema development (Ismail et al., 2013). It may be partially explained by difference in CD14 genotype as it has been recently shown that breastfeeding was associated with a decreased risk of atopic sensitization in children with CT/CC genotype (OR 0.667, 95%CI 0.463-0.960) (Lee et al., 2013). Jones also found lower amniotic fluid sCD14 levels were associated with later atopy.

For many of the immunomodulatory components present in BM, there is only limited direct evidence for a role in the development of infant intestinal immunity or allergic disease. However some BM immune components are known to be important factors in infant immune development, or important stimuli of immune responses generally. In *Table 1*, I have summarised the evidence that variations in levels of breast milk immune mediators are associated with altered allergic disease risk. The evidence is perhaps strongest for TGF beta, which has known biological effects on intestinal immune responses, is actively secreted into breast milk, and has been shown in several studies of breast milk composition to differ significantly between populations.

Table 1 Relationship between BM immune composition and infant immune development – observational studies

Author and year	Country and numbers	Immune modulators measured	Main Outcomes
Saarinen 1999 (Saarinen et al., 1999)	Finland 108 infants with cow's milk allergy; 207 healthy controls	IgA, IgM, Cow's milk specific antibodies, IL-6, IFN-γ, TGF-β1	Colostrum TGF-β1 *reduced* for infants with IgE-mediated cow's milk allergy. Other factors did not differ.
Kalliomaki 1999 (Kalliomaki et al., 1999)	Finland 27 infants with eczema	TGF-β1, TGF-β2	Colostrum TGF-β1 and TGF-β2 reduced for infants with pre-weaning onset of eczema compared with post-weaning onset
Jones 2002 (Jones et al., 2002a)	UK 8 infants with eczema; 21 without	sCD14	sCD14 levels reduced in infants with eczema.
Rautava 2002 (Rautava et al., 2002)	Finland (BM from 30 women taking probiotics and 32 taking placebo)	TGF-β1, TGF-β2	The risk of developing atopic eczema during the first two years of life in infants whose mothers received probiotics was significantly lower compared with infants whose mothers received placebo. Higher levels of TGF-β2 were found in BM of mothers receiving probiotics.
Bottcher 2003 (Bottcher et al., 2003)	Sweden 53 infants	IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, IFN-γ, TGF-β1, TGF-β2, RANTES, eotaxin, SIgA	No difference in mediator levels with respect to atopy, allergic symptoms or salivary IgA in the first 2 years of life

Oddy 2003 (Oddy et al., 2003)	Australia 243 infants	TGF-β1, IL-10, TNF-α, sCD14	Prolonged breast feeding and high TGF-β1 in breast milk at 2 weeks were associated with reduced infant wheezing
Reichardt 2004 (Reichardt et al., 2004)	Germany 218 infants	n-3 and n-6 polyunsaturated fatty acids	No association between fatty acid levels and infant atopic eczema. High colostrum linoleic acid (n-6) associated with high milk-IgE. Low colostrum docosapentaenoic acid (n-3) associated with high total IgE at 1 year.
Savilahti 2005 (Savilahti et al., 2005)	Finland 228 children	total IgA, IgA antibodies to cow's milk, casein, sCD14, and TGF-β1, TGF-β2	Low colostrum IgA casein antibodies and low sCD14 concentration were associated with atopy. Low levels of specific IgA antibodies to cow's milk and sCD14 in colostrum were associated with atopy and allergic symptoms (eczema, allergic rhinitis, conjunctivitis, asthma) by four years of age.
Rigotti 2006 (Rigotti et al., 2006)	Italy 22 infants	TGF-β1, IL-10	TGF-β and IL-10 did not influence immunological outcomes at the age of 6 months
Snijders 2006 (Snijders et al., 2006)	Netherlands 307 infants	TGF-β1, IL-10, IL-12, sCD14	None of the studied immune factors in breast milk at 1 month, were associated with infant's atopic outcomes.
Bryan 2007 (Bryan et al., 2007)	Australia (36 milk samples from mothers of infants hospitalized with bronchiolitis and 63 mothers of postpartum age-matched healthy controls)	IFN-γ, IL-2, IL-4, IL-8, IL-10, RANTES	IL-10 level in BM of mothers of infants with bronchiolitis was lower compared with mothers of healthy infants.

Bottcher 2008 (Bottcher et al., 2008)	Sweden (colostrum and mature milk from women treated with L. reuteri (n = 54) or placebo (n = 55) from 36 weeks gestation until labour)	Total IgA, SIgA, TGF-β1, TGF-β2, IL-10, TNF, sCD14, Na/K ratios	Infants receiving BM with low levels of TGF-β2 were less likely to become sensitized or developing eczema during their first 2 years of life.
Lowe 2008 (Lowe et al., 2008)	Australia 194 infants	n-3 and n-6 polyunsaturated fatty acids	High levels of n-3 fatty acid in colostrum were associated with increased risk of infantile atopic eczema, while total n-3 concentration in BM was associated with increased risk of non-atopic eczema. Higher levels of total n-6 fatty acid in colostrum were associated with increased risk of childhood rhinitis. There was no evidence of associations between fatty acid profile and risk of asthma.
Tomicic 2010 (Tomicic et al., 2010)	Sweden, Estonia 39 Estonian and 60 Swedish infants	SIgA, IL-4, IL-10, IL-13, IFN-γ, TGF-β1, TGF-β2	High levels of IL-13 in colostrum were associated with allergic sensitization during infancy in Swedish cohort, but not in the Estonian cohort.
Boyle 2011 (Boyle et al., 2011)	Australia (7 days and 28 days BM samples from 38 mothers receiving placebo and 35 receiving probiotic)	Total IgA, sCD14, TGF-β	Prenatal Lactobacillus rhamnosus GG supplementation did not have any effects on infants immunological outcomes. Maternal probiotic supplementation resulted in reduced levels of IgA and sCD14 in BM.
Thijs 2011 (Thijs et al., 2011)	Netherlands 315 infants	n-3 polyunsaturated fatty acids	Higher concentrations of n-3 PUFAs in breast milk at 1 month, are associated with atopic dermatitis, parent reported eczema and sensitization at one year of age.
Kuitunen 2012 (Kuitunen et al., 2012)	Finland 364 infants	Total IgA, IgA antibodies to cow's milk, casein, β -lactoglobulin, ovalbumin, TGF-β2, IL-10	High OVA IgA antibodies in colostrum were associated with the atopy development by 2 years of age. Low levels in BM were is a risk factor for eczema development by the age of 2. High levels of TGF- β 2 in BM at 3 months was associated with more allergic

	disease and eczema at 2 years.

1.5 Immune active molecules to be assessed in this Thesis

Data from recent proteomics studies show that BM contain hundreds of proteins (D'Alessandro et al., 2010), including cytokines, growth factors, signalling molecules and other factors which may interact with infants immunity. Previous research found some associations between a variety of maternal and environmental exposures and levels of a certain immune active molecules as well as associations with subsequent health outcomes in children. In the ideal world proteomics, lipidomics and analysis of cytokines and growth factors should be done at once and considered as a "gold standard" study design. Unfortunately, it is impossible to fulfil these criteria due to the following reasons:

a) Colostrum is very difficult to obtain and usually only small volumes of this biological fluid can be collected. It is especially prevalent in primiparous mothers and those women undergo caesarean section. This does not allow to provide colostrum analysis for all cytokines and growth factors of interest.
b) Financial limitations enforced me to restrict amount of cytokines analysed.

The final choice of cytokines was based on the data from other studies, I selected those immune active molecules which have been reported to be influenced by various exposures and/or been associated with short-term health outcomes (especially allergic disease) in children, even when existing evidence was somehow conflicting. I also considered methodological issues as some cytokines cannot be analysed on a single multiplex plate due to difference in protocols. A summary of the immune factors known to be present in human breast milk, and their association with infant health outcomes especially allergic outcomes, is shown below and has been published in the Clinical & Experimental Allergy Journal as a review article (Munblit et al., 2014).

It was not possible to add more immune active molecules into my laboratory experiments due to the lack of funds as well as feasibility of laboratory analysis.

The final list of immune active molecules included a selection of Th1, Th2 cytokines and growth factors involved in a number of immunological interactions and consisted of IL2, IL4, IL5, IL10, IL12, IL13, IFNγ, HGF, TGFβ1, TGFβ2 and TGFβ3.

1.6 Environmental influence on BM immune composition

Levels of factors with the potential to influence infant immune development, may be modifiable. There is evidence that a number of different exposures can influence maternal breast milk and colostrum components, and even a limited body of evidence that such interventions can alter infant immune outcomes. Below I have summarised some of the exposures/maternal variables which have been associated with altered breast milk or colostrum composition, with or without direct evidence of effects on infant immune outcomes.

1.6.1 Tobacco smoke

It is well established that tobacco smoke is immuno-toxic, but the effect of smoking on the immunological function of the mammary gland of smoking mothers has not been well studied. Current recommendations state that maternal smoking is not an absolute contraindication to breastfeeding (American Academy of Pediatrics, 2012).

Zanardo et al. (2005)suggested that exposure to tobacco smoke during pregnancy may affect the development of the immunologic function of the mammary gland, significantly influencing colostral milk provision of the proinflammatory cytokine IL-1α for breast-feeding infants. (Zanardo et al., 2005), and Agostoni and co-authors found that both

total lipid content and absolute amounts of the fatty acids linoleic, arachidonic, a-linolenic and docosahexaenoic acid in BM were lower in smoking vs. non-smoking mothers at 1 month. The latter study also showed that at 3 months after delivery, the BM of smoking mothers contained less docosahexaenoic than the BM of non-smoking mothers (Agostoni et al., 2003).

Ermis et al. found significant differences in breast milk malondialdehyde ($p=0.002$) levels, superoxide dismutase ($p=0.011$), but not glutathione peroxidase ($p=0.11$) activities, and antioxidant potential ($p=0.29$) in active-smoking, compared with passive-smoking, and nonsmoking mothers. This suggests that breast milk is vulnerable to oxidative stress and lipid peroxidation (Ermis et al., 2005). Maternal smoking may influence sleep/wake patterns in infants as they spent significantly less time sleeping during the hours immediately after their mothers smoked (53.4 minutes), in comparison to sessions when their mothers abstained from smoking (84.5 minutes) (Mennella et al., 2007).

Tobacco smoke contributes significantly to breast milk contamination by polycyclic aromatic hydrocarbons (PAH), organic chemicals formed in all the incomplete combustion processes of organic matter. The condensate contained in the cigarettes smoked daily by each subject was strongly related with the polynuclear hydrocarbon content of BM ($R^2=0.92$, $p<0.005$) (Zanieri et al., 2007).

1.6.2 Gestation

The effects of premature delivery on BM composition have been highlighted in the study done by Castellote et al. (Castellote et al., 2011) who found that levels of IL-6, TGF-β1 and TGF-β2 were higher in colostrum following preterm compared with term delivery.

The cohort size was small, with 10 prematurely and 22 term delivered women, and further studies are needed to confirm this finding.

1.6.3 Physical activity

Increasing metabolic-equivalent tasks, and thus caloric expenditures, can increase pro-inflammatory cytokines in the BM (Groer and Shelton, 2009). This is a novel idea but the study was cross-sectional, used self-reported data, and the exercise instrument used has not been validated. These observations require further elaboration. If there truly is a relationship between maternal physical activity and BM composition, then this is one possible mechanism through which modern lifestyles with reduced physical activity might lead to altered infant immune development.

1.6.4 Maternal diet

Maternal diet during pregnancy and lactation could significantly influence allergy development. Maternal diet can influence breast-milk fatty acid and immune mediator composition (Hoppu et al., 2012, Laiho et al., 2003, Lauritzen et al., 2006). A study done by Urwin et al. showed that salmon consumption during pregnancy leads to a higher proportion of individual and total n-3 PUFA in BM during early lactation and a lower ratio of n-6 : n-3 PUFA as well as lower sIgA levels (Urwin et al., 2012).

Dunstan and co-authors showed that giving fish oil to atopic pregnant women led in their infants to a lower cytokine response to common allergens and less severe atopic dermatitis by one year of age compared with a placebo group, taking olive oil (Dunstan et al., 2003). In contrast, Johansson and co-authors suggest that diet is not so important in that women with a combination of eczema and respiratory allergy had lower BM

levels of several PUFAs and lower ratio of long-chain n-3/n-6 PUFAs than healthy women, despite similar fish consumption (Johansson et al., 2011).

A recent study from Finland (Linnamaa et al., 2013) provides intriguing results suggesting that black currant seed oil supplementation during pregnancy leads to a lower level of IL-4 and higher level of IFN-γ in breast milk in comparison to control group having olive oil. However, there were no differences in the levels of IL-5, IL-10, IL-12 and TNF.

Probiotic supplementation during pregnancy and lactation also may influence atopy development. Prescott et al. showed that giving L. rhamnosus HN001 or Bifidobacterium lactis HN019 resulted in higher levels of TGF-β1 and IgA in BM compared with placebo (Prescott et al., 2008) and Dotterud and co-authors showed that probiotic milk containing Lactobacillus rhamnosus GG, L. Acidophilus La-5 and Bifidobacterium animalis subsp. lactis Bb-12 intake by women from 36 weeks of gestation to 3 months post-natally during breastfeeding, resulted in a lower incidence of eczema in their babies compared with placebo group (Dotterud et al., 2010). A recent dietary intervention study by Hoppu et al. on 125 Finish mothers suggests that diet (favourable fat intake (SFA, MUFA,PUFA)) on its own and diet in conjunction with probiotic intake can influence cytokine and fatty acid composition in BM. They found higher levels of n-3 fatty acids and α-linolenic acid in BM in both dietary intervention groups and concentrations of IL-2, IL-4, IL-10 and TNF-α was higher in BM of mothers in diet and probiotic group (Hoppu et al., 2012). Kuitunen and co-authors found that probiotic intervention has been associated with increased IL-10 levels and decreased casein IgA levels in BM and TGF-β2 levels in colostrum (Kuitunen et al., 2012). Boyle et al. (Boyle et al., 2011) found that prenatal treatment with Lactobacillus rhamnosus GG

was not sufficient to prevent eczema. However, total IgA levels at day 28 and sCD14 at day 7 in BM, were lower in the group receiving probiotics compared to placebo group.

Together these data all support the principle that maternal dietary composition can influence BM immune composition, although the precise nature of the effects of different dietary components and the mechanisms through which they influence BM composition need further investigation.

1.6.5 Parity

The “hygiene hypothesis” remains one of the most widely discussed explanations for the high prevalence of immune mediated diseases in the affluent world and the early-life origins of atopy (Strachan, 1989). The inverse relationship between the risk of atopic diseases, associated with a Th2 immune response, and early-life exposure to infections promoting Th1 responses, such as later birth order or sibship size, early attendance at day care, and early exposure to pets or other animals is well described (Strachan, 2000).

One study has suggested that the risk of scleroderma and systemic sclerosis increases with increasing birth order, which is the reverse of the reported risk associated with allergic and atopic disorders (Cockrill et al., 2010). These observations might suggest that increased Th1 activity in early life reduces allergy but increases the risk of auto-immune disease. However, other population studies have shown a higher risk of allergic problems in patients who also have the auto-immune disease, type 1 diabetes mellitus.

There are very few data on parity in relation to immune modulators in human milk. Higher concentrations of IgA and IgM have been found in maternal colostrum of primiparous women in comparison with multiparous (Striker et al., 2004)

It would be reasonable to hypothesise that if there is an effect of parity on BM immune composition, then microbial exposures which impact on BM immune mediator levels (Peroni et al., 2010) may explain these differences in mediator levels in relation to parity. Thus mothers breastfeeding higher birth-order infants will be exposed to a wider array of organisms from their other children and these will affect milk cytokine levels as shown in some probiotic trials.

1.6.6 Habitat

Environmental influences on BM immune composition and maternal atopy/allergy effect on BM composition are summarised in *Table 2*. Variability in BM composition has been shown to be important in other settings – for example BM from diabetic mothers can lead to a higher risk of obesity in offspring compared with BM from non-diabetic milk donors (Scharton-Kersten et al., 1996). In the context of allergy, it is clear that BM of mothers living in different settings, and even of mothers in the same setting but with different childhood exposures, differs significantly (Tomicic et al., 2010, Amoudruz et al., 2009). It is supposed that, the pathway for this latter finding is that enteral exposures of the mother during her own infancy prime her intestinal immune cell development such and when immune cells migrate from the maternal intestine to the mammary gland, there is altered BM composition (Brandtzaeg, 2010).

Table 2 Environmental influences on BM immune composition and maternal atopy/allergy effect on BM composition

Author and year	Country and numbers	Immune modulators measured	Exposure of interest	Main Outcomes
Islam 2006 (Islam et al., 2006)	Bangladesh (colostrum from 105 mothers)	IgA, IgM, IgG, peripheral immune cells	Age, BMI, Parity	Maternal age, BMI, parity and income level did not correlate with immunoglobulin concentration in colostrum.
Hoppu 2012 (Hoppu et al., 2012)	Finland (colostrum and/or 1 month BM samples from 125 mothers)	IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, n-3 polyunsaturated fatty acids	Fatty acid supplementation	The concentrations of TNF-alpha, IL-10, IL-4 and IL-2 were higher in both dietary intervention groups compared with controls and γ-linolenic acid in the diet/probiotic group was higher compared with the diet/placebo group.
Urwin 2012 (Urwin et al., 2012)	UK (BM from 123 mothers)	sCD14, TGF-β1, TGF-β2, sIgA, fatty acid composition	Fatty acid supplementation	Salmon consumption during pregnancy lead to a lower n-6 PUFA : n-3 PUFA ratio. Higher proportion of individual and total n-3 PUFA has been found and n-6 PUFA proportions did not differ Breast milk secretory IgA (sIgA) was lower in the salmon group. All breast milk immune factors decreased between days 1 and 28.

Amoudruz 2009 (Amoudruz et al., 2009)	Sweden (32 born in Sweden and 32 migrated from developing countries)	IL-6, IL-8, TGF-β1, TGF-β2, sCD14	Habitat	Mothers from developing countries had significantly higher levels of BM IL-6, IL-8 and TGF-β1.
Holmlund 2010 (Holmlund et al., 2010)	Sweden, Mali (30 from Mali, 32 Swedish immigrants and 33 native Swedish women)	TGF-β1, sCD14	Habitat	Women from Mali and immigrant women from Sweden contained higher levels of TGF-β1than milk from native Swedish women. Mali women also had significantly higher levels of sCD14 in BM compared with other two groups.
Peroni 2010 (Peroni et al., 2010)	Italy (colostrum and 1 month BM of 45 mothers from farming environment and 69 from urban environment)	TGF-β1, IL-10	Habitat	Exposure to farming environment has been associated with higher concentrations of TGF-β1 and IL-10 in BM compared with mothers from urban environment.
Tomicic 2010 (Tomicic et al., 2010)	Sweden, Estonia (colostrum and 1 month BM from 39 Estonian and 60 Swedish mothers)	SIgA, IL-4, IL-10, IL-13, IFN-γ, TGF-β1, TGF-β2	Habitat	Lower levels of TGF-β2 and higher levels of SIgA, IL-10, and IFN-γ have been found in BM from Estonian mothers compared with Swedish mothers.

Striker 2004 (Striker et al., 2004)	Brazil (colostrum from 82 mothers having vaginal delivery, caesarean section with labor or elective caesarean section)	IgA, IgG, IgM	Labour type	Occurrence of labor together with surgical stress induce higher IgA concentrations in colostrum of women submitted to emergency caesarean section.
Rudloff 1999 (Rudloff et al., 1999)	Germany (milk from 19 allergic and 23 non-allergic mothers)	IL-4, IL-10, IFN-γ, MIP-1α	Maternal Atopy/Allergy	Concentrations of proinflammatory markers and cytokines in BM did not differ significantly between allergic and non-allergic mothers.
Bottcher 2000 (Bottcher et al., 2000)	Sweden (colostrum and 1 month BM from 19 allergic and 20 non-allergic mothers)	IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-13, TGF-β1, TGF-β2	Maternal Atopy/Allergy	Concentrations of IL-4, IL-5 and IL-13 were higher in colostrum from allergic mothers compared with non-allergic.
Laiho 2003 (Laiho et al., 2003)	Finland (BM from 43 allergic and 51 non-allergic mothers)	TGF-β2, TNF-α, IL-4, IL-10, prostaglandin E2, cysteinyl leukotrienes, fatty acid composition	Maternal Atopy/Allergy	Allergic mothers had a lower level of TGF-β2 in BM compared with non-allergic. Other cytokines concentrations and fatty acid composition were not different between the groups. Positive association observed between TGF-β2 and proportion of polyunsaturated fatty acids.

Prokesova 2006 (Prokesova et al., 2006)	Czech Republic (colostrum and BM from 21 allergic and 21 non-allergic mothers)	IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TGF-β	Maternal Atopy/Allergy	No significant difference found in colostrum of healthy and allergic mothers. Higher levels of IL-4 and lower of IL-10 observed in mature milk of allergic mothers.
Rigotti 2006 (Rigotti et al., 2006)	Italy (BM from 13 allergic and 9 non-allergic mothers)	TGF-β1, IL-10	Maternal Atopy/Allergy	TGF-β1 in mature milk was lower in allergic mothers compared to non-allergic. IL-10 level did not differ much between allergic and non-allergic mothers.
Snijders 2006 (Snijders et al., 2006)	Netherlands (1 month BM from 182 allergic and 125 non-allergic mothers; 123 sensitised and 164 non-sensitised mothers)	TGF-β1, IL-10, IL-12, sCD14	Maternal Atopy/Allergy	Higher sCD14 levels detected in mothers with a positive vs. negative allergic history and in mothers who were sensitised vs. non-sensitised. IL-10 was not detected.
Sidor 2008 (Sidor et al., 2008)	Poland (colostrum and 1 month BM from 12 allergic mothers and 30 non-allergic mothers)	β-casomorphin-5, β-casomorphin-7	Maternal Atopy/Allergy	Content of β-casomorphin-5 in colostrum of women from control group was three times higher compared with an allergic group.

Marek 2009 (Marek et al., 2009)	Poland (colostrum from 30 allergic mothers and 46 non-allergic)	IL-4, IL-10, TGF-β1	Maternal Atopy/Allergy	TGF-β1 median concentration was higher in the allergy group than in the control. IL-10 was present in colostrum of all women and median IL-10 concentration did not differ between the allergy and control groups. Median IL-4 level did not differ significantly between the two groups but found more often in colostrum of allergic mothers compared with nonallergic.
Rautava 2002 (Rautava et al., 2002)	Finland (BM from 30 women taking probiotics and 32 taking placebo)	TGF-β1, TGF-β2	Probiotic	Giving probiotics to pregnant and lactating women increased TGF-β2 in their milk compared with mothers receiving placebo.
Bottcher 2008 (Bottcher et al., 2008)	Sweden (colostrum and mature milk from women treated with L. reuteri (n = 54) or placebo (n = 55) from 36 weeks gestation until labour)	Total IgA, SIgA, TGF-β1, TGF-β2, IL-10, TNF, sCD14, Na/K ratios	Probiotic	L. reuteri supplementation during pregnancy was associated with low levels of TGF-β2 and slightly increased levels of IL-10 in colostrum.
Prescott 2008 (Prescott et al., 2008)	New Zealand (1 week, 3 month and 6 month BM from 34 mothers receiving Lactobacillus rhamnosus HN001; 35 mothers - Bifidobacterium lactis	IL-6, IL-13, IFN-γ, TNF-α, IL-10, TGF-β1, IgA sCD14	Probiotic	TGF-β1 levels were higher in early 1 week BM from groups taking probiotics. IgA concentration was also higher in BM from both the B. lactis HN019 and the L. rhamnosus HN001 group.

	HN019 and 36 taking placebo beginning 2-5 weeks before delivery and continuing for 6 months into lactation)			
Boyle 2011 (Boyle et al., 2011)	Australia (7 days and 28 days BM samples from 38 mothers receiving placebo and 35 receiving probiotic)	Total IgA, sCD14, TGF-β	Probiotic	Prenatal probiotic (Lactobacillus rhamnosus GG) supplementation was associated with decreased BM soluble CD14 and IgA levels
Kuitunen 2012 (Kuitunen et al., 2012)	Finland (364 colostrum and 321 BM samples)	Total IgA, IgA antibodies to cow's milk, casein, β - lactoglobulin, ovalbumin, TGF-β2, IL-10	Probiotic	Probiotic intervention (*Lactobacillus rhamnosus* GG, *L. rhamnosus, Bifidobacteriumbreve, Propionibacterium freudenreichii* ssp. *shermanii* JS) was associated with increased IL-10 levels and decreased casein IgA levels in BM and TGF-β2 levels in colostrum.
Zanardo 2005 (Zanardo et al., 2005)	Italy (colostrum and 10-th day BM from 42 smoking and 40 non-smoking mothers)	IL-1α, β-endorphin, leptin	Smoking	IL-1α concentrations were lower in smokers' colostrum compared with non-smoking mothers. IL-1α, β-endorphin, leptin levels in transitional milk did not differ significantly.

1.7 Methodological issues of BM immune factor analysis

One of the modern approaches to list the proteomic profile in various biological samples is protein micro-array. There are convenient and commercially available multiplex techniques based on antigen–antibody binding. The cytokine array is a very useful tool that can give precise information on relative changes in cytokine concentrations, but they unfortunately do not provide specific quantitative information. For protein level quantification ELISA can be used. It is important to mention that the sensitivity for each protein of particular interest varies because of differences in antibody affinity (Kverka et al., 2007).

Mass spectrometry is a powerful method of immunity assessment and nutritional immune modulation. It is a relatively underused technology because it is mostly located in large institutions. This method allows very accurate and sensitive diagnostics in immunology and nutrition. It can lead to the detection of biomarkers relevant to immune status and human nutrition. Various food products have been investigated using mass-spectrometry, analysing their protein composition. Reviews by De Roos and McArdle reviewed proteomics use, as a basis for biomarker development in nutrition research (de Roos and McArdle, 2008, de Roos, 2008) They provide a comprehensive summary of various nutritional studies related to immunity using by mass spectrometry as a diagnostic tool.

At present, the vast majority of the studies on human's colostrum and/or breast milk composition, especially immune modulators were done using ELISA, but not mass-spectrometry. I am not aware of any studies so far, on the difference in the protein composition of colostrum and/or breast milk in allergic and non-allergic mothers or mothers of babies who subsequently developed atopy using proteomics tool.

The "gold standard" for breast milk collection is considered to be samples of all milk expressed over 24 hours, with collection of a large number of samples from the same person over time (Bauer and Gerss, 2011, Ballard and Morrow, 2013). This method is ideal but at the same time utopian as it is time consuming, associated with high costs and compliance issues. The usual approach in breast milk research is standardisation of collection at a specific time of a day and collection from the breast collateral from the last feed as well as collection on multiple occasions from the same mother (Geraghty et al., 2005).

It is essential to underline that different research groups use various sample preparations prior to lab tests and currently there are no “gold standards” in collection and processing samples. Investigators employed a range of non-standardised collection from their participants at different times of day; at different times within a feed; or at various stages of lactation (Ballard and Morrow, 2013). Centrifugation details given in rpm do not provide complete information because rotor radius is also very important in g calculation. The range of methodologies used in various studies are presented in *Table 3*.

Table 3 Methodology used for immune modulators measurement in colostrum and breast milk in various studies

Author and year	Collection process	Colostrum and/or breast milk centrifugation	Sample storage	Method used for analysis
Rudloff 1999 (Rudloff et al., 1999)	By manual expression at least 1 hour after the last feeding	In 2 steps for further analysis: 2000 rpm for 10 min at room temperature to remove the cells and 6000 rpm for 10 min at 4°C later on to remove fat	-20°C	ELISA (Genzyme, Cambridge, USA) and (R&D Systems, Wiesbaden, Germany)
Bottcher 2000 (Bottcher et al., 2000)	Mothers collected breast milk at home in sterile plastic tubes, using a manual breast-pump	In 2 steps for further analysis: 680×g for 10 min at 4°C, after which the supernatants were removed and centrifuged at 10 000 ×g for 30 min at 4°C (all samples were centrifuged after thawing)	-20°C	ELISA (Research Diagnostics Inc., Flandern, NJ) and (R&D Systems Inc., Minneapolis, MN); concentrations of IL-5 were analyzed on high-binding, half-area Costar 3690 plates, coated with monoclonal rat anti-human IL-5
Laiho 2003 (Laiho et al., 2003)	Infants were allowed to suckle for a few minutes before a breast milk sample was collected by manual expression, and then feeding continued	Once at 8832×g for 5 min, at room temperature (after thawing)	–70°C	ELISA (R&D Systems Europe Ltd, Abindgon, U.K.)
Savilahti 2005 (Savilahti et al., 2005)	No specific description is given	Once at 10000×g for 30 min, at unknown temperature regime, (after thawing)	-80°C	ELISA (IBL Inc. Hamburg, Germany) and Quantikine Human TGF-β1 and TGF-β2 Immunoassays (R & D Systems, Minneapolis, MN)

Ustundag 2005 (Ustundag et al., 2005)	By manual expression within 2 hours of the first feeding in the morning (8 AM to 11 AM)	Once at 690×g for 20 minutes at unknown temperature regime	-40°C	Chemilumminessence method, and BIODPC diagnostic kits (BIODPC, Istanbul, Turkey, DPC, Calif) with IMMULITE 2000 hormon auto-analyzer (BIODPC, Istanbul, Turkey, DPC, Calif)
Bryan 2006 (Bryan et al., 2006)	Samples were collected from mothers at home on three consecutive days and in hospital. Samples were collected in the morning (manual expression at home and using an electric pump at clinic)	On day 3-4, samples collected at home, were thawed and processed at 900×g for 5 min. Samples collected in clinic processed without freezing and centrifuged at 890×g for 30 min	Those collected at home stored then at -20°C until transport to the laboratory. Those collected in clinic and centrifuged stored at -80°C until ELISA	ELISA (R&D Systems, Abingdon, UK)

Snijders 2006 (Snijders et al., 2006)	In the morning, before breastfeeding the child, from the contra-lateral breast (since the last feeding)	Once at 400×g, for 12 min at 4°C	-80°C	ELISA (R&D Systems Europe Ltd., Abingdon, UK) and (Biosource Int., Camarillo, CA, USA)

1.8 Immunological changes during pregnancy

Changes in maternal immunity aim to tolerate the semi-allogeneic fetus. It involves changes in the mucosal lining of the pregnant uterus (decidua) (Trundley and Moffett, 2004), and changes in peripheral immune responses (Veenstra van Nieuwenhoven et al., 2003). When implantation occurs, trophoblast cells (fetally derived placental cells) infiltrate uterine endometrium; later the endometrium develops into decidua ensuring anchorage of the placenta and therefore proper fetal nutrition. This process needs to be regulated accordingly to protect the corporal integrity of the uterine wall of the mother (Herberts et al., 2010). There is some evidence suggesting that failure of human cytotrophoblasts to mimic vascular adhesion receptors may be associated with pre-eclampsia (Zhou et al., 1997).

The developing fetus requires maternal immunoglobulins for future passive immunity protection from infection during the fetal and newborn period (Poole and Claman, 2004). Fc-γ receptors (FcγRI, FcγRII, and FcγRIII) expressed by fetal macrophages, are involved in catching immune complexes and protecting the fetus from rejection (Simister and Story, 1997).

Two decades ago Wegmann and co-authors proposed that a shift from Th1 to Th2 is needed for a successful pregnancy (Wegmann et al., 1993). This concept may be seen clinically as about 70% of women experience some remission of rheumatoid arthritis symptoms (TH1 mediated) during pregnancy (Da Silva and Spector, 1992), while Th2 immunity disorders flare during pregnancy and after birth (Wegmann et al., 1993). A variety of immune active molecules are known to play active role in fetus protection (IL-4, IL-5) (Roberts et al., 2001, Wegmann et al., 1993) and growth and development of

trophoblast cells (IL-3, IL-10, GM-CSF, CSF-1) (Roth et al., 1996, Athanassakis et al., 1987).

Overall, current evidence indicates that anti-inflammatory cytokines at the maternal–fetal interface facilitate survival of the fetus (Poole and Claman, 2004). It is still unknown to what extend the "cytokine orchestra" in utero influences subsequent breast milk immunological composition, although few studies have approached this topic (Jones et al., 2001, Thornton et al., 2002, Jones et al., 2002b, Power et al., 2002, Thornton et al., 2003b, Thornton et al., 2003a, Thornton et al., 2009, Holloway et al., 2009).

In this thesis I will focus on assessment of cytokines and growth factors in human BM and colostrum, rather than immunoglobulins. Growth factors are critical for infant intestinal development, and the role of cytokines in human BM, which potentially may skew infant immune responsiveness to Th1 or Th2 depending on their specific composition, is controversial.

1.9 Aims and Hypothesis

1.9.1 Aims

- To investigate associations of exposures with the levels of immune modulators in colostrum and mature breast milk and the relation to the development of atopy in infancy
- To investigate the difference in colostrum and breast milk immune modulators depending on maternal and environmental factors
- To establish correlations between the immune modulator levels in mother's colostrum and breast milk and the prevalence of reported allergic events and disease during infancy
- To ascertain if the immune modulator levels in colostrum and breast milk of atopic mothers are significantly different when compared with non-atopic mothers

1.9.2 Hypothesis

Primary hypothesis: In lactating women maternal and environmental factors influence colostrum and breast milk immune modulator composition.

Secondary hypothesis: There is a relationship between the colostrum and breast milk composition and subsequent atopy, allergy, GI outcomes development and prevalence in children.

Tertiary hypothesis: Lactating women can be grouped according to a specific immunological profile of their breast milk (lacto-type).

2 Materials and methods

2.1 Study population and questionnaires

2.1.1 Ethics and Research and Development (R&D) Approval

The investigations and sample collection have been conducted following ethical approval by Ethics committee in three countries participating in the study: West London Rec 3 (UK) (Ref.number 10/H0706/32) and all paperwork has been completed according to the hospital R&D Joint Research Office (UK) (JROSM0072) policy; Ethical Committee of the Azienda Ospedaliera di Verona (Italy) (approval N°1288) and Moscow Institute of Paediatrics and Child Health of Ministry of Health of Russian Federation (Russia) (approval № 1-MS/11).

2.1.2 Study design and subject recruitment

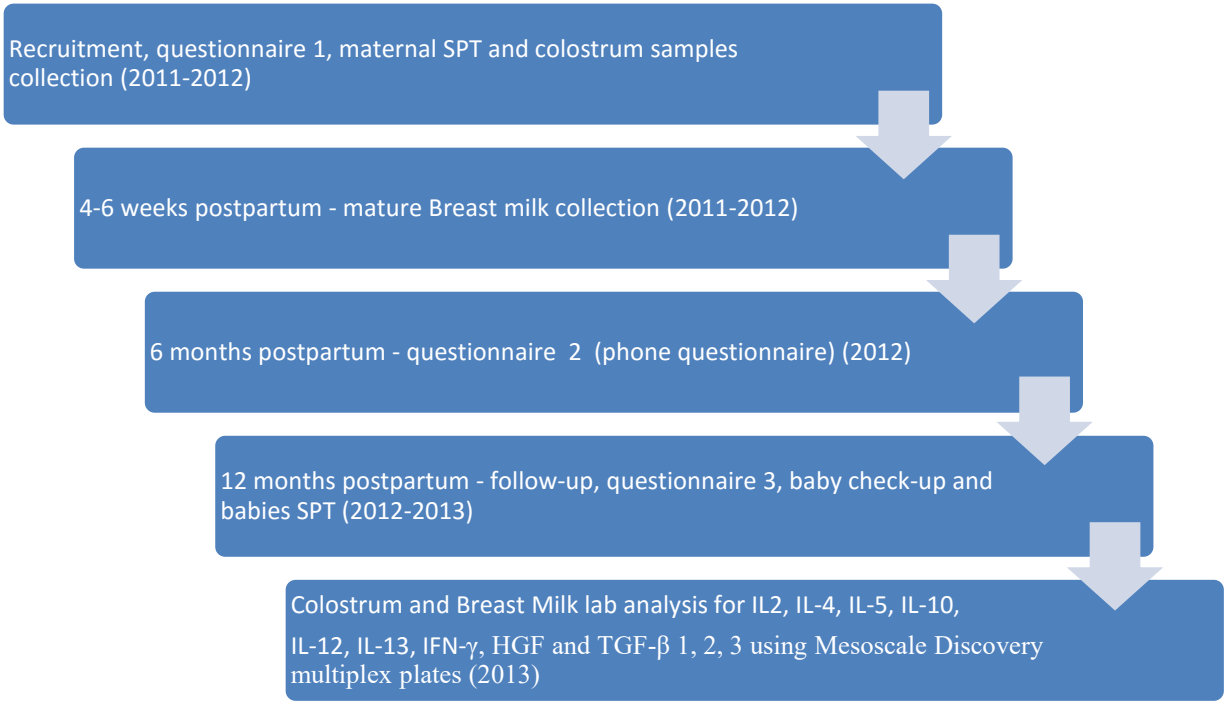
In this prospective study (*Figure 1*) of human participants and their biological samples women and their newborn babies living in London from St.Mary's Hospital were recruited. Collaborations were established with the Moscow Institute of Paediatrics, Russia and the University of Verona, Italy which facilitated recruitment of additional participants overseas.

Women and their newborn infants have been enrolled in the study from the postnatal unit and birth centre. Women who consented underwent an allergy skin prick test and answered a 10 minute questionnaire regarding their medical history, particularly for the presence of any allergic or other immunological disease or treatment. Their medical file has been reviewed to extract any relevant health information, and they have been asked to provide samples of breast milk as described below.

All mothers have been given a pot in which they collected colostrum in the first 6 days and mature breast milk 4-6 weeks postpartum.

Contact details have been recorded at the enrolment visit, and as the infant reached 6 months age participants have been contacted to ask about the infant's health. At 1 year of age we have begun follow-up assessments for allergy development which include skin tests on babies to egg, milk, peanut, cod and common aeroallergens.

Figure 1 Study timeline



2.1.3 Study Population

Total of 398 mothers (London n=101, Moscow n=221, Verona n=76) voluntarily provided the samples and participated in this study. Demographic data of the participants presented in *Table 4*.

All women were generally healthy, aged 19 to 50. None of them were taking any anti-inflammatory medication at the time of enrolment. Colostrum was collected from all mothers during the first 6 days postpartum and mature breast milk in interval between 4 to 6 weeks. All those mothers who have been able to produce and donate milk have been included into the study.

	London (all participants)	London (colostrum analysed)	Moscow (all participants)	Moscow (colostrum analysed)	Verona (all participants)	Verona (colostrum analysed)
Pos Maternal Allergy history (n) (%) (self-reported Allergy)	42/123 (34)	33/101 (33)	87/277 (31)	63/221 (29)	30/81 (37)	26/76 (34)
Pos Maternal confirmed Atopy		33/92 (36)		23/156 (15)		9/41 (22)
Maternal Age (Years)						
Mean (SD)	32.4 (4.98)	32.8 (4.78)	29.3 (4.49)	29.8 (4.45)	37.6 (5.58)	37.4 (5.38)
Mode of Delivery (n) (%)						
Vaginal	84/123 (68)	69/100 (69)	232/277 (84)	188/219 (86)	64/81 (79)	63/76 (83)
Caesarean Section	34/123 (28)	31/100 (31)	43/277 (15)	31/219 (14)	17/81 (21)	14/76 (17)
Gestational Age (weeks)						
Mean (SD)	40.0 (1.49)	40.1 (1.45)	39.5 (1.19)	39.6 (1.03)	39.1 (1.7)	39.7 (1.52)
Baby Gender (n) (%)						
Male	61/123 (49)	53/101 (53)	144/277 (52)	118/217 (54)	43/83 (52)	41/76 (54)
Baby Weight (grams)						
Mean (SD)	3455 (487.35)	3527 (535.37)	3509 (494.15)	3526 (438.97)	3319.5 (473.9)	3328.3 (476.95)
Previous Pregnancies (n) (%)						
Primigravida	53/123 (43)	42/97 (43)	99/277 (36)	70/217 (32)	33/81 (41)	26/75 (35)
Multigravida	65/123 (53)	55/97 (57)	172/277 (62)	147/217 (68)	47/81 (58)	49/75 (65)
Smoking (n) (%)						
Smokers	4/123 (3)	3/99 (3)	13/277 (4.5)	11/213 (5)	3/81 (4)	3/76 (4)
Exposed to smoke	34/123 (28)	30/99 (30)	176/277 (64)	135/218 (62)	25/81 (31)	25/76 (33)
Antenatal Medications (n) (%)						
Positive	39/123 (32)	34/100 (34)	169/277 (61)	131/212 (62)	46/81 (57)	56/76 (74)
Antenatal Infections (n) (%)						
Positive	17/123 (14)	16/100 (16)	73/277 (26)	61/211 (29)	30/81 (37)	29/76 (38)
Contact with Mould/Mildew (n) (%)						
Positive	25/123 (20)	22/95 (23)	49/277 (18)	38/218 (17)	28/81 (35)	28/76 (37)
Time of colostrum collection (hours)						
Mean (SD)		58.605 (33.2)		50.03 (14.34)		57.84 (26.52)

Table 4 Demographic data

2.1.4 Inclusion and Exclusion Criteria

Mothers had to meet inclusion criteria for the study in order to participate (*Table 5*).

Table 5 Inclusion and exclusion criteria

Inclusion Criteria	Exclusion Criteria
• Healthy term infants (full-term newborns) and their mothers, born at Birth centres and/or postnatal units of the hospitals in London, Verona and Moscow during the study period • Willingness to comply with the study procedures	• Premature infants (<37 weeks) • Mothers taking immunosuppressive agents during lactation • Major birth defects • Admission to NICU • Intrauterine growth restriction (weight below 2nd centile) • Significant maternal or infant illness, such that participation will place an unreasonable burden on the mother or child e.g. maternal postnatal depression

2.2 Informed Consent

All participants were given an information sheet to read. Mothers had about 60 minutes to think if they wished to participate in the study. They were also given time to ask questions. No pressure was exerted on the mothers to participate in the study. No financial benefits were offered or given, as the study is completely voluntary. Those who signed informed consent were recruited into the study.

2.3 Questionnaires

All participants were asked to fill in the questionnaire 1 (*Appendix A*) asking simple questions about their general health focusing on allergic conditions. At 6 months postpartum participants have been asked over the phone to answer simple questions from questionnaire 2 (*Appendix B*) on breastfeeding patterns and early development of their baby. At 12 months mothers have been asked to come into clinic with their babies. They have been asked questions according to questionnaire 3 (*Appendix C*) and measurements of maternal and child's height and weight have been performed alongside prick test in order to find out if baby is atopic to any of the allergens listed in questionnaire 3.

2.4 Clinical data collected

- Maternal self-reported Allergy
- Maternal Atopy (positive sensitivity according to skin prick test)
- Paternal Allergy History
- Country of the sample collection
- Infant gender
- Mode of delivery (Elective Caesarean/ Caesarean with labor/ Vaginal)
- Maternal Gravidity
- Maternal Parity
- Home pet ownership
- Mould at home
- Baby's weight, length, head circumference at birth and at 12 months
- Maternal BMI at 12 months
- Gestation at birth in weeks
- Maternal age (at infant birth)
- Maternal medications (antibiotics) intake during pregnancy and first month of lactation
- Maternal infections during pregnancy
- Maternal smoking (active/passive)
- Maternal alcohol consumption
- Maternal educational level (at recruitment)
- Mode of infant feeding – duration of exclusive breast feeding and any breastfeeding
- Time of non-human mammalian milk introduction
- Time of egg introduction

- Time of peanut introduction
- Cumulative incidence of reflux and/or vomiting (moderate or severe) in 12 months
- Cumulative incidence of colic before 12 months
- Self-reported eczema at 6 months
- Eczema presence at 12 months
- Sensitisation at 12 months – aeroallergen sensitization; food allergen sensitization; any allergen sensitisation
- Self-reported Food intollerance/allergy at 6 months
- Food Allergy at 12 months

2.5 Skin Prick Testing (SPT)

After completing the questionnaire mothers were skin prick tested to common allergens. We used the following allergen solutions for Skin Prick Test (SPT): Histamine Positive Control, Negative Control, House Dust Mite (*Dermatophagoides pteronyssinus*), Cat fur (*Felix domesticus*), 5 Grasses mix, Ryegrass, Peanut, Hazelnut, Whole hen's egg (Stallergens, SA 92160 Anthony, France) and Cow's milk raw (ALK-Abello, Hørsholm, Denmark). Skin pricking was performed using lancets (ALK-Abello, Hørsholm, Denmark).

A drop of solution was placed on the skin of the forearm and lancet pushed through the drop to prick the skin without drawing blood. After pricking the skin the remaining fluid has been removed using pieces of paper tissue. Results have been interpreted 10 -15 minutes later, using a ruler to measure the wheal diameter. Mothers were considered to be sensitised if the positive control wheal was ≥3 mm and any allergen wheals were ≥3 mm or in event of a reaction to the negative control >greater than negative control.

2.6 Laboratory methods

I used modern and ultra-sensitive kits in order to measure the levels of cytokines and growth factors. Three different plates have been used for laboratory analysis:

- Th1/Th2 7-plex Human cytokine kit
- HGF Human kit
- TGF-beta 1,2,3 prototype Human kit

An overview of technical information, relevant to the diagnostic tool is found in *Table 6*.

I decided to use a median for LLOD from all plates to avoid bias in results.

Table 6 Technical data of MesoScale 7-plex, TGF-beta and HGF plates

Cytokine	Average lower limit of detection (LLOD)	Standard curve range
IFN-γ	3,49	0-2500 pg/ml
IL-2	2,06	
IL-4	1,83	
IL-5	2,89	
IL-10	1,50	
IL-12p70	3,50	
IL-13	4,60	
HGF	73,00	0-100000 pg/ml
TGF-β 1	8,73	
TGF-β 2	265,00	
TGF-β 3	8,37	0-50000 pg/ml

2.6.1 Electrochemiluminescence Multiplex

For the study I used Human TH1/TH2 7-Plex Assay Ultra-Sensitive Kit (MesoScale Discovery). This diagnostic kit can provide a rapid method for measuring the levels of proteins within a single small-volume sample, which is very convenient. In the multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well.

The Human TH1/TH2 7-Plex Assay detects IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, and IL-13 in a sandwich immunoassay format (*Diagram 1*). The same principle applies to HGF and TGF-beta triplex plates.

The plate was pre-coated with capture antibody on spatially distinct spots – antibodies for IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, and IL-13. Using this kit I added the sample and a solution containing the labelled detection antibodies - anti-IFN-γ, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-10, anti-IL-12p70, and anti-IL-13 labelled with an electrochemiluminescent compound, MSD SULFO-TAG™ label - over the course of one or more incubation periods.

Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the labelled detection antibodies by bound analytes completes the sandwich. After read buffer addition, which provides the appropriate chemical environment for electrochemiluminescence, the plate can be loaded into an MSD SECTOR® instrument (Figure 2) for analysis.

Figure 2 MSD Sector Imager 2400

Inside the SECTOR instrument, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of

emitted light to afford a quantitative measure of IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, and IL-13 present in the sample.

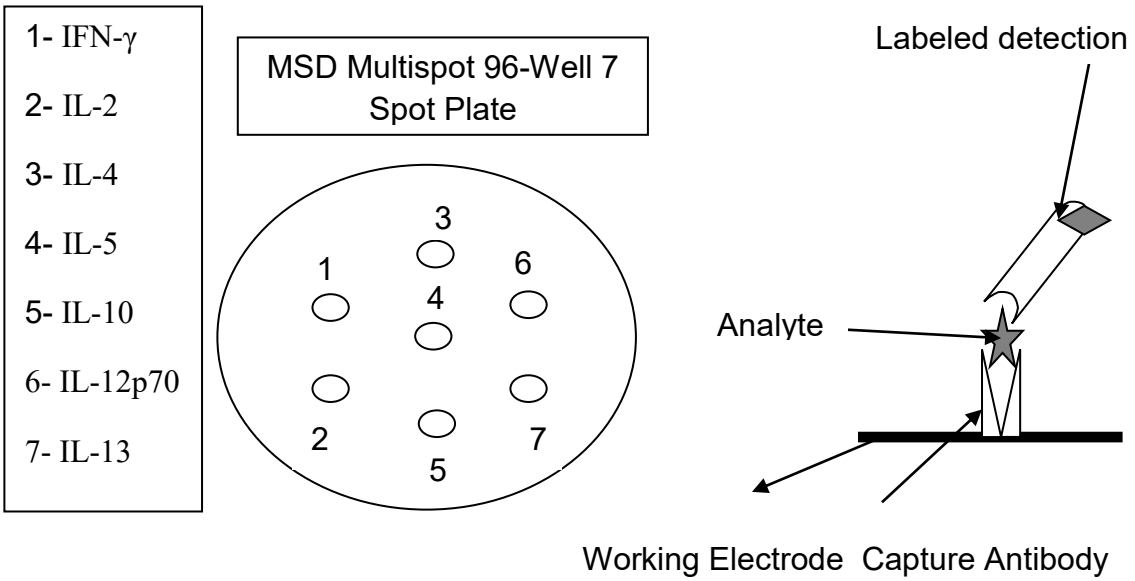


Diagram 1 ***Diagram showing placement of analyte capture antibody. (Adapted from MDS 7-plex Manual)***

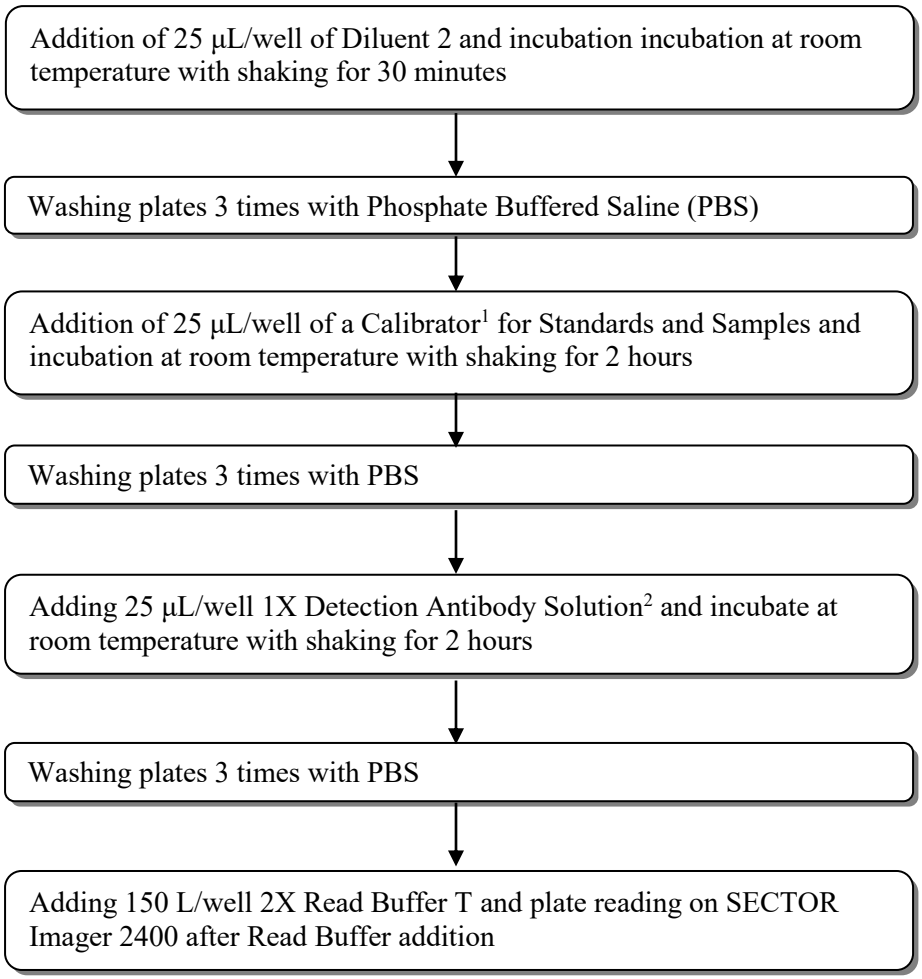
2.6.2 Total protein and sodium assesment in breast milk

In an attempt to correct for time of collection influence on growth factor levels we used Abbott Architect Analyser in order to detect the levels of total protein and sodium in colostrum. The details of this method are present in the relevant section of chapter 5.

2.7 Colostrum and breast milk samples analysis

Samples from all three centres are stored at -80°C in the freezer in Paediatric Research Unit, St.Mary's hospital. After thawing samples were centrifuged at 1500 ×g for 15 minutes at 4°C. The lipid layer is trimmed off with a pipette and aqueous fraction is analysed for immune modulators later on, according to protocols described below.

2.7.1 Protocol for Th1-Th2 7-plex plate



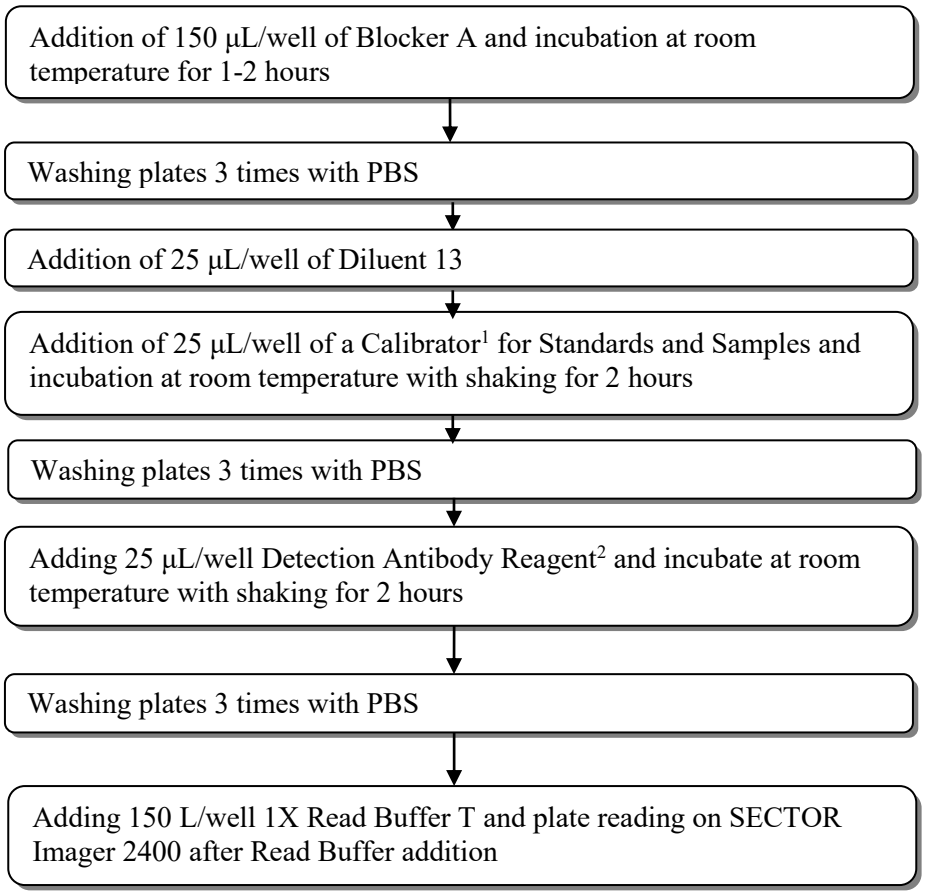
Calibrator for the Human TH1/TH2 7-Plex Assay is supplied at 400-fold higher concentration than the recommended highest calibrator. For Calibrator preparation it is needed to dilute the stock Calibrator 100-fold in Diluent 2. MSD recommends the preparation of an 8-point standard curve. Each well requires 25 µL of Calibrator. For this assay, MSD recommends 4-fold serial dilution steps and Diluent 2 alone for the 8th

point. It is needed to prepare the diluted stock Calibrator by transferring 10 µL of the Human TH1/TH2 7-Plex Calibrator Blend (Ultra-Sensitive) to 990 µL of Diluent 2. Then it is needed to prepare the highest Calibrator point by transferring 50 µL of the Human TH1/TH2 diluted stock Calibrator to 150 µL Diluent 2. Repeat 4-fold serial dilutions 6 additional times to generate 7 Calibrators. For 8th Standard just Diluent 2 is used as a zero Calibrator.

[2]Detection Antibody Reagent preparation:

MSD 7-plex Detection Antibody Blend is provided at 50X stock solution. The final concentration of the working Detection Antibody Solution should be at 1X. For each plate used it is needed to dilute a 60 µL aliquot of the stock Detection Antibody Blend into 2.94 mL of Diluent 3.

2.7.2 Protocol for HGF plate



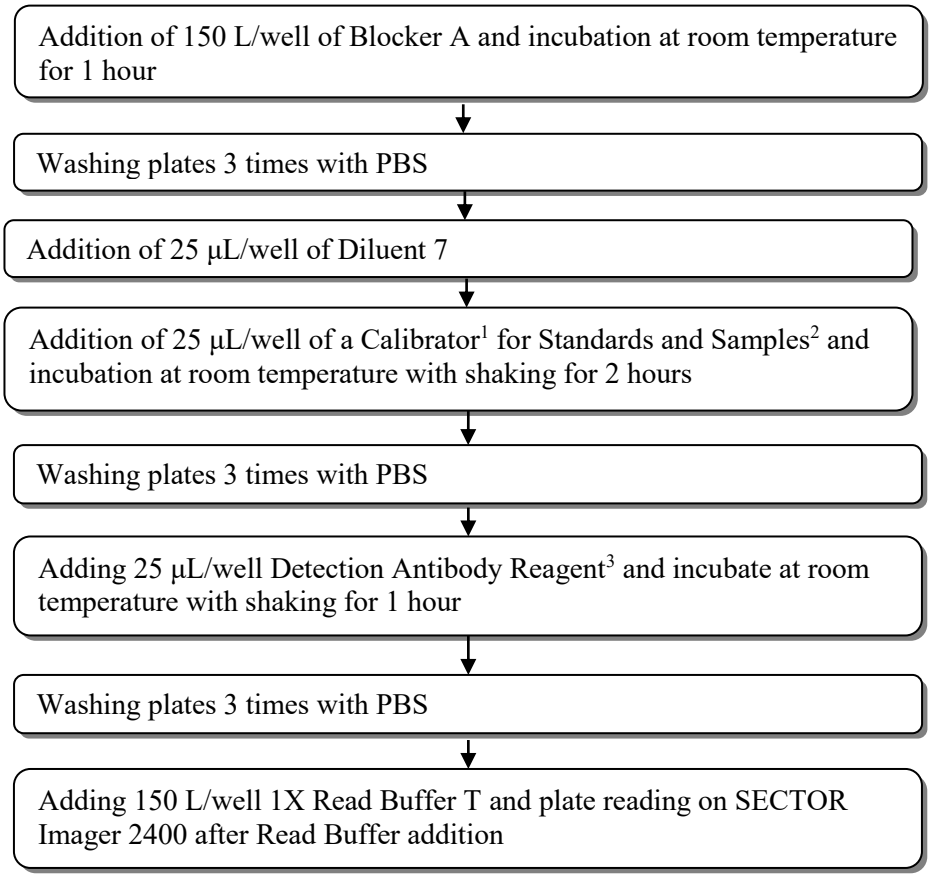
[1]Calibrator preparation:

Each well requires 25 µL of Calibrator. Upon thawing of Human HGF Calibrator stock solution I vortex it and prepared the required concentrations by serially diluting Calibrator stock into Diluent 13. I followed a recommended Calibrator dilution procedure preparing an initial high Calibrator at100000 pg/ml by adding 10 µL of Human HGF Calibrator at 1 µg/mL to 90 µL Diluent 13, and then prepare six additional 1:10 serial dilutions. I used Diluent 13 alone as the zero Calibrator concentration as recommended. The resulting Calibrator levels are: 100000, 10000, 1000, 100, 10, 1, 0.1, and 0 pg/mL HGF.

[2]Detection Antibody Reagent preparation:

Each well requires 25 µL of Detection Antibody Reagent. I prepare 3 mL per plate combining in a 15 mL tube 2.97 mL of Diluent 8 and 30 µL of 100X SULFO-TAG Anti-hHGF Antibody to reach a final concentration of 1X.

2.7.3 Protocol for TGF-β triplex plate

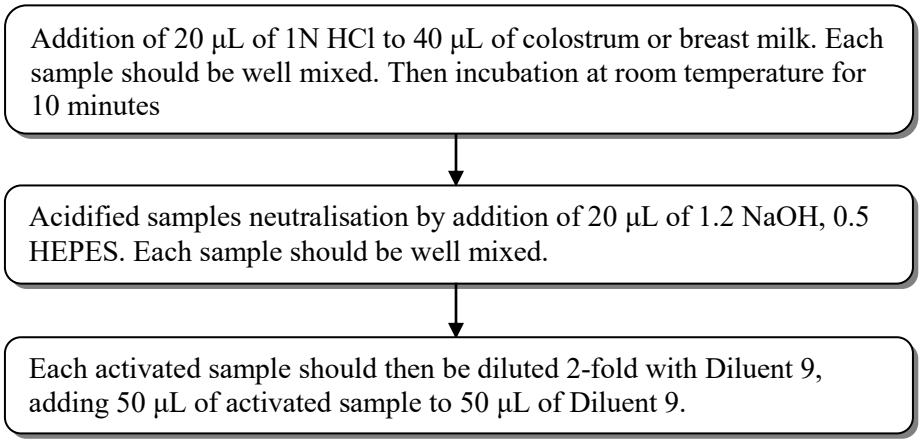


[1]Calibrator preparation:

Each well requires 25 µL of Calibrator. Upon thawing of Human TGF-β 1,2 and 3 Calibrator stock solutions I vortex it and prepared the required concentrations by serially diluting Calibrator stock into Diluent 9. I prepare an initial highest Calibrator point by transferring 20 µL of the 1000000 pg/ml of Human TGF-β1 Calibrator stock, 20 µL of the 1000000 pg/ml of Human TGF-β2 Calibrator stock, and 10 µL of the 1000000 of Human TGF-β3 Calibrator stock to 150 µL of Diluent 9, and then prepare six additional 1:4 serial dilutions. I used Diluent 9 alone as the zero Calibrator concentration as recommended.

[2]Samples preparation for TGF-β triplex analysis:

Analysis of samples for TGF-β 1,2 and 3 requires preliminary sample preparation. Samples should be treated and neutralised before they may be used in the assay analysis.



[3]Detection Antibody Reagent preparation:

Each well requires 25 µL of Detection Antibody Reagent. I Combine 60 µL of 50X SULFO-TAG Human TGF-β1 Detection Antibody, 60 µL of 50X Biotinylated Human TGF-β2 Detection Antibody, 60 µL of 50X Biotinylated Human TGF-β3 Detection Antibody, and 6 µL of 500 µg/mL SULFO-TAG Streptavidin into a final volume of 3 mL in Diluent 100.

2.7.4 Reagents used

All reagents used in experiments are listed in *Table 7*.

Th1/Th2 7-Plex	HGF	TGF-β1,2,3
Diluent 2 and Diluent 3	Diluent 8 and Diluent 13	Diluent 7, Diluent 9 and Diluent 100
Human Th1/Th2 7-plex Calibrator blend (for Ultra-Sensitive plates)	Human HGF Calibrator	Human TGF-β1 Calibrator Human TGF-β2 Calibrator Human TGF-β3 Calibrator
SULFO-TAG Detection Antibody blend	SULFO-TAG Anti-hHGF Antibody	SULFO-TAG TGF-β1 Detection Antibody Biotinylated TGF-β2 Detection Antibody Biotinylated TGF-β3 Detection Antibody
PBS		
Read Buffer T		
Blocker A		

Table 7 Reagents used in laboratory experiments

2.7.5 Immune active molecules in Breast milk and maternal blood serum

When breast milk immune profile is analysed a reasonable question arises, whether immune molecules present in breast milk are just a mirror of those cytokines and growth factors found in maternal serum. I supervised work done by Dr. Gabriella Feketea for her MSc in Allergy thesis; she collected paired colostrum (day 2), transitional milk (day8) and maternal serum samples from twenty healthy women from Amaliada, Greece, who had given birth by caesarean section to term neonates and breastfed their infants. Her data (*Table 8*) suggests that neither the level of cytokines, nor growth factors concentration correlate with levels present in the maternal blood serum (Feketea, 2014).

Table 8 Correlations between breast milk and serum for different mediators, in day 2 and day 8 samples

	Day 2		Day8	
	Breast milk: Serum		Breast milk: Serum	
	r	p	r	p
HGF	0.31	0.19	-0.01	0.96
IL-2	-0.55	0.11	0.19	0.61
IL-4	0.16	0.66	0.54	0.11
IL-5	-0.13	0.73	0.36	0.31
IL-10	-0.006	1	0.36	0.31
IL-12p70	-0.19	0.61	-0.09	0.81
IL13	0.52	0.13	0.55	0.10
IFN-γ	0.66	0.04*	-0.12	0.76

2.8 Statistical analysis

Statistical analysis methods varied for different outcomes and are described in detail in relevant sections of the result chapters.

In principle, the following methods were used:

Chapter 3: Standard descriptive statistics. Non-parametric tests were used as data was not normally distributed to compare independent observations of different populations for unadjusted analyses. Mixed-effect regression model were used for the data analysis.

Chapter 4: Spearman rank coefficient have been calculated in order to establish correlations between the growth factors level and time of collection.

Chapter 5: Descriptive statistics, cross tables, correlation and statistical tests were performed. "Best" statistical model using GL Multi and LASSO for each health outcome was used.

Chapter 6: Principal component analysis of environmental and maternal data; colostrum immune profile and infant health outcomes have been performed.

All results have been adjusted to Parity, Maternal Atopy, Maternal age, Site (Country) of collection, Mode of delivery labour versus no labour, Mould presence at home, Pets at home or regular contact; Exposure to tobacco smoke ie smoker or living in household with smoker or self-reported passive smoker, at recruitment; At least 1 self-reported maternal infections during pregnancy; Maternal diet - fish intake at least once per week

versus less often; daily fresh fruit versus less often; daily probiotic versus none/less often.

3 Determinants of growth factor levels and cytokines detectability in colostrum and breast milk of mothers from Moscow, London and Verona

3.1 Abstract

Background: Cytokines and growth factors in colostrum and breast milk (BM) play an important role in infant immunity maturation and potential beneficial effects include protection against infections and atopy/allergy development. Breast milk composition varies between populations, but the environmental and maternal factors influencing this are still not clear. I examined the relationship between maternal and environmental factors and levels of immune modulators in colostrum and breast milk of women from 3 separate international cohorts.

Objectives: To examine whether environmental and/or maternal factors influence levels of immune active components in colostrum and breast milk.

Methods: A prospective cohort study of mother/infant pairs in London (N=105), Moscow (N=200) and Verona (N=80). Participants underwent allergy testing, and questionnaire interview. Colostrum samples (days 0-6) and Mature Breast Milk (4-6 weeks) were analysed in duplicate at Imperial College London using electrochemiluminescence (Meso Scale Discovery®) for level of TGF-β 1,2,3; HGF, IL2, IL4, IL5, IL10, IL12, IL13, IFN-γ. Analyses used mixed models adjusting for study site, mode of delivery, parity, maternal age, maternal diet, maternal atopy, smoking exposure, contact with pets, mould exposure and time of sampling to identify determinants of human milk composition. All P values were adjusted for multiple comparisons using mixed models.

Results: There was an inverse correlation between time after birth and all growth factors levels in colostrum: HGF ($p<0.001$); TGF-β1 ($p=0.01$); TGF- β3 ($p<0.001$). HGF was lower in the colostrum/milk of Verona mothers (Beta=-0.63, $p<0.001$), and TGFβ2 and 3 were higher in London mothers (Beta from 0.33 to 0.74, $p<0.001$). TGFβ1 was higher in London for colostrum only (Beta=0.27, $p=0.01$). Primiparous mothers had a trend to higher HGF ($p=0.05$), TGF-β3 ($p=0.06$) in colostrum only. Fish consumption at least once a week was associated with lower concentrations of TGFβ1 ($p=0.04$). Ability to detect IL2, IL5, IL10, IFN-γ and IL13 in colostrum negatively correlated with the time of colostrum collection (p from 0.04 to <0.001), with a similar trend for IL4 and IL12 ($p=0.22$ and $p=0.12$ respectively). IL5 was less detectable in mothers having infections during pregnancy ($p=0.04$)

Conclusions: Growth factor levels in colostrum drop rapidly after birth so assessment must be corrected for time after birth in future studies. There were similar trends with cytokines in colostrum, and detection diminishes over time. Results of this study, combined with existing knowledge provide additional data to highlight a degree of difference between colostrum and mature milk. It seems that colostrum is almost a "different substance" but larger cohorts needed to address this question in a future research. Cytokines in colostrum and BM are detected in a very low concentration, close to the lower level of detection for most of the samples, which questions their biological importance at this early stage of infant development. There were significant differences between breast milk immune profiles of mothers living in different countries but these cannot be explained by variations in mode of delivery, parity, maternal age, maternal diet, maternal atopy, smoking pet or mould exposure between sites. Further work is needed to understand the reasons for variations between sites in human milk composition.

3.2 Introduction

There have been few attempts to investigate relationships between maternal and environmental factors and immune active profiles of BM. Data from a variety of studies suggest that factors, such as country of maternal origin, diet, exercise, exposure to smoke or farming environment in early life may influence colostrum and BM constituents (Hoppu et al., 2012, Urwin et al., 2012, Amoudruz et al., 2009, Holmlund et al., 2010, Peroni et al., 2010, Tomicic et al., 2010, Striker et al., 2004, Bottcher et al., 2000, Laiho et al., 2003, Prokesova et al., 2006, Rigotti et al., 2006, Marek et al., 2009, Sidor et al., 2008, Snijders et al., 2006, Bottcher et al., 2008, Boyle et al., 2011, Prescott et al., 2008, Rautava et al., 2002, Kuitunen et al., 2012, Zanardo et al., 2005). There is still no certainty which particular factors influence specific immune active molecules and how they may affect infant development long-term.

Inverse relationships between the risk of atopic diseases, associated with T-helper lymphocyte type 2 (TH2) immune response, and indicators of early-life exposure to infections, such as high birth order or sibship size, early attendance at day care, and early exposure to pets or other animals are well described (Strachan, 2000). The “hygiene hypothesis ” remains one of the most popular current hypotheses on early-life exposures and allergy risk (Strachan, 1989). It has been suggested that farming environment (Peroni et al., 2010), higher bacterial exposure (Tomicic et al., 2010) or maternal country of origin (Holmlund et al., 2010) may have a significant impact on BM immune composition , and such effects on BM composition may be an important pathway through which early variations in microbial exposures influence risk of allergy development.

This study aimed to prospectively investigate the relationship between maternal and environmental factors and levels of HGF, TGFβ 1,2,3 and detectability of TH1 and TH2 cytokines using colostrum and BM samples collected from birth cohorts in three countries, representing UK, continental Europe and eastern Europe respectively.

3.3 Methods

3.3.1 Statistical analysis

Maternal factors and characteristics of cytokines or grown factors were summarised using standard descriptive statistics. As response variables were not normal distributed, non-parametric tests such as paired Mann–Whitney U test, was used to compare independent observations of different populations for unadjusted analyses. Univariate analysis have been performed prior to multivariate approach. Data from univariate analysis is not presented in this thesis as these data was a preliminary step for the multivariate analysis and does not provide any definitive and accurate information when not adjusted for a number of potential confounders.

Since concentration of grown factors and cytokines were assayed twice for a number of specimens, mixed-effect regression model have been used to account for the absence of independence between those two measures and to manage a broad range of variable types. Model selection was a key part of the analysis, this aimed to choose a parsimonious model from a large set of candidate statistical models, therefore compared model selection methods based on major approaches such as Akaike information criterion (AIC) and Bayesian information criterion (BIC). These criteria represent a measure of the relative quality of statistical models for a given set of data. (Burnham and DR, 2004).

Difference between group effects (maternal and environmental factors) as well as within subject effects were evaluated using the following multilevel mixed-effect regression model:

$$\log(y_{ik}) = \beta_0 + \mu_{0i} + \beta_1(\mathrm{Time})_{ik} + \beta_2 X_{2ik} + \ \ldots + \ \beta_p X_{pik} + e_{ik}$$

where $\log(y_{ik})$ is the log of concentration level of each cytokine investigated for the i^{th} subject on the k^{th} time of measurement; β_0 is the overall intercept; β_1 is the regression coefficient for time; $\beta_2 \ldots \beta_p$ are the regression coefficients for the covariates, these are fixed effects and included both maternal and environmental variables; μ_{0ik} is the random intercept at the subject level, and e_{ik} is the residual error.

To optimize power, the analysis evaluated the relationship between concentrations of grown factors and both environmental and maternal exposures by fitting the models based on prior knowledge. All tests were two-sided, and $P<0.05$ were considered significant.

For the purpose of this analysis, data on cytokines are presented as detectable or undetectable. Upon statistical analysis it became evident that this approach provides much clearer results and facilitates good quality statistical analysis.

Factors analysed and used as a covariates in mixed models were: Parity, Maternal Atopy, Maternal age, Site (Country) of collection, Mode of delivery - labour versus no labour, Mould presence at home, Pets at home or regular contact; Exposure to tobacco smoke ie smoker or living in household with smoker or self-reported passive smoker, at recruitment; At least 1 self-reported maternal infection during pregnancy; Maternal diet - fish intake at least once per week versus less often; daily fresh fruit versus less often; daily probiotic versus none/less often.

3.4 Results

3.4.1 Population characteristics

Mothers from London had the highest rates of allergic sensitisation in comparison to Verona and Moscow ($p<0.01$), with more than a third having a positive SPT result to one or more allergen. Median age of women from Verona was higher than in both Moscow and London. Rate of caesarean mode of delivery was highest in London with more than 30% of women from UK having c/section. Mothers from Moscow were exposed to smoke during pregnancy significantly ($p<0.01$) more often than women from London and Verona. Mean colostrum collection time was earlier in Moscow in comparison to London and Verona. Detailed demographic statistic on our cohort is presented in *Table 4*.

3.4.2 Influence of time on colostrum and breast milk immune composition

Among the environmental and maternal factors analysed, the time of colostrum collection postpartum had the most significant influence on growth factors (HGF, TGFβ1 and TGFβ3) level and cytokine (IL2, IL5, IL10, IFNγ) detectability with no significant influence but similar trends for TGFβ2, IL4, IL12 and IL13 (

Table 10). Levels of HGF, TGFβ1, 2 and 3 were significantly (p<0.001 for every growth factor) higher in colostrum in comparison to BM. IL10 was more commonly detected in colostrum than in BM (p<0.001) with an opposite trend for IL4 (p=0.04) and IFNγ (p<0.001) and no significant difference for other cytokines (

Table 10).

3.4.3 Other influences on colostrum and breast milk immune composition

There was a significant influence of country of residence on growth factors concentration with HGF to be lowest in the colostrum/milk of Verona mothers (Beta=-0.63, p<0.001), whilst TGFβ2 and 3 were higher in London mothers (Beta from 0.33 to 0.74, p<0.001) and TGFβ1 was higher in London for colostrum only (Beta=0.27, p=0.01) (*Table 9*).

Significantly higher levels of HGF (p=0.05) were present in primiparous mothers compared with multiparous, with a similar trend for TGFβ3 (p=0.07).

Mothers who reported fish consumption less than once a week had significantly higher levels of TGFβ1 (p=0.03) when compared with women having fish more often.

Women reporting infections during pregnancy had detectable IL5 less often in comparison to mothers reporting no antenatal infections (p=0.04).

Levels of HGF, TGFβ1, 2 and 3 were significantly ($p<0.001$ for every growth factor) higher in colostrum in comparison to BM. IL10 was more detectable in colostrum than in BM ($p<0.001$) with an opposite trend for IL4 ($p=0.04$) and IFNγ ($p<0.001$). Information on changes from colostrum to BM provided in *Table 11*.

All detailed data on mixed models of environmental and maternal factors influencing growth factors level and cytokine detectability are presented in *Appendix D*.

Table 9 Concentration (pg/ml) of Growth Factors in Colostrum and Breast Milk. Exposures significantly influencing Growth Factors level in Colostrum and Breast Milk are presented in the right column.

All analyses were adjusted to the following factors: Parity, Maternal Atopy, Maternal age, Site (Country) of collection, Mode of delivery - labour versus no labour, Mould presence at home, Pets at home or regular contact; Exposure to tobacco smoke ie smoker or living in household with smoker or self-reported passive smoker, at recruitment; At least 1 self-reported maternal infection during pregnancy; Maternal diet - fish intake at least once per week versus less often; daily fresh fruit versus less often; daily probiotic versus none/less often.

	Median (IQR) pg/ml	Important growth factor level difference between the groups
	Colostrum	
HGF	2055.31 (964.825 - 6239.798)	***UK > Italy*** β=0.599 ; SE=0.152; p<0.001 ***Russia > Italy*** β=0.609; SE=0.141; p<0.001 ***Primiparous > Multiparous*** β=0.174; SE=0.090; p=0.05
TGFβ1	731.534 (505.266 - 1142.029)	***UK > Italy*** β=0.276 ; SE=0.094; p=0.01 ***Fish consumption*** ***< Once a week > ≥ Once a week*** β=0.152; SE=0.072; p=0.034
TGFβ2	42209.88 (23847.86 - 98597.95)	***UK > Russia*** β=0.328; SE=0.119; p=0.016 ***UK> Italy*** β=0.393; SE=0.128; p=0.006
TGFβ3	1535.081 (847.555 - 3395.457)	***UK > Russia*** β=0.342; SE=0.118; p=0.010 ***UK > Italy*** β=0.741; SE=0.130; p<0.001 ***Russia > Italy*** β=0.398; SE=0.134; p=0.008

Breast Milk		
HGF	784.041 (508.175 - 1189.644)	***Primiparous > Multiparous*** β=0.174; SE=0.090; p=0.05
TGFβ1	493.514 (375.595 - 653.208)	***Russia > UK*** β=0.396; SE=0.126; p=0.005 ***Russia > Italy*** β=0.495; SE=0.128; p<0.001
TGFβ2	14040.62 (10080.95 - 27262.09)	***UK > Russia*** β=0.328; SE=0.119; p=0.016 ***UK> Italy*** β=0.393; SE=0.128; p=0.006
TGFβ3	279.41 (183.132 - 395.768)	***UK > Russia*** β=0.342; SE=0.118; p=0.010 ***UK > Italy*** β=0.741; SE=0.130; p<0.001 ***Russia > Italy*** β=0.398; SE=0.134; p=0.008

Table 10 Detectability of Th1 and Th2 Cytokines in Colostrum and Breast Milk and exposures influencing cytokines detectability.

All analyses were adjusted to the following factors: Parity, Maternal Atopy, Maternal age, Site (Country) of collection, Mode of delivery - labour versus no labour, Mould presence at home, Pets at home or regular contact; Exposure to tobacco smoke ie smoker or living in household with smoker or self-reported passive smoker, at recruitment; At least 1 self-reported maternal infection during pregnancy; Maternal diet - fish intake at least once per week versus less often; daily fresh fruit versus less often; daily probiotic versus none/less often.

Immune Modulator	Colostrum	Breast Milk	Factors influencing cytokines detectability
IL2	Detectable - 49/342 (14%)	Detectable - 38/190 (20%)	No significant influence
IL4	Detectable - 35/342 (10%)	Detectable - 30/190 (16%)	No significant influence
IL5	Detectable - 77/342 (23%)	Detectable - 27/190 (14%)	Antenatal infections OR 0.49 (95% CI 0.25 - 0.98)
IL10	Detectable - 225/342 (66%)	Detectable - 69/190 (36%)	No significant influence
IFNγ	Detectable - 66/342 (19%)	Detectable - 92/190 (48%)	No significant influence
IL12	Detectable - 63/342 (18%)	Detectable - 31/190 (16%)	No significant influence
IL13	Detectable - 86/342 (25%)	Detectable - 58/190 (31%)	No significant influence

Table 11 Time of colostrum collection postpartum influence on concentration of growth factors and detectability of cytokines in colostrum and changes from colostrum to breast milk.

All analyses were adjusted to the following factors: Parity, Maternal Atopy, Maternal age, Site (Country) of collection, Mode of delivery - labour versus no labour, Mould presence at home, Pets at home or regular contact; Exposure to tobacco smoke ie smoker or living in household with smoker or self-reported passive smoker, at recruitment; At least 1 self-reported maternal infection during pregnancy; Maternal diet - fish intake at least once per week versus less often; daily fresh fruit versus less often; daily probiotic versus none/less often.

Immune Modulator	Difference between Colostrum and Breast Milk	Time of colostrum collection influence
HGF	Conc. Col > BM β=1.35; p<0.001**	β=-0.01; p<0.001**
TGFβ1	Conc. Col > BM β=0.93; p<0.001**	β=-0.003; p=0.01*
TGFβ2	Conc. Col > BM β=1.12; p<0.001**	β=-0.003; p=0.12
TGFβ3	Conc. Col > BM β=2.03; p<0.001**	β=-0.011; p<0.001**
IL2	Detect. BM > Col β=0.32; p=0.30	β=-0.02; p=0.02*
IL4	Detect. BM > Col β=0.72; p=0.04*	β=-0.01; p=0.22
IL5	Detect. Col > BM β=0.54; p=0.09	β=-0.03; p<0.001**
IL10	Detect. Col > BM β=1.66; p<0.001**	β=-0.02; p=0.001**
IFNγ	Detect. BM > Col β=1.20; p<0.001**	β=-0.012; p=0.04*
IL12	Detect. Col > BM β=0.11; p=0.73	β=-0.01; p=0.12
IL13	Detect. BM > Col β=0.09; p=0.72	β=-0.01; p=0.09

3.5 Discussion

It is evident that breast milk composition may be influenced by a number of maternal and environmental factors. There are many reasons such as small cohort size, limited number of immune modulators assessed, heterogeneity of the population, and difference in methodology which create uncertainty and controversy as to which factors may suppress or stimulate particular cytokine secretion into breast milk.

This study is one of the first which attempts to assess breast milk samples from different countries distinct in population genetics, diet and environmental influences. I have confirmed earlier findings of significant differences in milk composition between sites, but was not able to explain these differences by maternal or environmental factors. Further work is needed to understand differences between sites in human milk composition, and the influence that such variations have on infant health. In particular, it would be important to assess maternal diet in more detail as this has previously been suggested to influence BM composition especially the lipid fraction (Urwin et al., 2012).

It has been previously shown that breast milk immune active constituents differ between mothers living in different environments in two cohorts from neighbour populations, although with significant differences in lifestyle, environment, and allergy prevalence (Tomicic et al., 2010). A recent study by Orivuori and co-authors attempted to address differences in BM composition collected in a few European Union countries (Orivuori et al., 2014). This study has assessed how maternal and environmental factors influenced immune active molecule concentrations in breast milk of mothers from an international cohort recruited in UK, Mediterranean region and Eastern Europe. This has allowed evaluation of country of residence influence, taking into account multiple environmental and maternal factors.

3.5.1 Parity

It is a well established fact that family size and birth order are related to an allergy prevalence (Strachan, 1989). It was originally used to explain what has become known as the hygiene hypothesis. However, it has not been established if birth order influences breast milk composition and thus affects immune system maturation and subsequent risk of allergy development. A number of studies suggest that "in utero" environment changes with each subsequent pregnancy. Karmaus et al. stated that there may be a decreasing risk of having raised cord blood IgE levels with increasing birth order (Karmaus et al., 2001), as Bergmann et al. found earlier that IgE levels decreased with increasing parity (Bergmann et al., 1995).

The risk of scleroderma and systemic sclerosis increases with increasing birth order, which is the reverse of the reported risk associated with allergic and atopic disorders (Cockrill et al., 2010). While this might be assumed to be explained mechanistically by the mutual exclusivity of Th1 and Th2 responses it fails to explain how early childhood exposure to multiple infectious agents and/or other mechanisms changes the risks of autoimmune disease.

Grulich et al. studying risk of non-Hodgkin lymphoma in relation to parity (Grulich et al., 2005) found that first-born children had an approximately fifty percent reduction in non-Hodgkin lymphoma risk compared with fourth or later-born children. Another study done by Becker and co-authors is consistent with earlier observations and showed an inverse relationship between atopic disorders and risk of lymphomas (Becker et al., 2007).

Various explanations of the parity influence on atopic diseases and other immune related conditions development have been proposed. Cullinan suggested that variations in constituents of breast milk (Cullinan, 2006) may be related to birth order or family size and could be due to different exposures to domestic allergens (Atkinson et al., 1999). Rangaraj and Doull assumed that immunological shift from Th1 to Th2 during pregnancy takes place because of the hormonal factors (WHO/UNICEF, 2003) and has less relation to “hygiene”. I assume that breast milk composition may be also partially responsible for this phenomenon assuming that each subsequent pregnancy and birth changes breast milk immune profiles.

Wegienka and co-authors proposed that pregnancy “educates” the immune system in a way that persists into the postpartum period and that these pregnancy-associated immune changes may affect the immune status of women in their subsequent pregnancies. They found that change in percentages of Treg cells may vary depending on the number of previous births (Wegienka et al., 2011). Lagadari and co-authors demonstrated that parity influences the number of placental macrophages, finding much higher levels in multiparous women (Lagadari et al., 2004). Assumed that maternal immunology changes with each pregnancy it is possible that it also influences the immunological composition of breast milk and consequently allergy development in children.

Some studies have demonstrated that parity may have a significant effect on milk composition, with primiparous women having higher concentrations of protein and fat, and multiparous women having a reduction in the content of major components in milk with each subsequent pregnancy (Bachour et al., 2012). The effect was most prominent in the group of women who had third and fourth babies, increasing lipid content by

103% and 72% respectively. Unfortunately, sample size of this study was very small and doesn't allow firm conclusions.

Significantly higher neutrophil – macrophage cells count in colostrum was observed with increase of parity (Islam et al., 2006). In the earlier works some authors suggested that this difference may be explained by specific anti-fetal cell-mediated priming as a result of repeated pregnancy (Narula et al., 1982).

Kim and co-authors reported increased IL-4 levels in maternal serum of primigravida and suggested an inverse relationship between parity and the degree of Th2 polarization. This may explain, in part, allergic prevalence with different birth order independent from the hygiene hypothesis (Kim et al., 2008),unless the presence of young children with frequent infections in the house-hold of pregnant women moderates their Th2 activity. Groer et al. previously found that IL-10 showed big differences in relation to parity, with the highest levels found in primiparas and was undetectable in the breast milk of mothers with parity higher than two (Groer and Shelton, 2009). Primiparous mothers with less previous live births had higher concentration of IL7 in their BM when compared with multiparous women (Walter 2007).

Amoudruz et al. found that a larger number of previous pregnancies was associated with significantly lower levels of sCD14 and IL-8 (Amoudruz 2009) with parity having a trend towards being a weak negative predictor for sCD14 and TGF-b2 (Urwin 2012). My results show similar patterns for HGF and TGF-β3, with significantly higher levels of HGF with a similar trend seen for TGF-β3 in colostrum of primiparous mothers in comparison to multiparous.

Data from other studies suggests that parity does not influence BM composition significantly (Urwin et al., 2012, Ismail et al., 2013). In agreement to these results I failed to highlight any significant difference in detectability of any cytokine in colostrum and breast milk of primiparous and multiparous women.

My data (*Figure 3*) in conjunction with results of other studies (*Table 12*) suggests that there is a trend in higher levels of certain markers in primiparous mothers which may be an additional mechanism to explain decreased allergy risk with an increase of birth order. Pregnancy is a significant challenge to the maternal immune response as tolerance to the fetus is crucial for a successful outcome. Current hypothesis suggests that Th1 to Th2 shift (Saito et al., 1999, Saito and Sakai, 2003) provides in utero defense for the fetus and possibly the first pregnancy primes the maternal immune response such that it is more adaptable for subsequent pregnancies.

It is still poorly understood what happens with maternal immunity with an increased number of pregnancies. No data on how Th1/Th2 ratio varies between multiparous and primiparous mothers during pregnancy are available (Luo et al., 2007). More work is needed to assess parity/gravidity influence on maternal immunity during ante and postnatal periods.

Figure 3 Parity influence on HGF concentration (pg/ml) in colostrum and breast milk, mean (CI) is shown in the figure. Multiparous mothers have lower levels of HGF in their colostrum (p=0.05) but not breast milk.

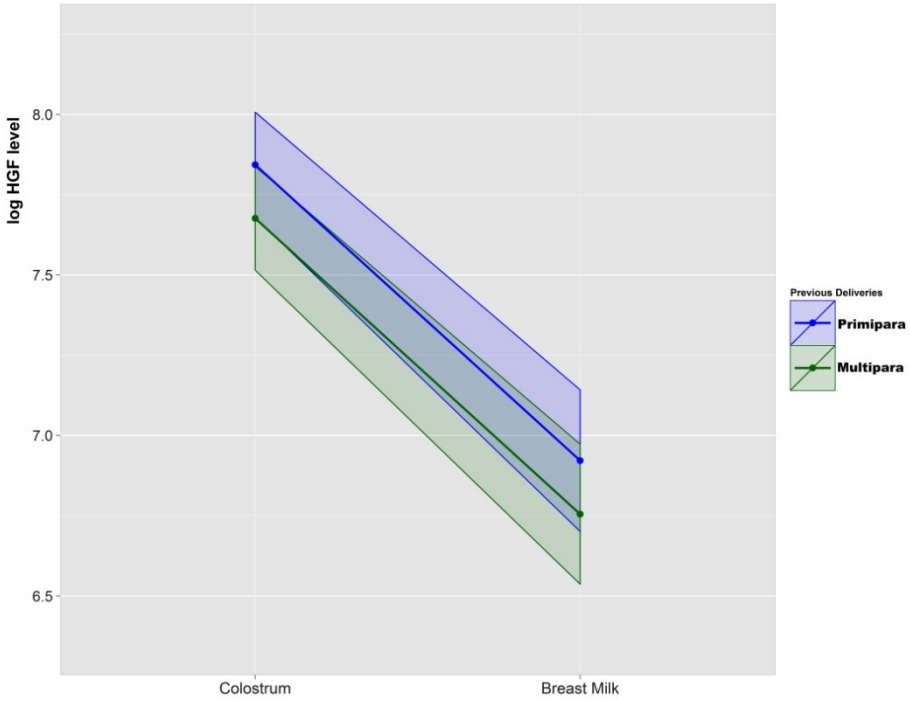


Table 12 Outcomes of the studies which accounted for parity influence on Breast Milk composition

Study	Outcomes
Walter 2007	**↑IL7 Primiparous**
Groer 2009	no significant influence on IL1a, IL1b, IL2, IL4, IL5, IL6, IL7, IL8, IL12, IL13, IL15, IL17, Ip10, TNFα, IFNγ, Eotaxin, MCP1, MIP1a, GMCSF **↑IL10 Primiparous**
Amoudruz 2009	no significant influence on IL-1b, IL-6, IL-10, IL-12p70,TNF **↑sCD14 and IL-8 in Primiparous**
Urwin 2012	no significant influence on sCD14, TGF-β1, TGF-β2, sIgA and fatty acids
Ismail 2013	no significant influence on TGF-β1, sCD14 and total IgA
Our data	no significant influence on concentration of TGFβ 1 and 2, detectability of IL2, IL4, IL5, IL10, IL12, IL13, IFN-γ **↑HGF and a trend for ↑TGFβ3 in Primiparous**

3.5.2 Maternal Atopy

Existing data suggests that breastfeeding has a certain protective effect against allergy with the most prominent effect seen on asthma development (Gdalevich et al., 2001b, Dogaru et al., 2014), in both allergic and non-allergic mothers, but it is still unclear which immunological factors within breast milk are responsible for this. At present we do not possess strong evidence on allergic maternal status influence on qualitative and quantitative immunological constituents in human breast milk. Does maternal atopy lead to changes within BM composition copying similar patterns seen in serum? Few attempts have been undertaken in order to assess allergy/atopy influence on BM immunological profile. Studies done up to date have produced conflicting results in regards to both immunological profile and PUFA composition.

Women with a history of eczema and respiratory allergy had lower levels of PUFA's as well as lower ratio of long-chain n-3 PUFAs/n-6 PUFAs in their breast milk compared with non-allergic (Johansson et al., 2011). Two other studies found no influence of maternal atopy on PUFA composition (Laiho et al., 2003, Lauritzen et al., 2006).

Snijders et al. found higher sCD14 levels, in atopic mothers (Snijders et al., 2006). Savilahti et al. reported no difference in total IgA concentration, sCD14 and cow's milk-specific IgA levels between allergic and non allergic mothers (Savilahti et al., 2005).

No difference in TGF-β2 (Bottcher et al., 2000, Savilahti et al., 2005, Prokesova et al., 2006, Kondo et al., 2011) and TGF-β1 (Snijders et al., 2006, Marek et al., 2009, Ismail et al., 2013, Orivuori et al., 2014) levels between allergic and non-allergic mothers have been reported in a number of studies done in different parts of the world. Data from a single study suggested allergic mothers have lower levels TGF-β2 in BM compared with non-allergic (Laiho et al., 2003). Rigotti and co-authors reported the same trend for

TGFβ1 levels in mature milk of allergic mothers (Rigotti et al., 2006) while Urwin et al., in contrast, found higher both, TGF-β1 and TGF-β2 concentrations in the milk of allergic women (Urwin et al., 2012). The limitation of both studies was small sample size.

In a study done in Czech Republic allergic mothers had substantially higher IL-10 concentration in 3 months BM and reduced levels of IL10 and IL-13 in 6 months BM comparing to healthy mothers (Prokesova et al., 2006). In contrast other studies revealed no significant difference in the levels of IL-10 in BM (Rudloff et al., 1999, Laiho et al., 2003, Rigotti et al., 2006, Snijders et al., 2006, Marek et al., 2009, Kuitunen et al., 2012). Recent data from the study done by Linnamaa et al. suggests that mothers having atopic dermatitis have significantly reduced levels of IL-10 in their BM compared to healthy women (Linnamaa et al., 2013) adding more uncertainty to the topic.

Bottcher et al., in a small cohort recruited in Sweden (Bottcher et al., 2000), observed higher IL-4 concentrations in colostrum but not in mature milk but no changes for IL-5, IL-6, IFN-γ. IL-4 was detectable more often in the colostrum of allergic mothers in a study done in Poland (Marek et al., 2009).

In a recent study done in Japan levels of 26 cytokines, including IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13 and IFN-γ did not differ between breast milk from allergic and non-allergic mothers (Ochiai 2013).

I did not find any relationship between the levels of growth factors and/or detectability of cytokines and maternal allergy (*Table 13*). Analysis of data from other studies suggest that there is no obvious trend in BM composition of allergic mothers compared to non allergic.

Differences observed in some studies may be explained by analysis simplification and most probably would disappear if adjusted for appropriate confounders.

Table 13 Outcomes of the studies accounted for atopic status influence on Breast Milk composition.

Study	Outcomes
Rudloff 1999	no significant influence on IL4, IL10 and IFN-γ
Bottcher 2000	no significant influence on IL-5, IL-6, IL-10, IL-13, IFN-γ and TGF-β ↑**IL-4 in Allergic mothers in colostrum** but not in mature milk
Laiho 2003	no significant influence on TNF-α , IL-4, IL-10, prostaglandin E2, cysteinyl leukotrienes and fatty acids ↓**TGFβ2 in BM Allergic mothers**
Savilahti 2005	no significant influence on IgA TGF-β1, TGF-β2, sCD14 and cow's milk-specific IgA levels
Ustundag 2005	no significant influence on IL-1β, IL-2, IL-6, TNF-α ↑**IL-8 in BM of Allergic mothers**
Prokesova 2006	no significant influence on IL-4, IL-5, IL-6, IFN-γ, TGF-β **Allergic mothers ↑IL-10 in 3 month BM**
Rigotti 2006	no significant influence on IL-10 ↓**TGFβ1 in BM of Allergic mothers**
Snijders 2006	no significant influence on IL-12, TGF-β1, IL-10 ↑**sCD14 levels in Atopic mothers**
Johansson 2011	↓ **PUFAs in BM of Allergic women**
Kondo 2011	no significant influence on TGFβ2
Urwin 2012	↑ **TGF-β1 and TGF-β2 in early BM**
Kuitunen 2012	no significant influence on IgA, casein, β-lactoglobulin, ovalbumin, IL10 ↓**TGFβ2 in BM ↑ IgA to casein in BM**
Ismail 2013	↑**sCD14 in Allergic mothers**
Linnamaa 2013	↓**IL-10 in BM of mothers with Atopic Dermatitis**
Orivuori 2014	no significant influence on TGFβ1 and sIgA
Our data	We did not reveal any significant influence of Atopic maternal status on growth factors concentration and cytokines detectability

3.5.3 Dietary preferences

With the development of 'hygiene hypothesis' many researchers focused their research on the protective effects of environmental exposures during pregnancy and early life, during a period of time when infant gut colonization and immunity development normally takes place. Breast milk PUFA and immunological composition in relation to maternal dietary preferences has been assessed in a number of observational and intervention studies (Dunstan et al., 2003, Prescott et al., 2008, Hoppu et al., 2012, Urwin et al., 2012, Linnamaa et al., 2013). Accumulated knowledge (*Table 14*) on diet influence on

breast milk composition is still very inconclusive but suggests that certain dietary changes may influence breast milk profile.

In a large cohort study done in Denmark PUFA composition of the breast milk was associated with PUFA intake, energy intake and macronutrient composition (Lauritzen et al., 2006). Fish and shellfish consumption did not influence levels of long-chain PUFA's in breast milk (Johansson et al., 2011).

Prescott et al. found that higher levels of TGF- β1 and IgA levels in week one breast milk of mothers receiving B. lactis HN019 probiotics, and higher IgA levels alone in those receiving L. rhamnosus HN001. In contrast probiotics supplementation did not seem to have an effect on the rest of BM immunological profile (IL-13, IFN-γ, IL-6, TNF-a, IL-10 and sCD14) (Prescott 2008).

Finnish scientists studied the effect of black currant seed oil on breast milk composition and found lower levels of IL-4 and increased IFN-γ in breast milk, with no differences in IL-5, IL-10, IL-12 and TNF levels, in comparison to an olive oil fed group (Linnamaa 2013).

Rist and co-authors showed that levels of rumenic acid and trans-vaccenic acid in breast milk were higher in mothers following a diet containing organic dairy and meat products, in comparison with women on a conventional diet (Rist 2007).

Two studies reported mothers receiving probiotics during pregnancy had no effect on TGFβ levels in breast milk (Boyle et al., 2011, Kondo et al., 2011) with opposite results shown by Rautava and co-authors (Rautava et al., 2002).

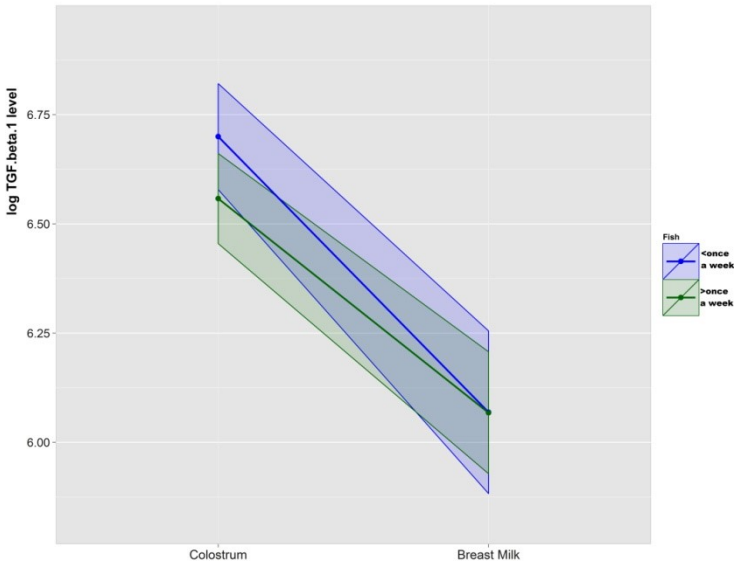
My study did not employ an intervention but it has been possible to analyse the data from the questionnaires on the frequency of fresh fruits, and pro-/prebiotic yoghurts

intake reported by the mother. The only significant difference was that fish intake influenced TGFβ1 levels in colostrum, with higher levels reported in women eating fish less than once a week (*Figure 4*). A limitation of the study is that it was not possible from the questionnaire to distinguish lean from oily fish. It is not clear why less regular fish consumption resulted in lower TGFβ1 levels but existing data is conflicting, with Urwin and co-authors reported TGF-β1 levels to be highest in the colostrum of women residing in the river and lake region of China , well known for high fish consumption and Hawkes et al failed to find any influence of fish consumption on TGFβ1 levels (Hawkes et al., 2001).

Table 14 Outcomes of the studies accounted for dietary influence on Breast Milk composition.

Study	Outcomes
Hawkes 2001	no significant influence of fish consumption on TGF-β1 and TGF-β2
Dunstan 2004	**↑n-3 in BM from women supplemented with fish oil** **↓n-6 in BM from women supplemented with fish oil** no significant influence on IgA, sCD14 levels
Bottcher 2008	**supplementation of *L. reuteri* during pregnancy ↓TGF-β2 in colostrum** **supplementation of *L. reuteri* during pregnancy ↑IL-10 in colostrum** no difference in IgA, SIgA, TGF-b1, TNF, sCD14, and Na/K ratio in BM
Prescott 2008	no significant influence on IL6, IL10, IL13, IFN-γ, TNF-α, sCD14, total IgA **↑TGF-β1 in 7 days BM from *Bifidobacterium lactis HN019* group**
Boyle 2011	no significant influence of *Lactobacillus rhamnosus GG* on TGF-β **↓ *sCD14 and IgA levels in BM***
Hoppu 2011	no significant influence on IFN-γ ↑IL-2, IL4, IL10 TNF-α in probiotic group
Kondo 2011	probiotics use no difference in TGF-β2
Ribeiro 2011	**↓ratio of n-6/n-3 PUFA in BM lipids of fish oil group compared with the control group**
Kuitunen 2012	**↑IL-10 in probiotics group (*Lactobacillus rhamnosus, Bifidobacterium breve, Propionibacterium freudenreichii*) in BM** **↓casein IgA antibodies in BM**
Urwin 2012 b	**salmon group ↑ EPA (80%), docosapentaenoic acid (30%), andDHA(90%) in day 5 BM compared with controls** **↓n-6 PUFA : n-3 PUFA ratio and sIgA in salmon group** no significant influence on TGF-b1, TGF-b2 and sCD14
Ismail 2013	no significant influence of *Lactobacillus rhamnosus GG* consumption during pregnancy on TGF-β1, sCD14 and total IgA
Linnamaa 2013	no significant influence on IL-5, IL-10, IL-12 and TNF levels **↓IL-4 in blackcurrant seed oil group** **↑IFN-γ in blackcurrant seed oil group**
Orivuori 2014	raw milk consumption no influence on TGF-β1 and sIgA
Our data	**↓TGFβ1 in colostrum of mothers eating fish more than once a week** no significant influence on concentration of HGF, TGF-β 2 and 3 and detectability of IL2, IL4, IL5, IL10, IL12, IL13, IFN-γ

Figure 4 Fish consumption influence on TGFβ1 concentration (pg/ml) in colostrum and breast milk, mean (CI) is shown in the figure. It can be seen that levels of TGFβ1 is significantly higher (p=0.03) in colostrum of mothers having fish less than once a week. There is no significance associated with the levels of TGFβ1 in breast milk.



3.5.4 Changes in the levels of immune modulators in breast milk over time and between colostrum and Breast Milk

It is well established that colostrum is particularly rich in immunologically active molecules and that levels of immunological markers usually drop when compared to mature BM (Takahata et al., 2001, Ustundag et al., 2005, Rigotti et al., 2006, Peroni et al., 2010). This may be explained by a higher requirement of the developing infant in immunologically active molecules and growth factors in particular, as they may stimulate rapid gut immunity maturation. I also observed a predictable, very significant, drop in the concentration of all growth factors analysed from colostrum to breast milk, depending on the geographical location (*Figure 5*).

Apart from well known changes of BM immune composition from colostrum to mature milk there was also a rapid decline in growth factors levels and detectability of cytokines in colostrum with each subsequent hour. This fact could well explain the diversity of results between studies because few authors define the timing of colostrum collection.

Soto-Ramirez et al. found none of the immune markers to correlate with the time of milk collection in a study done in USA (Soto-Ramirez et al., 2012). In contrast, I found a significant decline in HGF, TGFβ1, TGFβ3, concentration and detectability of IL2, IL5, IL10, IFNγ over time with a similar pattern for the rest of immune modulators (*Table 11*).

The steady decline of immunological markers over time has not been consistently demonstrated (*Table 15*). Striker et al. reported negative correlation between IgA levels and time of colostrum collection (Striker et al., 2004). In a study done in China river/lake, coastal and inland regions levels of sCD14, TGF-b1, TGF-b2, sIgA decreased over time (Urwin 2012). Lauritzen and co-authors detected significantly higher concentrations of n-6 and lower of n-3 PUFA's in breast milk of atopic mothers in their study (Lauritzen et al., 2006), however upon adjustment to time of collection and dietary preferences this difference lost its significance. This highlights importance of multivariate analysis in breast milk research as adjustment to crucial maternal or environmental factors may change the final result. Ignorance of important confounding factors may lead to inappropriate interpretation of the data.

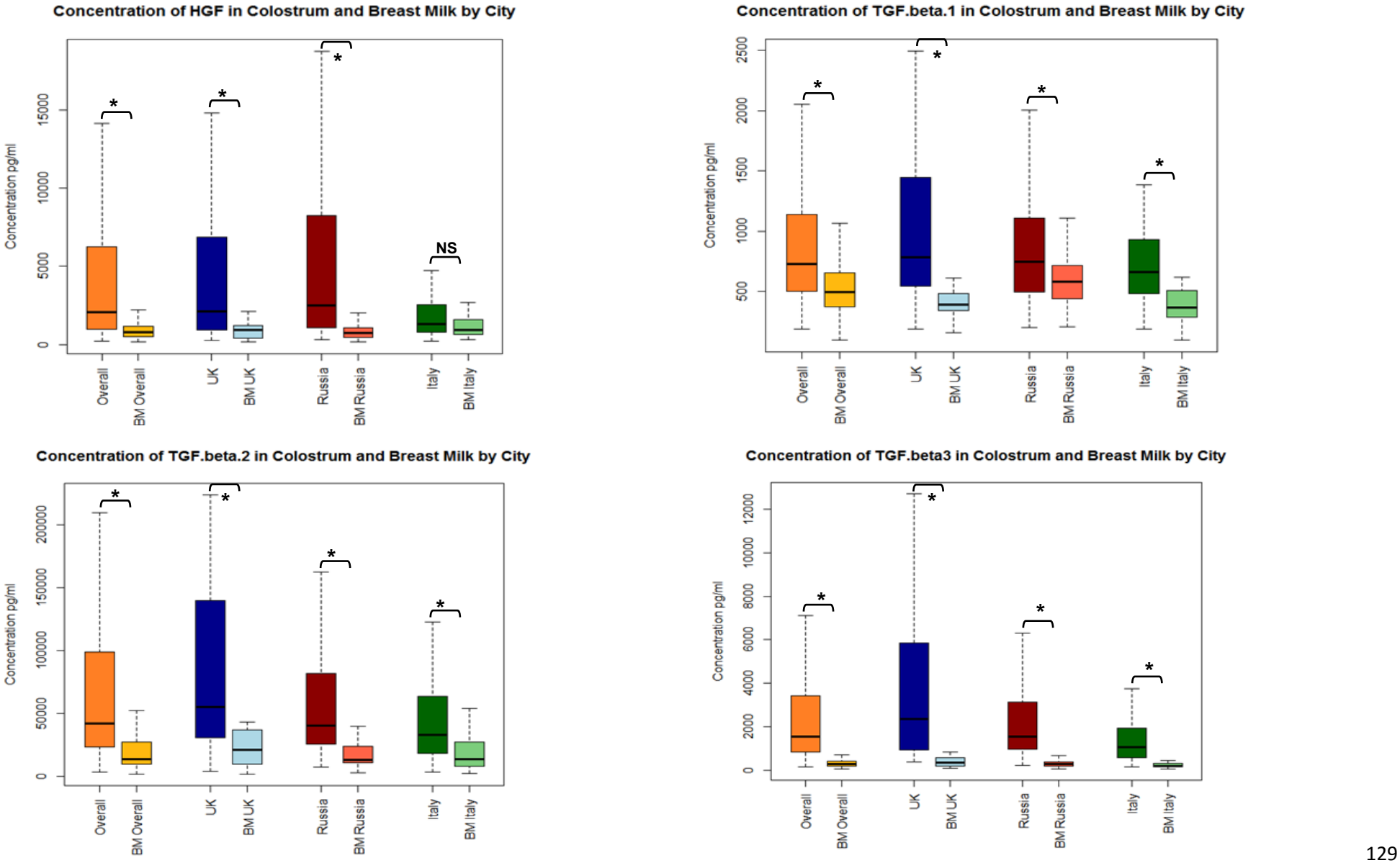
In addition assessing the concentration of immune modulators in milk does not provide an assessment of the absolute amount reaching the infants intestinal mucosa. As the volume of feed increases it is possible that absolute quantity remains the same despite a lower concentration. Whether or not this makes any difference to the biological effects of modulators is unknown.

Considering the importance of collection time I conducted an additional analysis attempting to correct for the stage of lactation. Protein and sodium were used as a correction factors. Results of this study are presented in the following chapter.

Table 15 Outcomes of the studies taking into account changes in breast milk composition over time in their analysis.

Study	Outcomes
Takahata 2001	**↑levels of IL-18 in colostrum than early milk, mature milk and maternal serum**
Ustundag 2005	**↑IL-2 and TNF-α levels were higher in colostrum, but reduced in later stages of lactation** no changes for IL-1, IL-6 and IL-8
Lauritzen 2006	**Time of collection influences PUFA profile of breast milk**
Rigotti 2006	**↑TGF-β1 in colostrum than in mature milk in samples from allergic mothers**
Kobata 2007	**↓VEGF, HGF, and EGF over time**
Peroni 2010	**↑TGF-b1 concentrations in colostrum than in mature milk In farming mothers** no significant changes in TGFβ1 and IL10 concentration from colostrum to BM
Urwin 2012 b	**↓sCD14, TGF-b1, TGF-b2, sIgA over time**
Soto-Ramirez 2012	IL-1β, IL-4, IL-5, IL-6, CXCL8, IL-10, IL-12(p70), IL-13, CXCL10, CCL11, and IFN-γ did not correlate with the time of milk collection
Our data	**↓ HGF, TGFβ1, TGFβ3, concentration and detectability of IL2, IL5, IL10, IFNγ over time** no significant influence on TGFβ2concentration and IL4, IL12, IL13 detectability

Figure 5 Growth factors concentration (pg/ml) in colostrum and breast milk in average, across all sites (overall) and at each site of collection (UK, Russia and Italy)



3.5.5 Site of collection

Recent work by Sozanska et al. highlights the importance of the local environment influence on the allergy development (Sozanska et al., 2014). Their study showed that an increase in atopy prevalence over a very short period of time in a Polish population was associated with a decrease in farming exposures due to a new European Union agricultural rules implementation.

Some researchers suggest that breast milk composition "from women from different geographic, ethnic, and socioeconomic backgrounds is remarkably similar, particularly in reference to the macronutrients. Any differences observed are more likely to be the result of dietary variation, rather than genetic modification of composition" (Darragh, Lonnerdal 2011).

Data from a variety of studies suggests that colostrum and breast milk constituents may be influenced by the country of origin and a number of environmental conditions which may differ significantly from one location to the other (*Table 16*).

Amoudruz and co-authors showed that immigrant women, living their first ten years of life in a developing country but giving birth in Sweden, had statistically significantly higher levels of breast milk interleukin-6 (IL-6), IL-8 and TGF-β1 than native Swedish women (Amoudruz et al., 2009). Later another study done by the same research team highlighted Mali women having higher levels of TGF-β1 in comparison with women born in Sweden and higher levels of sCD14 in comparison to both, immigrants and local Swedish women (Holmlund et al., 2010). In agreement with this Peroni et al. reported higher concentrations of TGF-β1 and IL-10 in breast milk of women living in a farming environment with a higher bacterial exposure in comparison to mothers living in the city (Peroni et al., 2010). Similar trends have been observed by Tomicic and co-authors with

Estonian mothers have lower levels of TGFβ in total and TGFβ2 in particular, but higher levels of SIgA, IL-10, and IFN-γ in their breast milk than Swedish mothers (Tomicic et al., 2010) which the authors explained by the differences in microbial load.

Local dietary habits may also have a strong influence on breast milk composition as it has been demonstrated by Urwin and co-authors reporting higher concentrations of sCD14, sIgA and TGFβ1 in the colostrum from the river and lake region of China compared with the coastal and inland regions (Urwin et al., 2012).

Recent study by Orivuori et al. assessed breast milk samples collected in four countries of continental Europe and Finland and showed TGFβ1 levels to be highest in Finland and sIgA lowest in Germany (Orivuori et al., 2014). The reasons behind these differences are unclear.

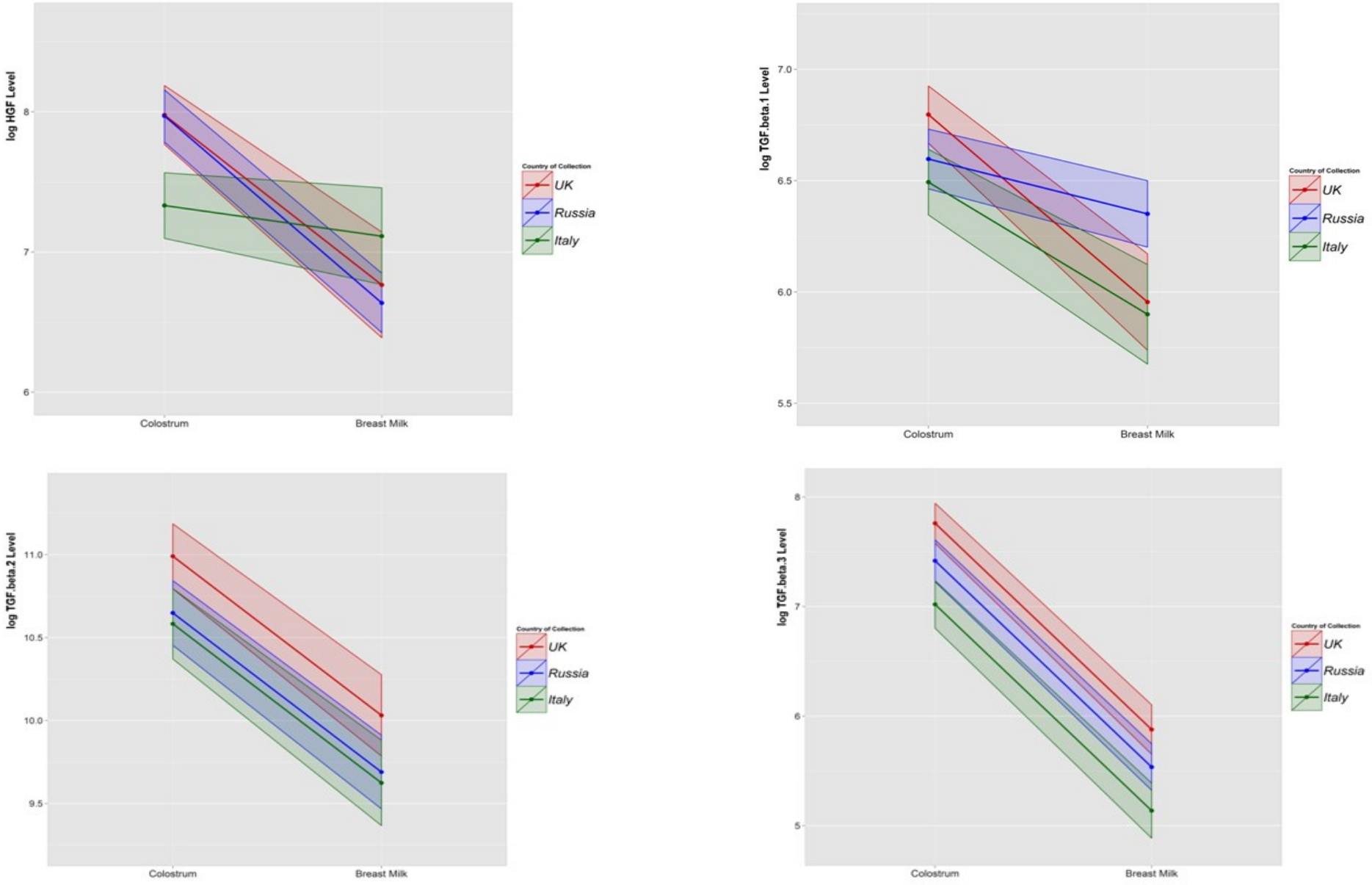
My data suggests that HGF levels were lower in the colostrum/milk of Verona mothers and TGFβ2 and TGFβ 3 were higher in colostrum/milk of London mothers with the same significant difference for colostral TGFβ1 (*Figure 6*). London, Moscow and Verona do not differ much in regards to microbial load. It could be explained by genetic variations between the populations but most the probable explanation of these differences is heterogeneity of London population in comparison to Moscow and especially Verona, where the populations' ethnic origins are much more homogeneous.

It becomes more evident that future breast milk research should be focused on multicentre studies involving different countries to evaluate genetic and epigenetic influence on breast milk composition.

Table 16 Outcomes of the studies assessing differences in breast milk immunological composition between sites of collection.

Study	Outcomes
Amoudruz 2009	no significant influence on IL-1β, IL-10, IL-12p70,TNF and sCD14 **↑ IL-6, IL-8 TGF-β1 of in BM from Immigrants from a developing country**
Holmlund 2010	**↑TGF-b1 in Mali women vs. native Swedish women** **↑sCD14 in Mali women vs. immigrating to Sweden and native women from Sweden**
Peroni 2010	**↑TGF-b1 in colostrum and BM of farming mothers and ↑IL10 in BM of farming mothers compared with BM of women from urban population**
Tomicic 2010	**↑ TGF-β (especially TGF-β2) in Swedish mothers compared to Estonian mothers** **↑ sIgA, IL-10, and IFN-γ in BM of Estonian mothers compared to Swedish mothers** no significant influence on IL-4 and IL-13
Urwin 2012	**↑sCD14, sIgA and TGF-b1 in colostrum from the river and lake region compared with the coastal and inland regions of China**
Orivuori 2014	**↑TGF-β1 levels in breast milk of mothers from Finland (compared to Austria, France, Germany and Switzerland)** **↓sIgA levels in breast milk of mothers from Germany (compared to Austria, France, Finland and Switzerland)**
Our data	**↓HGF levels in colostrum/milk of Verona mothers (compared to Moscow and London)** **↑TGFβ2 and TGFβ3 in colostrum/milk of London mothers (compared to Moscow and Verona)** **↑ TGFβ1 in colostrum of London mothers (compared to Moscow and Verona)**

Figure 6 Site of collection influence on Growth factors concentration (pg/ml) in colostrum and breast milk, mean (CI) for each country is shown in the figure. Significant differences can be seen in both, colostrum and breast milk. β and p values for this figure can be found in Table 9.



3.5.6 Other factors

I found that maternal infections during pregnancy reduced the likelihood to detect IL5 in colostrum. While the underlying cause remains unclear, this suggests that maternal health will also influence breast milk composition.

Few studies assessed smoking influence on breast milk profiles. Two studies showed conflicting results of smoking influence on IL1α levels in colostrum and breast milk (Zanardo et al., 2005, Szlagatys-Sidorkiewicz et al., 2013). Smoking supplementation use did not influence TGF-β2 levels in the study done in Japan (Kondo et al., 2011). In the international multicentre study done in continental Europe and Finland, Orivuori and co-authors reported higher levels of TGFβ1 in the smokers BM. In contrast to this data I did not find any statistically significant differences in growth factors concentration and cytokine detectability (*Table 17*).

Breast milk of teenage girls has a significantly lower concentration of lactose and some macro-minerals compared to adults. Authors suggest that diet may play an important part in this difference in that teenagers diets are often suboptimal when compared to adults (Darragh, Lonnerdal 2011). In a single study maternal age was positively correlated with eosinophil count in breast milk (Islam 2006). From my data there was no influence of age on growth factors concentration and cytokines detectability.

Table 17 Outcomes of the studies which included potential influence of smoking exposure on breast milk immunological composition.

Study	Outcomes
Agostoni 2003	no significant influence on colostrum Total Lipid content **↓absolute amounts of linoleic, arachidonic, a-linolenic and docosahexaenoic acid in 1 month BM of smokers** **↓docosahexaenoic acid in 3 month BM in smokers**
Zanardo 2005	**↓IL-1α in the colostrum of smokers**
Kondo 2011	no significant influence on TGF-b2
Bachour 2012	no significant influence on sIgA levels **↓ lipids and proteins in BM of smokers**
Szlagatys-Sidorkiewicz 2013	no significant influence on IL-1β, IL-6, IL-8, IL-10, TNF-α **↑IL-1α in BM of smokers**
Orivuori 2014	**↑TGFβ1 in BM of smokers**
Our data	We did not reveal any significant influence of smoking on growth factors concentration and cytokines detectability

3.6 Conclusion

Colostrum and mature human milk composition research is important to identify the reasons for conflicting results from observational studies of the protective effects of breast feeding on health outcomes in the infants. Growth factor levels in colostrum drop hourly after birth and the decline continues from colostrum to mature milk. Thus it is important to correct for time after birth in any further research. A similar trend occurs with other immune active molecules, as they are less detectable in colostrum over time after birth. Cytokines in colostrum and BM are detected in tiny concentrations, within the border line for the lower level of detection for most of the samples, making their detection reliable solely on the sensitivity of the plate. Extremely high levels of growth factors and border line detectable cytokines questions biological importance of the latter at the early stage of infants' development. My results accord with limited existing data suggesting that country of residence may have a significant influence on breast milk immune profile, highlighting the importance of defining populations in multicentre international prospective studies. However, there are no specific maternal or environmental exposures which significantly and consistently influence BM composition.

Future studies should explore alternative environmental and maternal factors, particularly maternal genotype, microbiome and diet – in order to better understand the underlying factors which determine BM composition. Further work is also needed to optimize the collection and analysis of BM components, especially the correction of colostrum component levels for stage of lactation. Some preliminary experiments on correction of colostrum levels for stage of lactation presented in Chapter 4.

4 Protein and Sodium as a potential correction factors for immune active molecules over time changes in colostrum

4.1 Abstract

Background: Colostrum is an important source of nutrients and immunologically active components which facilitate normal development in early infancy. Recent data adds some evidence showing a significant decline in levels of cytokines and growth factors over time postpartum. Even a few hours difference may lead to substantial changes in breast milk immune composition. This may explain discrepancies in both, breast milk constituents as well as health outcomes, seen in many studies published from around the globe. In an attempt to normalize data between studies by the application of a correction factor in relation to the post-partum timing of sample collection, I analysed levels of total protein and sodium in colostrum from the international cohort.

Objectives: To assess a potential approach to correct for the stage of lactation using total sodium and protein levels in colostrum.

Methods: Colostrum samples (day 0-6) from 298 mothers (n=160 Moscow, n=74 Verona, n=64 London) were analysed by Abbott Architect Analyzer using turbidimetric procedure for protein and ion selective electrodes for sodium levels. I used Spearman's correlation to assess the relationship between the concentration of growth factors (HGF, TGFβ1,TGFβ2 ,TGFβ3) and time of colostrum collection postpartum. The same method was applied to the levels of growth factors, when corrected for total protein and sodium.

Results: Growth factors levels declined over time after birth (HGF r= -0.39, p<0.001; TGFβ1 r=-0.21, p<0.001; TGFβ2 r=-0.16, p=0.01; TGFβ3 r=-0.35, p<0.001). Total protein and sodium levels also declined over time (protein r= -0.42, p< 0.001; sodium r=

-0.38, p<0.001 respectively). When growth factor levels were corrected for total protein or sodium levels, significant relationships with time (either positive or negative) persisted HGF (r=-0.39, p<0.001 protein; r=-0.19, p=0.003 sodium), TGFβ1 (r=0.21, p<0.001protein; r=0.25, p<0.001 sodium),TGFβ2 (r=0.16, p=0.01 protein; r=0.20, p=0.004 sodium) and TGFβ3 (r=-0.35, p<0.001 protein; r=-0.06, p=0.34 sodium).

Conclusions: Growth factor levels markedly decline in concentration in human colostrum over time after birth, and so do total protein and sodium levels. However correction of growth factor levels for total protein or sodium levels did not account for the effect of time, suggesting that these are not useful correction factors for maturation of colostrum in comparisons between human populations. The kinetics of decline of each growth factor differed significantly, suggesting that more complex approaches are needed to account for maturation of colostrum when comparing human populations. Differences in correlation of absolute and relative concentrations of a particular growth factor suggest that they have different biological importance at the first days of life. Although all growth factors decline over time, proportion of TGFβ1 and 2 within the protein content of colostrum increases over time. This tendency needs to be studied further.

4.2 Introduction

Human breast milk contains a large variety of immune active molecules and we know that it provides the developing infant with a range of bioactive factors influencing immune system maturation, physical and cognitive development and the infant intestinal microbiome (Borsutzky et al., 2004). A large number of cytokines, growth factors, signaling molecules and other factors playing their roles in immunity, are found in colostrum and BM (D'Alessandro et al., 2010).

Many authors reported decline in cytokine concentration in colostrum over time and levels of immunological markers to be higher in colostrum when compared to mature BM (Takahata et al., 2001, Ustundag et al., 2005, Rigotti et al., 2006, Peroni et al., 2010, Striker et al., 2004, Lauritzen et al., 2006, Urwin et al., 2012, Prescott et al., 2008). It has been assumed that the higher levels will stimulate rapid gut immune maturation in the critical few days after birth. I also observed this significant, drop in the concentration of all growth factors analysed over time. One US study failed to find an association between time and immune markers (Soto-Ramirez et al., 2012),which may be partially explained by a wide range in collection times (week one to eight).

The aim of this study was to determine if protein and/or sodium concentrations could be used as a correction tool for stage of lactation, to facilitate comparison of human early breast milk (colostrum) composition between populations.

4.3 Materials and Methods

4.3.1 Study population

Study population has been described in detailed in the main Methods section of this thesis. Colostrum samples (day 0-6) from 298 mothers (n=160 Moscow, n=74 Verona, n=64 London) were analysed by Abbott Architect Analyzer using turbidimetric procedure for protein and ion selective electrodes for sodium levels.

4.3.2 Protein and Sodium analysis

Abbott Architect Analyser (*Pic. 1*) have been used to determine total protein and sodium levels in colostrum. The laboratory analysis of protein and sodium was carried by fourth year medical students Ms. Shreya Sheth (Sheth, 2014) and Ms. Priya Abrol (Abrol, 2014) for their BSc project, under my supervision.

Picture 1 Abbott Architect c8000 Clinical Chemistry Analyser

Abbott Architect Analyser has been assessed and evaluated to match the analytical performance of a highly precise Beckman Array nephelometer technique and has been shown to improve laboratory efficiency. Standard urine protein assay was used for colostrum protein analysis.

Colostrum proteins were denatured by benzethonium chloride, forming a fine suspension, which is then measured turbidimetrically at 404nm.

During the validation step, 24 colostrum samples were randomly selected from the breast milk bank. Samples were centrifuged at 1500g for 15 minutes at 4°C. The fat layer was trimmed off and supernatant transferred in the tubes and loaded into the analyser.

As colostrum is highly lipaemic, mass spectrometry was conducted to examine suitability of the assay. Supernatant was manually diluted at 1:10 in normal saline prior to analysis. The Architect device was programmed to generate on board 1:2 dilutions for samples where protein levels were above the detection range. 4 of the 24 samples required a dilution factor of 40 for protein levels to be within the detectable range (*appendix E*).

To assess the nature of the dilutions and achieve an ideal protein detection range, serial dilutions were carried out on two of the samples, ranging from 1:2 to a 1:32 dilution using saline solution. This established that an on board dilution factor of 30 would allow an appropriate protein detection range (*appendix F*).

Sodium levels were measured at the same time in these 24 samples, using ion-selective electrodes in the Abbott Architect Analyser®. A voltage is generated between the reference and measuring electrodes according to the Nernst equation. This voltage is then compared to previously determined voltages and converted into sodium concentrations in mmol/L.

4.3.3 Protein analysis

Following a successful validation step, all colostrum samples (n=298) were prepared for analysis. All samples were thawed and centrifuged at 3000g for 15 minutes at 4°C. 300µl of supernatant were carefully transferred to the tubes and loaded into the Architect device for the analysis.

An on board 1:30 dilution was programmed into the Architect to avoid the risk of errors with manual dilutions, allowing a protein detection range of 3 –60g/L. For this dilution, the analyser picked up 5µl of sample and dispensed it into a cuvette with 145µl of saline, analysing a sample from that dilution. Therefore the residual samples were left unaffected by the dilution, and could undergo further analysis whilst in the system.

Remaining colostrum from the 24 samples analysed in the pilot study were analysed again for protein levels, allowing assessment of method reproducibility.

The protein values measured for all colostrum samples in g/L were converted into pg/mL, aligned with growth factors concentration.

4.3.4 Sodium analysis

Sodium levels were detectable in all colostrum samples using the same laboratory system, and sample dilution was not required. The lower limit of detection was 20mmol/L,and samples with sodium levels below this were assigned a value of 10mmol/L for data analysis.

4.3.5 Statistical analysis

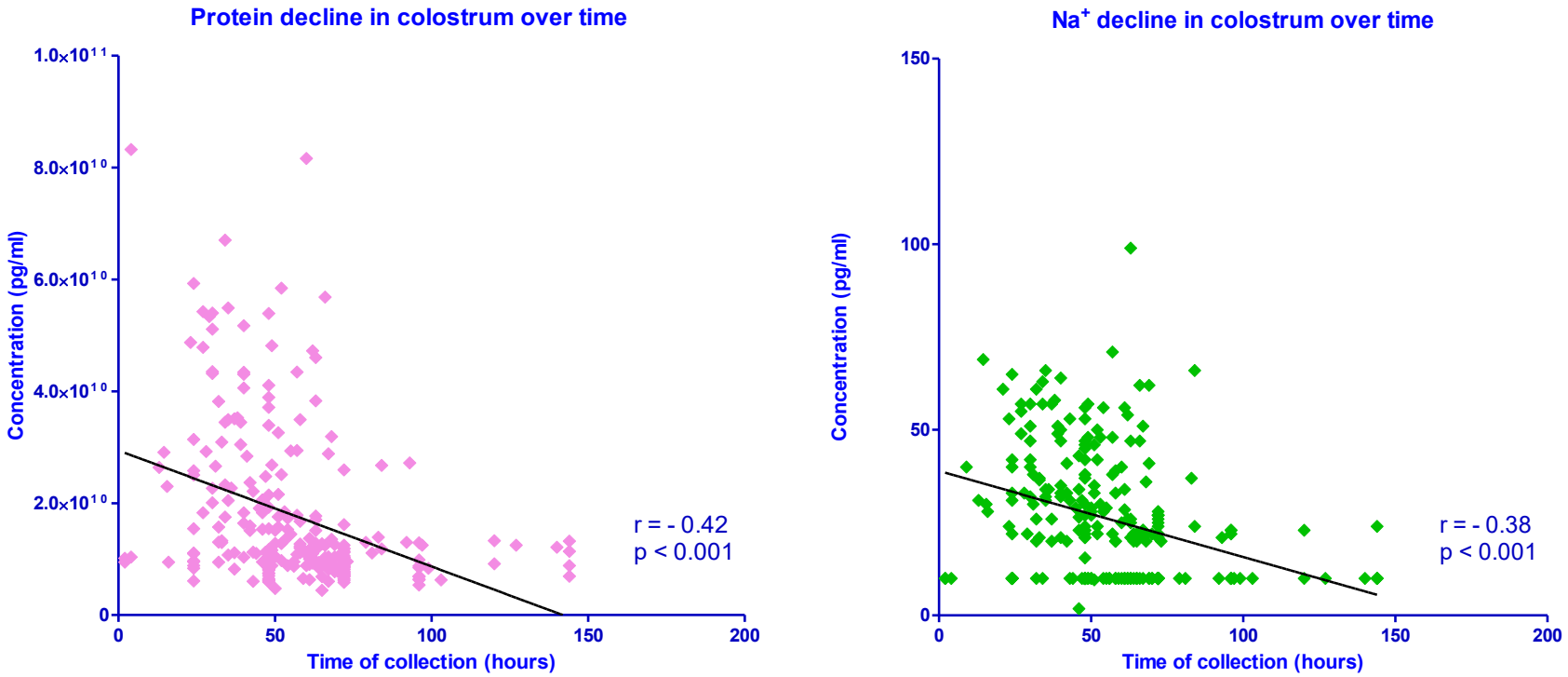
For the purpose of this chapter I provide a graphical representation of HGF, TGFβ1,2 and 3 and HGF/Protein, TGFβ1/Prorein, TGFβ2/Protein and TGFβ3/Protein decline, using non-linear and linear regressions. Since different models are used for the assessment of absolute and relative values of the growth factors analysed, it is impossible to compare them. Linear regression models showed better strength and that is the reason for them to be the model of choice for this Chapter analysis. Spearman rank correlation coefficient has been calculated for all correlations assessed. Results were considered significant when p-values were reported at a level less than 0.05. Correlations between absolute or relative concentrations of growth factors with time of collection allow assessment of the patterns of decline for both. Statistical analysis was carried out using SPSS Software Version 22.0 and GraphPad Prism Version 6.0.

4.4 Results

4.4.1 Protein and Sodium in colostrum

Levels of protein and sodium in human colostrum are highly correlated ($r=0.72$, $p<0.001$) and their concentrations decline over time in a very similar fashion ($r=-0.42$ and -0.38 respectively, with $p<0.001$ for both) (*Figure 7*). Sodium levels were undetectable in 107 samples out of 298, which meant that protein was more suitable for correction purposes. Correction for sodium is presented in *Appendix G.*

Figure 7 Protein and Sodium concentration in colostrum decline over time.



4.4.2 Growth factors in colostrum changes over time

All growth factor levels demonstrated a significant decline over time (*Figure 12*). The declines of HGF and TGFβ 3 are almost linear; TGFβ1 and 2 are non-linear. Upon correction the pattern became very linear, as it can be seen from the *Figures 8 - 11*.

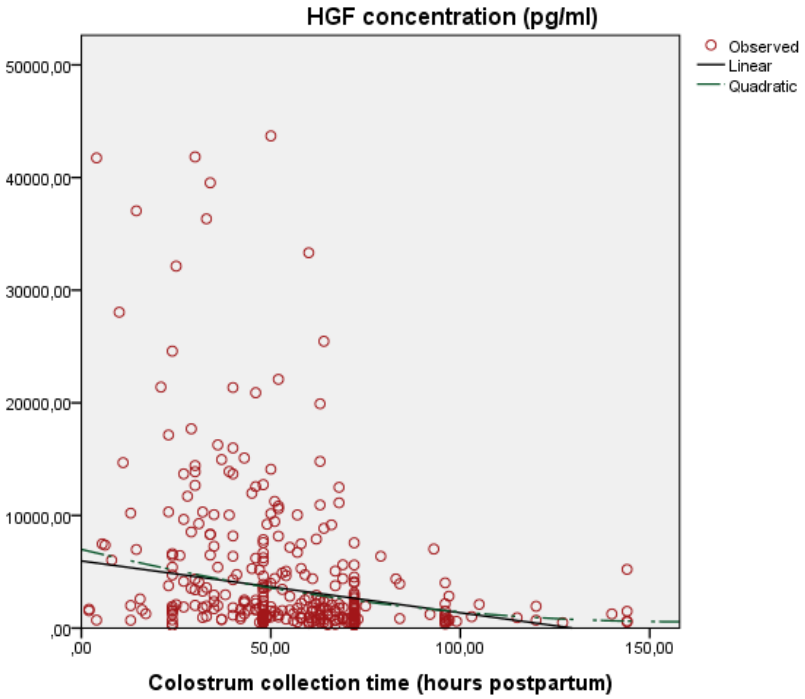
I assessed correlation between the levels of growth factors and time of colostrum collection. Same approach has been used for growth factors/protein ratio. As it can be seen from the table below correction for protein allows us to see the pattern of changes of absolute and relative growth factors concentration over time.

Absolute concentration/ Time of collection	Relative concentration (Protein)/ Time of collection
HGF concentration/ Time of collection r=-0.39, p<0.001 ↓	HGF/Protein ratio/ Time of collection r=-0.19, p=0.003 ↓
TGFβ1 concentration/ Time of collection r=-0.21, p<0.001 ↓	TGFβ1/Protein ratio / Time of collection r=0.25, p<0.001 ↑
TGFβ2 concentration/ Time of collection r=-0.16, p=0.01 ↓	TGFβ2/Protein ratio / Time of collection r=0.20, p=0.004 ↑
TGFβ3 concentration/ Time of collection r=-0.35, p<0.001 ↓	TGFβ3/Protein ratio / Time of collection r=-0.06, p=0.34 ↓

↑ - Positive correlation with time of collection

↓ - Negative correlation with time of collection

Figure 8 HGF absolute concentration (pg/ml) and relative (as a HGF/Protein ratio) concentration changes in colostrum over time



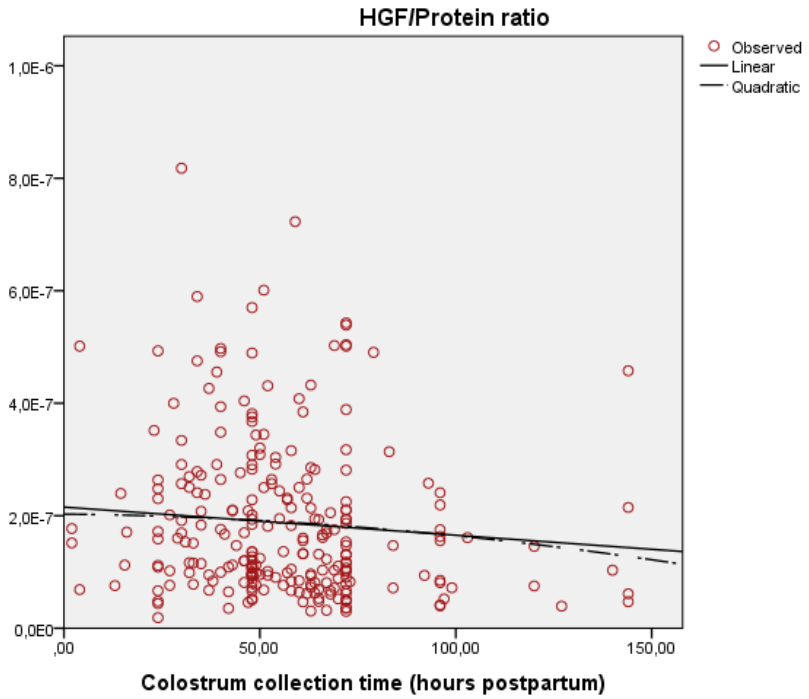


Figure 9 TGFβ1 absolute concentration (pg/ml) and relative (as a TGFβ1/Protein ratio) changes in colostrum over time

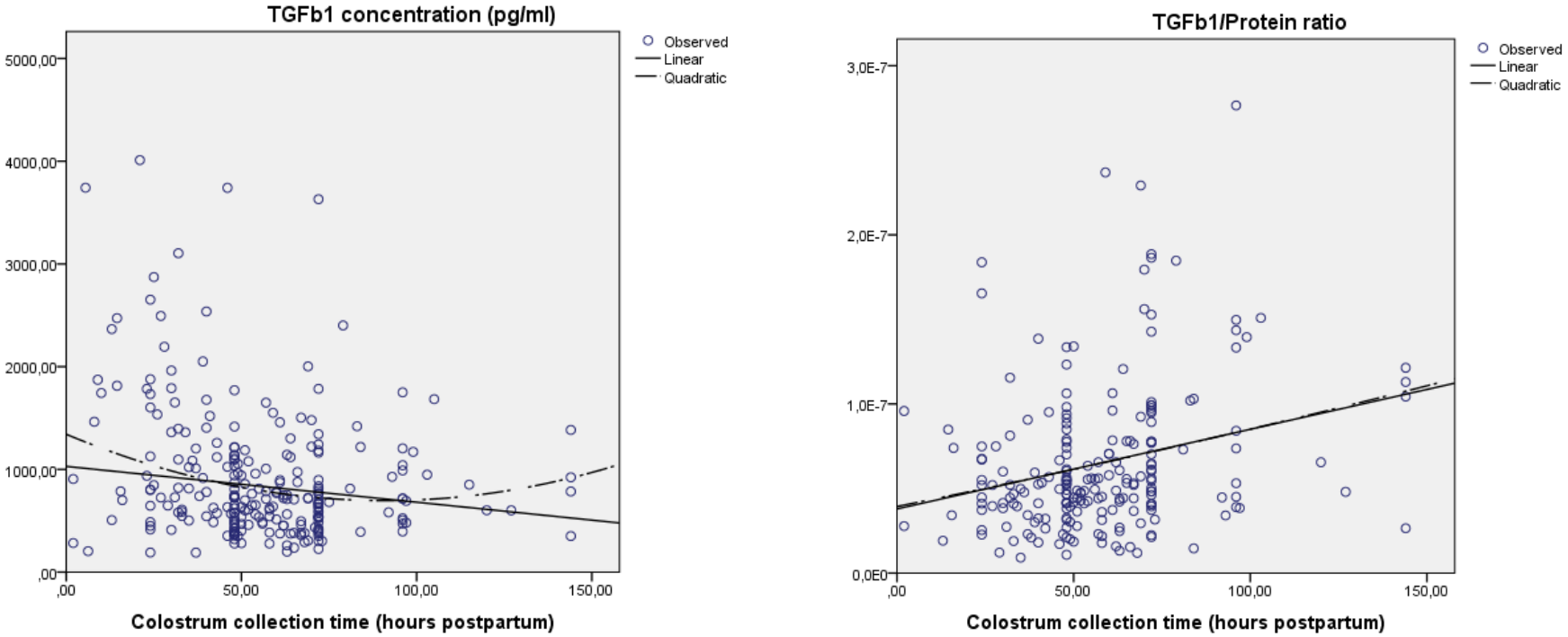


Figure 10 TGFβ2 absolute concentration (pg/ml) and relative (as a TGFβ2/Protein ratio) changes in colostrum over time

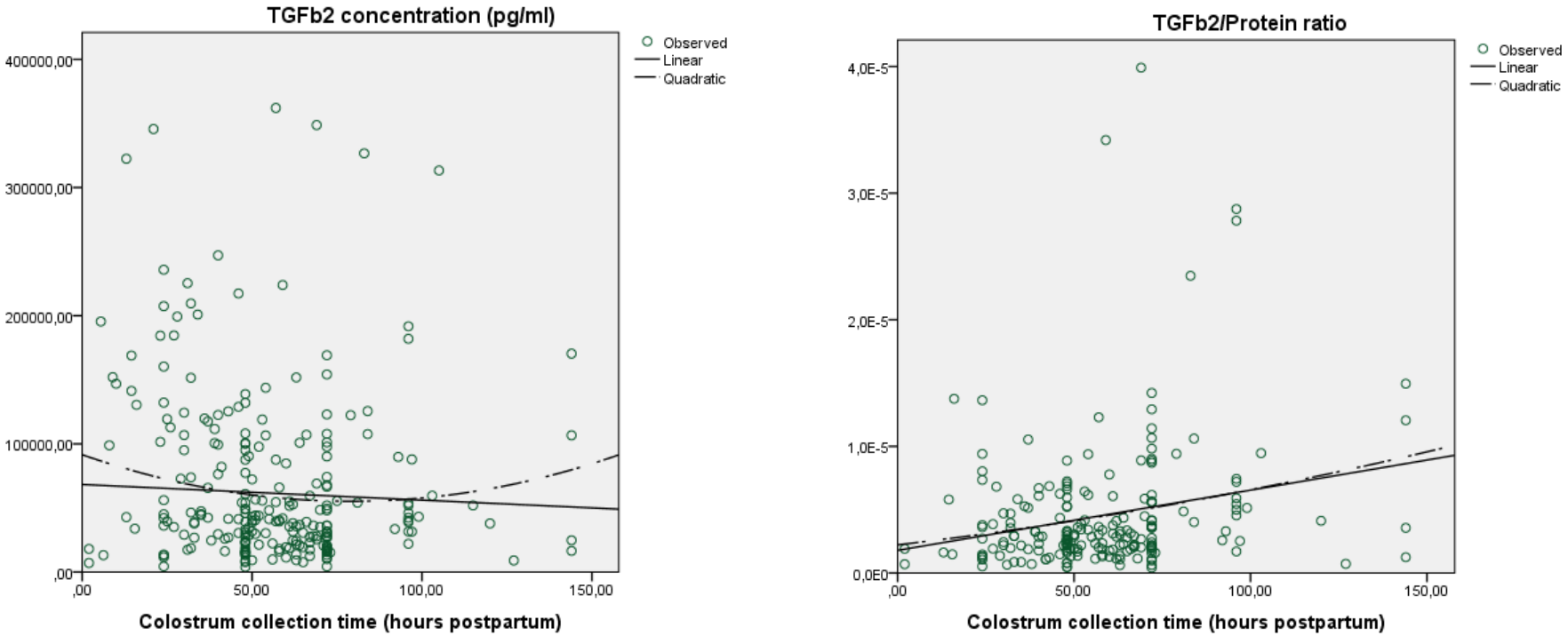


Figure 11 TGF-β3 absolute concentration (pg/ml) and relative (as a TGFβ3/Protein ratio) changes in colostrum over time

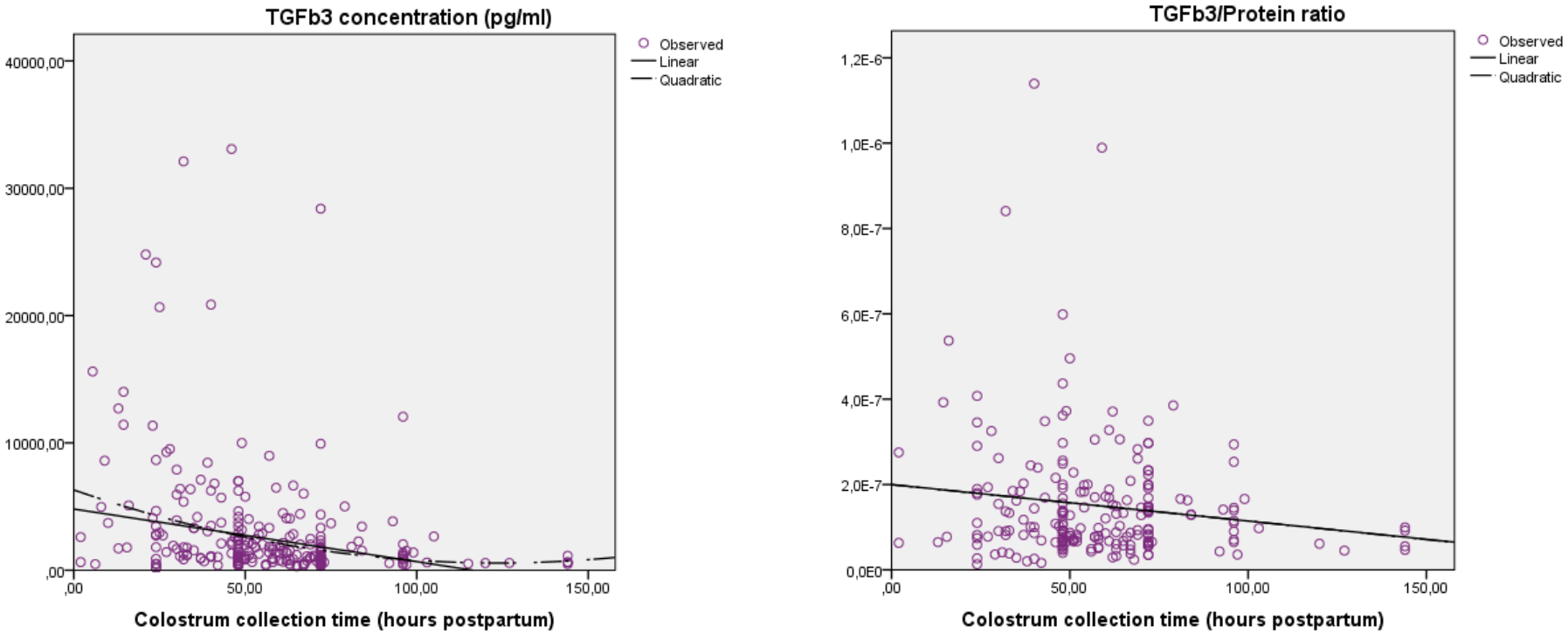
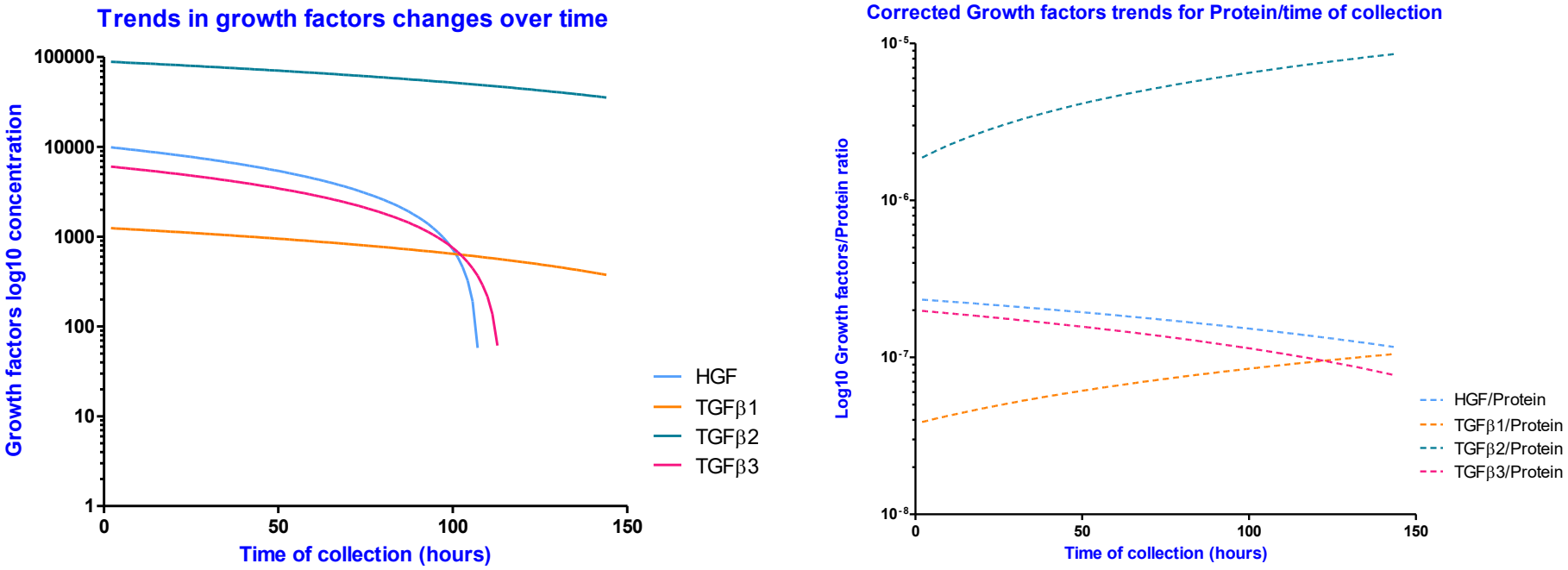


Figure 12 Trends in growth factors change over time



4.5 Discussion

It is well established that levels of total protein as well as Na^+ in human breast milk decrease over time, with the opposite trends seen for lactose and K^+ with the most prominent changes seen from colostrum to transitional milk (Kulski and Hartmann, 1981). The natural decline may be due to a simple dilution as the infants volume requirements increase. It would be possible if this was the case to correct for maturation of milk. We found that the kinetics of decline in different constituents in early human milk differ markedly, even between closely related growth factors, such that a unifying correction factor cannot be found. This means that much of the decline in growth factor levels is independent of dilution effects in colostrum, and that more sophisticated methods are needed to account for time changes in colostrum.

4.5.1 Protein in human breast milk

Protein content of breast milk has a multifunction role, providing amino acids essential for growth and development, immunoglobulins representing passive immunity transmission (Hurley and Theil, 2011), oligosaccharides having pre-biotic properties involved in protection against infections, and a variety of immunologically active molecules possessing different immunological functions (D'Alessandro et al., 2010).

On average, mature breast milk contains 0.9 - 1.2 g/dL of protein, 3.2 - 3.6 g/dL of fat and 6.7 - 7.8 g/dL of lactose (Ballard and Morrow, 2013). Breast milk protein levels undergo a steady decline over the first 4 to 6 weeks (Bauer and Gerss, 2011) and existing data suggest that composition differs between preterm and term milk, with higher protein and fat content in preterm milk (Ballard and Morrow, 2013).

Existing evidence suggests that breast milk nutritional composition does not differ in mothers from different geographic, ethnic, and socioeconomic backgrounds and any variations are more likely due to dietary preferences rather than genetic differences (Darragh and Lönnerdal, 2011).

There is some evidence that maternal weight may have an influence on protein and lipid concentration in breast milk, and Nommsen et al. reported decreasing levels associated with post pregnancy weight loss (Nommsen et al., 1991) or in those women, producing higher breast milk volume. Authors did not reveal any influence of maternal diet on protein content.

Immunologically active part of breast milk protein composition is presented by cytokines, signalling molecules and growth factors (D'Alessandro et al., 2010). These factors play an important role during early infant immunity development but we still do not possess enough data to draw proper conclusions regarding importance of each particular immune active molecule during a particular phase of infant development.

Immune active constituents of colostrum and breast milk represent a minor component but the most biologically active part of the breast milk total protein. Most studies concentrate on a particular part of the human breast milk composition, completely ignoring other important constituents. Most studies assessing protein in breast milk are interested in nutritional composition, ignoring the immunologically active components and vice versa.

4.5.2 Growth factors concentration changes over time

As it has been discussed in Chapter 2, existing knowledge suggests that there is a steady decline in the levels of immunologically active factors in colostrum over time. There are a variety of the potential explanations behind this phenomenon. The neonates' immune immaturity may be compensated by high concentrations of modulators in colostrum (Ehlers and Smith, 1991). This is illustrated by the observed direct correlation between the levels of TGFβ in human milk and concentration of IgA in the infants serum (Ogawa et al., 2004). As the infants' immune response matures there is likely to be less need for an extrinsic supply of immune stimulants.

Another potential explanation is dilution. During the first week of life the infant's volume requirements are low. Later levels of the immune active molecules decrease as the infants volume and nutritional requirements increase.

In this study I attempted to address growth factors decline over time, in order to facilitate merging of data. The uncorrected results showed visible and likely biologically significant decline of HGF, TGFβ 1, 2 and 3 levels over time. Correction for colostrum total protein resulted in a significant differences in the results in that the decline in the levels of TGFβ 1 and 2 disappeared and transformed into a steady rise when evaluated as a ratio with total protein levels *(Figures 9 and 10)*. This implies more active transport of these growth factors. If we assume biological relevance, then these results suggest that infants need relatively higher amounts of TGFβ1 and 2 for longer than that of other immune mediators. TGFβ3/Protein ratio on the other hand, had a different pattern with decreasing ratio over time. This difference may be explained by TGFβ3 being significantly different from TGFβ1 and 2 in its detailed tertiary structure of the active domain despite homology in amino acid sequence (Laverty et al., 2009). There is some evidence (Laverty et al., 2009) that TGFβ3 isoform may be distinct in its functions to

other TGFβ isoforms. It is known that TGFβ3 is up-regulated by milk stasis, and not by circulating concentration, and induces apoptosis in mammary gland epithelium during involution, in contrast to TGFβ1 and 2 (Nguyen and Pollard, 2000), which may explain variations in TGFβ isoforms decline over time. Another classic example of TGFβ isoform differences have been observed in cutaneous scarring experiments done on animal models (Ferguson and O'Kane, 2004), mammalian embryos showed healing with no scarring and full skin recovery (Whitby and Ferguson, 1991b) with expression of high levels of TGFβ3 seen and contrasting, low levels of TGFβ1 and 2 (Whitby and Ferguson, 1991a) and similar TGFβ3 function have been shown in human scarring physiology (Ferguson et al., 2009).

4.5.3 Limitations of the study

The main limitation is that this was a cross-sectional study with single specimens from each subject. The only way to elaborate on the dynamics of transport of each constituent over time is to collect breast milk longitudinally from the same mother.

There are currently no markers to utilize which will correct for time of colostrum collection. As this one of the most important factors influencing the levels of immune markers it is required to normalize data from diverse studies. v. As there are no "gold standard" methods of colostrum maturity I attempted to provide a correction using total protein and sodium levels in BM. The limitation of the study is that no subgroup analysis has been performed because of limited numbers. There is a chance that time of collection postpartum would have a more prominent effect in breast milk of the multiparous mothers who delivered by lower segment c/section. Furthermore the analysis covered only a small number of growth factors.

4.6 Conclusion

Despite the lack of a correction factor for the time of collection influence on the levels of immune active molecules concentration, correction for total protein level shows some potential. It gives a different perspective on the absolute and relative declines of immune active molecules over a period of time, highlighting the potential importance of a certain immune modulators during the very early period of infant development. More work on a larger sample size, preferably collected longitudinally, is needed in order to address existing pitfalls. The results suggest that breast milk research should be very diverse, multi-functional and aim to cover as many breast milk constituents as possible.

5 Growth factor levels and cytokines detectability in colostrum and breast milk of mothers from Moscow, London and Verona and infant health outcomes at six months and one year of age

5.1 Abstract

Background: The role of breastfeeding in improving outcomes in early childhood is still unclear. Cytokines and growth factors in colostrum and breast milk (BM) could play an important role in infant immune maturation and potential beneficial effects include protection against GI and respiratory infections and atopy/allergy development. Environmental and maternal factors may influence the levels of immune active molecules in breast milk but there is uncertainty over which factors are responsible. I examined the relationship between maternal and environmental factors and levels of immune modulators in colostrum and breast milk of women from a multi-national cohort and assessed outcomes in their children at 6 and 12 months of age.

Objectives: To identify associations between environmental and/or maternal factors and levels of immune mediators in colostrum and breast milk and the influence on outcomes at 6 and 12 months of age.

Methods: A prospective cohort study of pregnant mother/infant pairs in London (N=105), Moscow (N=200) and Verona (N=80). Participants underwent allergy testing, and questionnaire interview. Colostrum samples (days 0-6) and Mature Milk (4-6 weeks) were analysed in duplicate at Imperial College London using electrochemiluminescence (Meso Scale Discovery®) for level of TGF-β 1,2,3; HGF, IL2, IL4, IL5, IL10, IL12, IL13, IFN-γ. Statistical analyses used mixed models adjusting for the site of collection, parity and maternal atopic status. Fever, cough or wheeze, food allergy/sensitivity/intolerance, recurrent eczematous rash presence, were assessed at 6 months. Symptoms of upper respiratory tract infection symptom frequency, reflux and/or vomiting were assessed

both at 6 and 12 months and infant atopy and stool consistency using Bristol chart at 12 months of age. The "best" statistical model for each outcome has been used for health outcomes analysis. All models included adjustment for site of collection.

Results: Higher TGFβ2 concentration in BM was associated with more eczematous rash reported at the age of 6 months OR 1.04 (95% CI 1.01 - 1.06), with detectable IL13 in BM showing protective effect OR 0.18 (95% CI 0.04 - 0.92). Boys had less eczematous rash at the age of 6 months compared with girls OR 0.2 (95% CI 0.05 - 0.84).

Food allergy, sensitivity, intolerance events reported by the mother were negatively associated with detectability of IL13 in colostrum OR 0.1 (95% CI 0.01 - 0.83).

No significant association of any factor analysed with cough or wheeze development at the age of 12 months was demonstrated.

Mothers living in Moscow reported lower fever incidence at the age of 12 months OR 0.2 (95% CI 0.06 - 0.74) and runny nose or cold by the age of 12 months OR 0.02 (95% CI 0.01 - 0.23). HGF in breast milk showed some protective effect in regards to runny nose or cold incidence at first year of life OR 0.19 (95% CI 0.04 - 0.92). Runny nose or cold events have been reported more often in boys OR 4.27 (95% CI 1.08 - 16.90).

Conclusions: Data from this study suggests that differences in individual immune composition of breast milk may have an influence on early life infant health outcomes. The data shows that increased TGFβ2 levels in breast milk are associated with a higher incidence of reported recurrent eczematous rash, with detectable IL13 in colostrum showing protective effects. Detectable IL13 in breast milk is associated with less food allergy, sensitivity, intolerance events reported by the mother. HGF is associated with some protective effect on cold and runny nose incidence at one year of age. Although

several different potential relationships were explored in this project, the associations reported were significant despite adjustment for multiple comparisons. However the data are observational in nature, so that it is not possible to make definitive conclusions that the associations are causal in nature, due to the possibility of hidden confounding. Finally we did not assess gene-environment relationships, which are an area of interest for future work. Future studies should be focused on maternal genotype, breast milk microbiome and diet as influences on breast milk immune composition and both, short and long term health outcomes in the infant. More studies on babies at high risk of allergy development are needed to highlight the most important immune active molecules playing a role in allergy prevention.

5.2 Introduction

Breast milk (BM) is known to contain a large variety of immune active components (D'Alessandro et al., 2010) which are present in differing concentrations (Agarwal et al., 2011). Despite many published studies little is known about the human milk immune composition's influence on short and long term health outcomes.

Which factors influence short and long term health outcomes in infants is still a matter of discussion, despite a number of studies aimed to address this question (*Table 18*). Most of the authors focused on allergic sensitisation, eczema, early wheezing and/or asthma and allergic rhinitis development as the main phenotypic outcomes. Breast milk immune active molecules, which potentially could influence immunological outcomes in infancy and early childhood included not only cytokines, growth factors, soluble receptors and but also polyunsaturated fatty acids and other nutritional factors.

The aim was of this component was to assess the impact of maternal and environmental factors in relation to colostrum and BM immune active components composition and health outcomes at the age of six and twelve months.

Table 18 Studies done up to date, assessing Breast milk immune composition and immunological outcomes in children.

Study	Immunological Outcomes	Relationship between breast milk composition and outcomes
Kalliomaki 1999	Eczema (up to 12 months)	**↑TGF-β1 and TGF-β2 in colostrum in infants with postweaning-onset atopic disease**
Jones 2002	Eczema (up to 6 months)	**↓sCD14 levels in 3 month BM lead to a higher eczema incidence at 6 months of age**
Bottcher 2003	Allergic sensitisation (up to 2 years) Salivary IgA (up to 2 years) Eczema (up to 2 years)	No influence of IL4, IL5, IL6, IL8, IL10, IL13, IL16, IFN-γ, TGF-b1, -b2, RANTES, eotaxin or SIgA on atopy and/or allergy
Oddy 2003	Asthma-like symptoms (up to 12 months)	TNF-α, sCD14 and IL10 had no significant association with infant wheeze **↑TGF-β1 lower odds of wheeze in infancy**
Ogawa 2004	Serum IgA levels assessment	**↑ IgA in newborns serum during 1 month of life correlated significantly with levels of TGF-β1 and TGF-β2 in colostrum**
Savilahti 2005	Allergic sensitisation (up to 4 years) Eczema (up to 4 years)	**↓IgA casein antibodies and sCD14 in colostrum of mothers of babies who developed atopy**
Snijders 2006	Eczema (up to 12 months) Allergic sensitisation (up to 2 years) Wheezing (up to 2 years)	TGF-β1, IL-10, IL12 and sCD14 had no significant association with any of the atopic manifestations
Bryan 2007	Infant bronchiolitis (up to 12 months)	IL2,IL8,IFNγ had no significant association **↓IL-10 in BM of mothers of infants subsequently developed bronchiolitis**
Zanardo 2007	Neonatal jaundice	**↑IL-1β in colostrum from breast-feeding mothers whose infants had Neonatal Jaundice**
Bottcher 2008	Allergic sensitisation (up to 2 years) Eczema (up to 2 years)	**↓TGF-b2 reduced incidence of sensitisation during the first 2 years of life and a trend of protective effect on eczema development**
Lowe 2008	Eczema (up to 2 years) Asthma (up to 7 years) Allergic rhinitis (up to 7 years)	**↑n-3 fatty acids in colostrum were associated with higher risk of atopic eczema** **total n-3 fatty acids concentration in breast milk was associated with increased risk of non-atopic eczema** **↑total n-6 fatty acids in colostrum were associated with increased risk of childhood rhinitis**
Walter 2009	HIV transmission	**↑IL-15 concentrations were associated with a decreased risk of HIV transmission**

Thijs 2011	Eczema (up to 2 years) Allergic sensitisation (up to 2 years)	**↑n-3 PUFAs as well as ruminant fatty acids were associated with lower risk of parent-reported eczema, eczema, and sensitisation at 12 months of age**
Kuitunen 2012	Allergic diseases (up to 5 years) Eczema (up to 5 years) Allergic sensitisation (up to 2 years)	**↑TGF-β2 concentration in 3 month BM was associated with more allergic disease and eczema at 2 years of age** IL10 had no significant association with allergic manifestations at 2 years of age TGF- β2 and IL-10 were not significantly associated with allergy outcomes at 5 years
Soto-Ramirez 2012	Asthma-like symptoms (up to 12 months)	**↑total n-6 PUFAs in BM were associated with an increased risk of asthma-like symptoms** **↑ total n-3 PUFAs decreased the risk of atopy**
Soto-Ramirez 2012 b	Asthma-like symptoms (up to 12 months)	**Infants in the highest quartile of IL5 and IL-13 in BM were at a higher risk of asthma-like symptoms development**
Ismail 2013	Eczema (up to 12 months) Allergic sensitisation (up to 12 months)	TGF-β1, sCD14, total IgA had no significant association with any of the atopic manifestations
Orivuori 2014	Eczema (up to 4 years) Asthma (up to 6 years) Allergic sensitisation (up to 6 years)	TGF-β1 had no significant association any of investigated health outcomes **sIgA levels were inversely associated with AD up to the age of 2 years** sIgA had no significant association with atopy or asthma up to the age of 6

5.3 Methods

5.3.1 Study population

Demographic data has been described in detail in the Methods section of the Thesis.

5.3.2 Colostrum and breast milk analysis

Laboratory approaches used has been described in detail in the Methods section of the Thesis.

5.3.3 Outcome definitions

Babies health outcomes were assessed at the age of six months by means of a phone questionnaire and at one year of age by completing a questionnaire during the follow-up visit. All questions were carefully explained to the mothers. All health outcomes were self-reported by the mother of the infant except atopic sensitization which was assessed by skin prick test. Fever, cough or wheeze, runny nose or cold, eczematous rash, reflux and vomiting cumulative incidence, food allergy/sensitivity or intolerance incidence have been assessed.

Runny nose or cold was defined as at least one episode of runny nose or cold lasting for a minimum of 3 days.

Cough or wheeze outcome was defined as at least one episode of recurrent cough or wheezing prior to assessment at one year of age. Current atopic eczema symptoms were considered present if the child had ever had an eczematous rash intermittently at any time during the last 6 months.

Eczematous rash incidence was defined as children with at least one episode of an intermittent eczematous rash.

A baby was considered to be atopic if s/he had positive control wheal of ≥3 mm and any of the allergen induced wheals being ≥3 mm greater than negative control.

For exclusive breastfeeding the WHO recommended definition "that the infant receives only breast milk" was used. No other liquids or solids are given – not even water – with the exception of oral rehydration solution, or drops/syrups of vitamins, minerals or medicines" (WHO, 2009a).

5.3.4 Statistical analysis

Statistical analysis was performed using R statistical package version 3.1.0.

Factors included into the models were:

levels of growth factors in colostrum and breast milk, detectability of cytokines in colostrum and breast milk, site of collection, maternal atopic status, delivery type.

Descriptive statistics, cross tables, correlation and statistical tests were performed on each explanatory variable to describe the importance of each variable and identify which may be the most useful in explaining and predicting the outcome variable.

Outcomes assessed using models were:

fever, food allergy/sensitivity/intolerance, recurrent eczematous rash presence have been assessed at 6 months. Runny nose or cold incidence, reflux and/or vomiting, cough or wheeze have been assessed both at 6 and 12 months.

Since a model with a large number of predictors can 'overfit' the data, a "parsimonious model" or "best model" can be evaluated using selection methods such as forward, backward, and stepwise selection. The aim was to maximize explanatory power and minimise the number of predictor variables in the model in order to increase statistical power and obtain more valid parameter estimates.

Prior to multivariate analysis univariate analysis and correlation matrix has been performed. Then model selection methods (LASSO and glmulti) have been applied.

LASSO selection technique performs variable selection and estimates coefficients for predictors in linear regression. Although it does handle multicollinearity and group structure, it cannot select more predictors than the sample size. LASSO provides a point estimate of coefficients for relevant and selected predictors but not confidence interval and p-values unless bootstrapping or cross validation procedure are used.

For the analysis the candidate predictors, previously defined, were included in the model along with the interaction terms between colostrum collection time and growth factors.

The key feature of LASSO is the tuning parameter which controls the amount of shrinkage and so the selection of variables; for this reason, the tuning parameter lambda was selected by repeating 10 fold cross validation 100 times in order to reduce randomness and to average the error curves. In addition, 1000 bootstrap samples were used to estimate SE, p-values and CI.

Bootstrap is a simulation method which enables approximation and estimation of the LASSO distribution providing measures of accuracy such as bias, variance and confidence interval. However, such technique has some limitations as explained in many research papers.

The glmulti package in R provides an automated model selection and multi-model inference with GLMs. This technique enables fitting of all possible candidate models and selection of only one which satisfies IC profile and AIC criteria. Although this method can be time consuming, it allows the inclusion of uncertainty into the model and so in the statistical inference.

It converged very efficiently on the actual best models. However even the convergence is not perfect, but the procedure appears to be robust. This is consistent with what is known of IC-based model selection: the identity of the single “best” model or of the few best models may be subject to model-selection bias and are random variables over samples. Synthetic statistics of the confidence set of models (e.g., multi-model parameter estimates, relative support for the variables, etc.) are expected to be more robust.

An important characteristic of the GLM model selection framework is that all terms are equally frequent in the candidate models, so that the candidate set of variables is ”balanced”.

A faster genetic algorithm approach was used as allowed me to include a huge number of candidate predictors, perform model selection and estimate coefficients of relevant predictors. The aim of this statistical analysis is to explain the probability of babies

having several conditions (each of those are binary variables) from predictors, using a binomial GLM.

Interaction terms between growth factors and colostrum collection time were excluded to make the computation faster, increase the power of the analysis and to avoid discarding further missing values.

The resulting term importance was plotted in order to graphically visualize the effects of predictors. Such plots estimate the importance or relative evidence weight of candidate predictors, these weights are computed as the sum of the relative evidence weights of all models in which the term appears.

Sensitivity analysis was performed for both methods and all models; a different number of variables were included to make sure that those selected had a real and sensible effect on the outcome.

5.4 Results

Influence of immune active molecules in colostrum and breast milk in conjunction with environmental and maternal factors was analysed in relation to a number of health outcomes at the age of 6 and 12 months. The "best" statistical model for each outcome highlighted those factors having the most significant influence on a particular outcome. All results are adjusted for the site of collection as a potential confounder. The average Importance of the variables for a particular health outcome across all possible models are visualised on the graphs.

All factors found to be important at least in a single model and any significant associations with health outcomes are presented in the table below.

	BM TGFβ2	Detectable BM IL13	Detectable Col IL 13	Sex baby - Male	Verona	Moscow	BM HGF
Eczematous rash	**OR 1,04 (95% CI 1,01 - 1,06)**	**OR 0,18 (95% CI 0,04 - 0,92)**	Not important	**OR 0,2 (95% CI 0,05 - 0,84)**	Not important	Not important	Not important
Food Allergy/Sensitivity/ Intollerance	Not important	Not important	**OR 0,1 (95% CI 0,01 - 0,83)**	Not important	**OR 0,13 (95% CI 0,01 - 1,46)**	**OR 2,07 (95% CI 0,47 - 9,02)**	Not important
Fever	Not important	Not important	Not important	Not important	**OR 1,56 (95% CI 0,36 - 6,76)**	**OR 0,2 (95% CI 0,06 - 0,74)**	Not important
Cough or wheeze	Not important	Not important	Not important	Not important	Not important	Not important	**OR 1,89 (95% CI 0,94 - 3,78)**
Runny nose or cold 6 months	Not important	Not important	Not important	Not important	Not important	Not important	Not important for the model
Runny nose or cold 12 months	Not important	Not important	Not important	**OR 4,27 (95% CI 1,08 - 16,90)**	Not important	**OR 0,02 (95% CI 0,01 - 0,23)**	**OR 0,19 (95% CI 0,04 - 0,92)**
Baby atopy	Not important	Not important	Not important	Not important	Not important	Not important	Not important
Reflux and/or vomiting 12 months	Not important	**OR 0,21 (95% CI 0,04 - 1,04)**	Not important	Not important	Not important	Not important	Not important

Important for the model, statistically significant and OR < 1.0

Important for the model, statistically significant and OR > 1.0

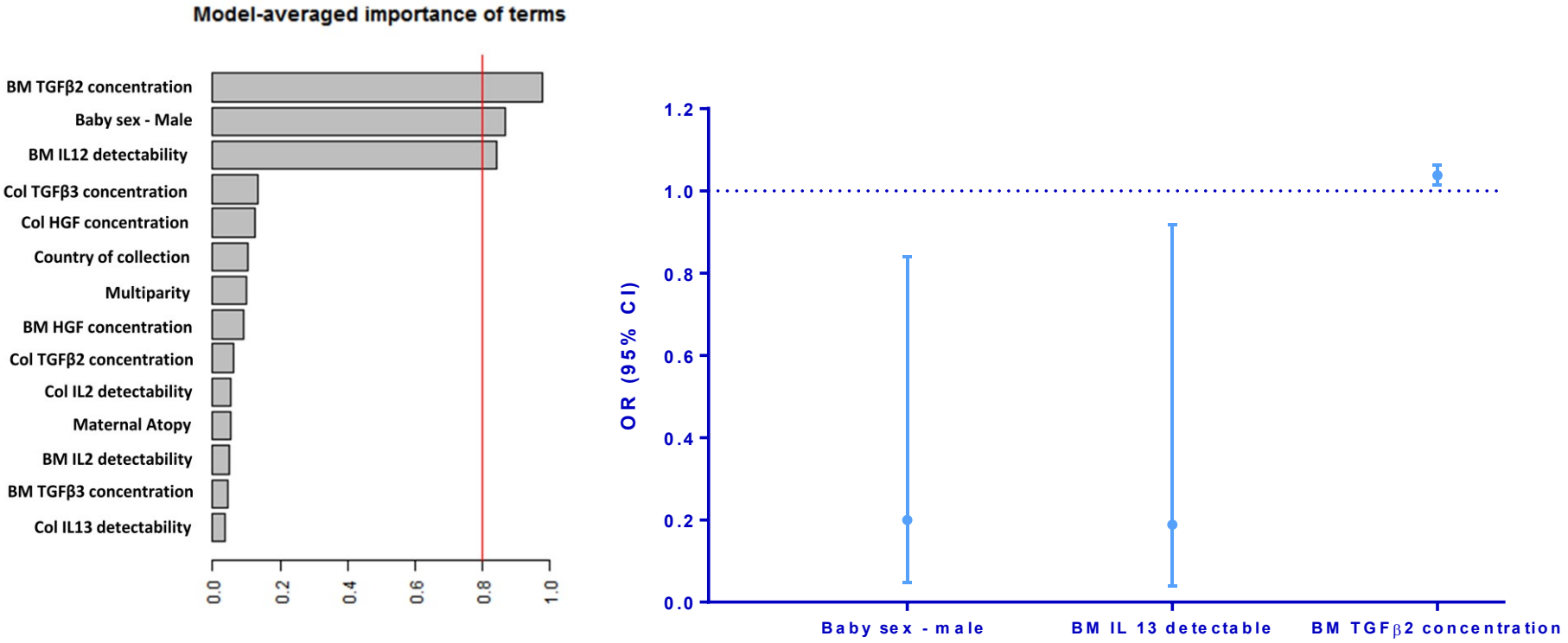
Important for the model but statistically not significant

Important for the model, statistically not significant, but there is a trend to report

5.4.1 Eczematous rash reported at 6 months of age

Three variables had an impact on eczematous rash reported by the mother at the age of six months. As it is shown in the *Figure 7*, TGFβ2 concentration in BM was the most important. Infants having breast milk with higher levels of this growth factor were at significantly higher risk OR 1,04 (95% CI 1,01 - 1,06) of eczematous rash development. In contrast, detectable IL13 in breast milk showed some protective effect OR 0,18 (95% CI 0,04 - 0,92) on eczematous rash development. Boys tended to have eczematous rash at the age of 6 months less often OR 0,2 (95% CI 0,05 - 0,84) compared with girls.

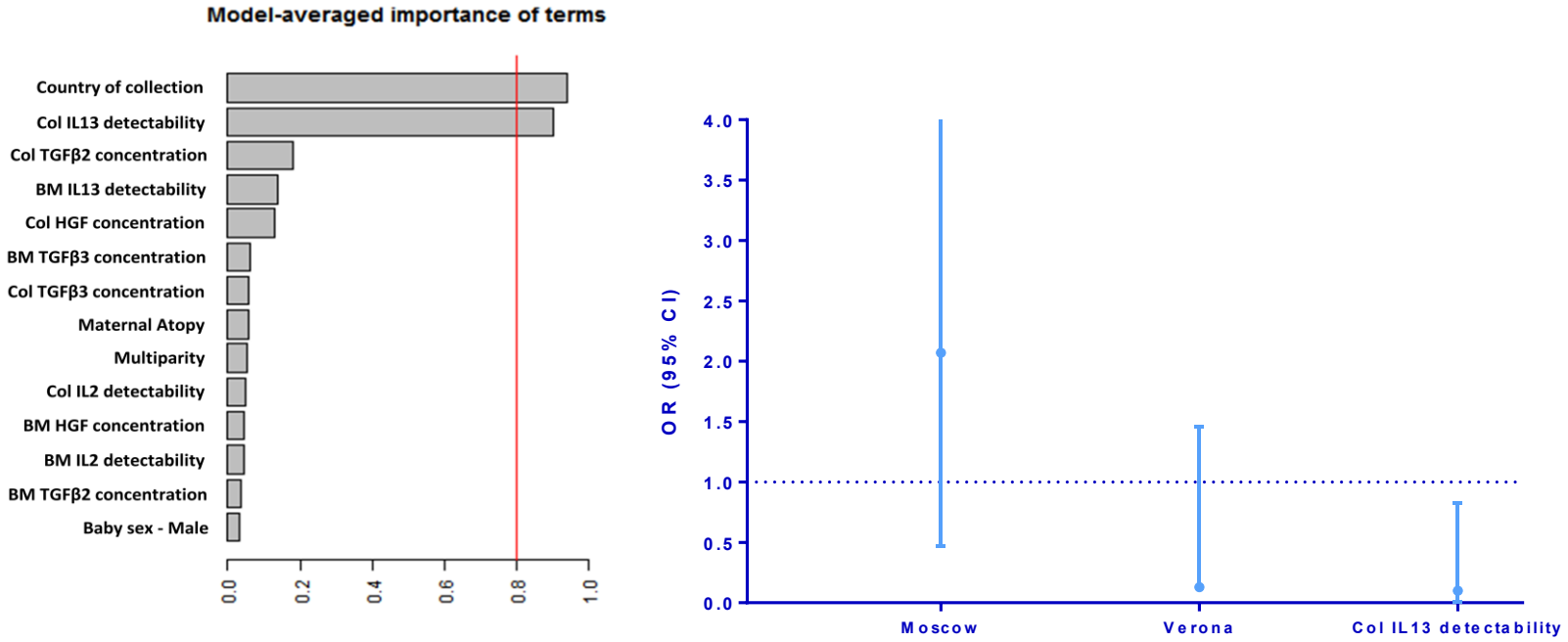
Figure 7 Importance of variables influence on eczematous rash development and OR of eczematous rash development due to the most important factors.



5.4.2 Food Allergy/Sensitivity/Intolerance reported at 6 months of age

Country of collection and detectability of IL13 in colostrum were found to be the most important for any form of allergy, sensitivity or intolerance reported by the mother at the age of 6 months (*Figure 8*). IL13 detectability in colostrum showed protective effects OR 0,1 (95% CI 0,01 - 0,83) on these health outcomes, whilst country of collection effect failed to reach significance OR 0,13 (95% CI 0,01 - 1,46) and OR 2,07 (95% CI 0,47 - 9,02) for Verona and Moscow respectively.

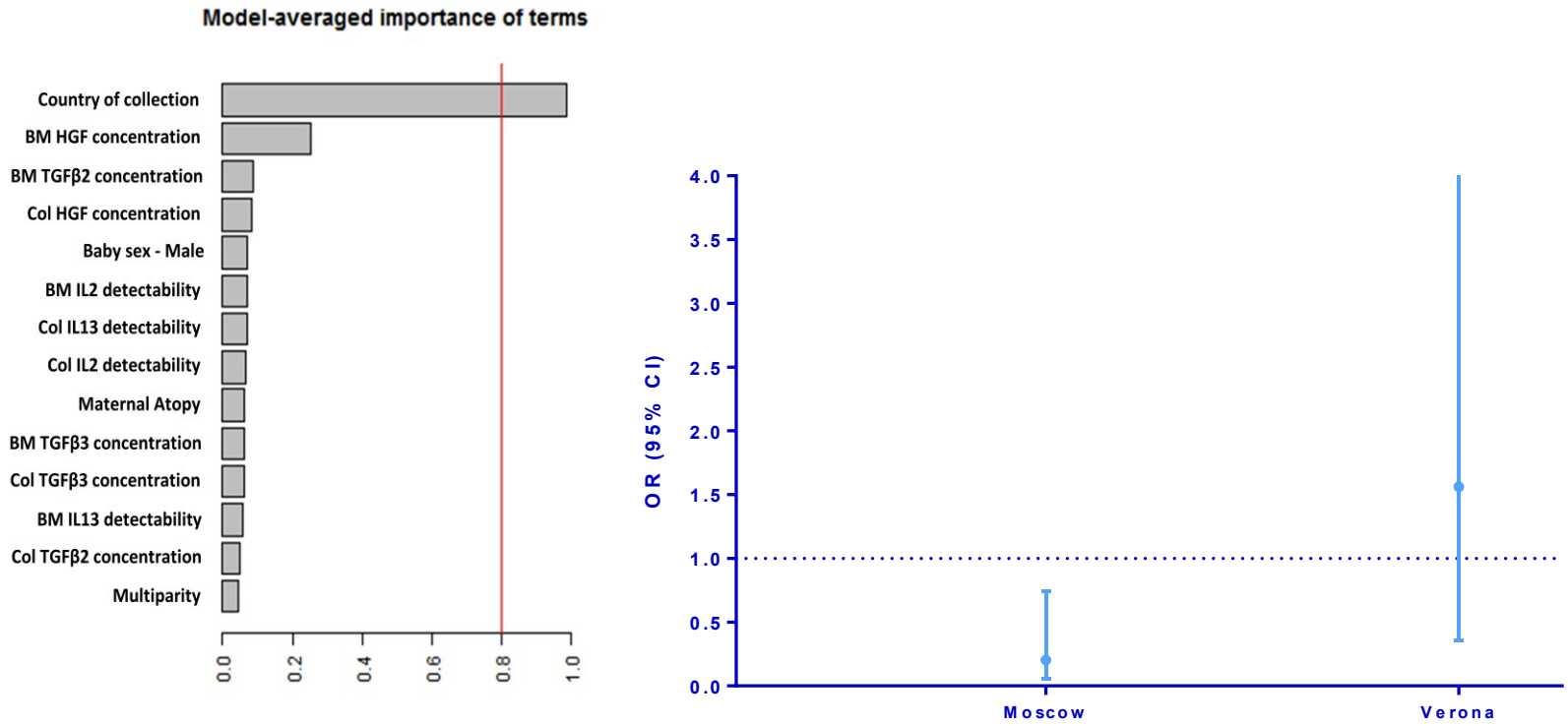
Figure 8 Importance of variables influence on food allergy/sensitivity/intolerance reported at 6 months and OR of food allergy/sensitivity/intolerance development due to the most important factors.



5.4.3 Fever incidence reported by mother at 12 months

The only factor associated with a higher incidence of fever reported by one year of age was country of collection (*Figure 9*). Mothers of infants born in Moscow reported episodes of fever significantly less OR 0,2 (95% CI 0,06 - 0,74). Women from Verona tend to report fever more often, but failing to reach significance OR 1,56 (95% CI 0,36 - 6,76).

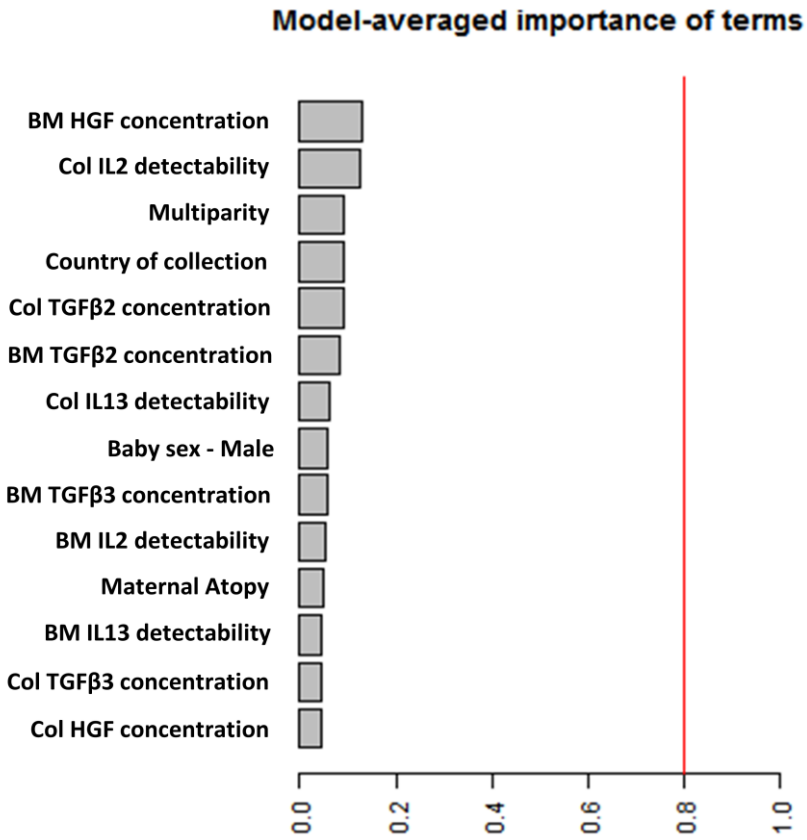
Figure 9 Importance of variables influence on fever incidence reported at 12 months and OR of fever incidence due to the most important factors.



5.4.4 Runny nose or cold reported by mother at 6 months

There were no associations of factors with maternal reports of at least one episode of runny nose or cold at the age of 6 months (*Figure 10*).

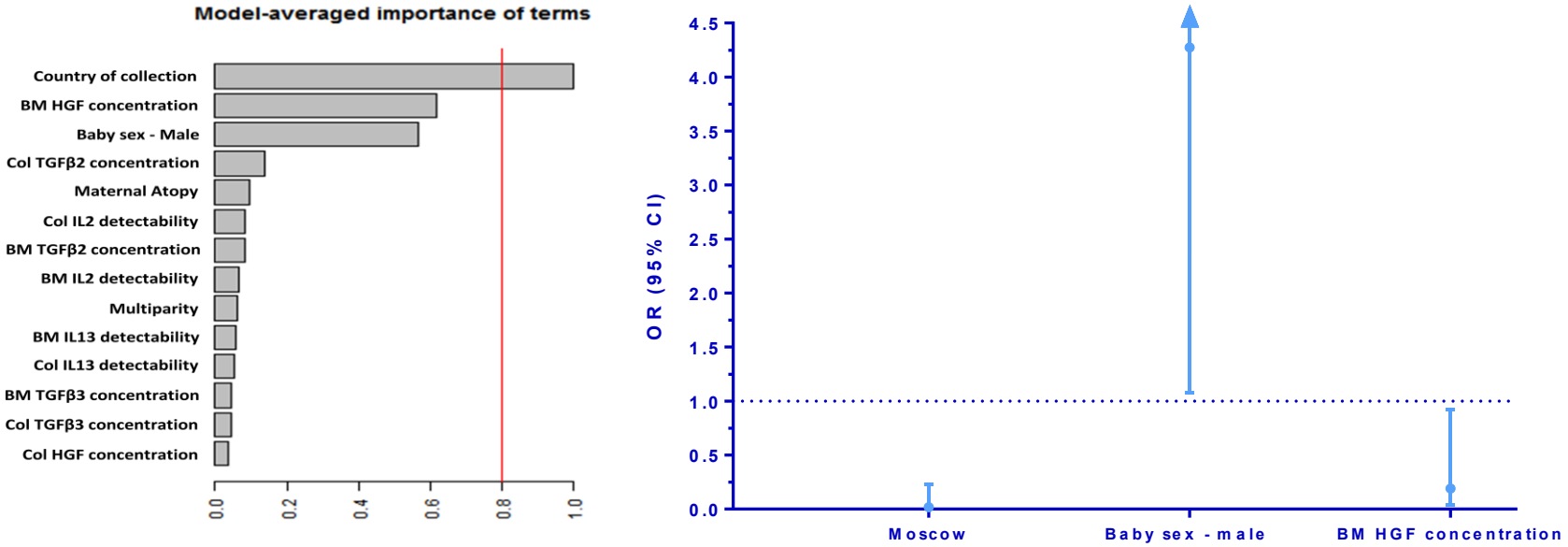
Figure 10 Importance of variables influence on runny nose or cold incidence reported at 6 months.



5.4.5 Runny nose or cold reported by mother at 12 months

Three variables seem to be important (*Figure 11*) for runny nose or cold development in children participated in the study, and the infants gender . At least one episode of runny nose or cold at the age of 12 months was reported less often OR 0,02 (95% CI 0,01 - 0,23) by mothers living in Moscow. HGF concentration in breast milk was associated with less runny nose or cold development OR 0,19 (95% CI 0,04 - 0,92) with boys tend to develop it significantly more often than girls OR 4,27 (95% CI 1,08 - 16,90).

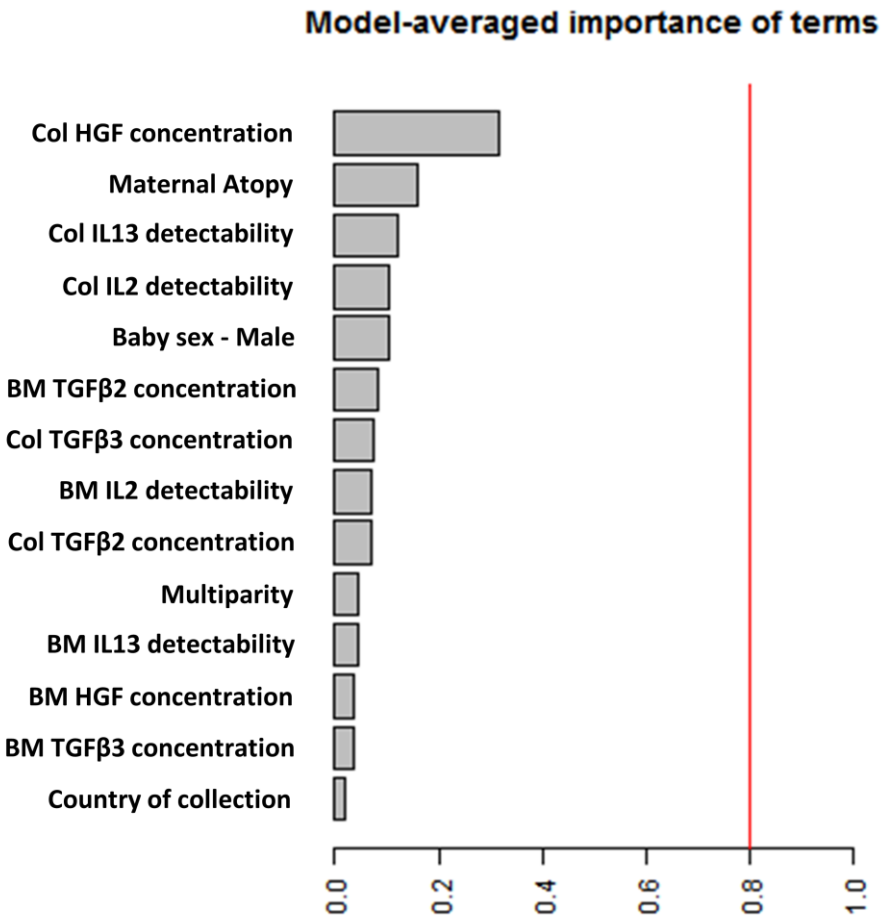
Figure 11 Importance of variables influence on runny nose/cold reported at 12 months and OR of runny nose/cold episodes incidence due to the most important factors.



5.4.6 Infant allergic sensitisation (at 12 months)

There was no association between any factor and infant allergic sensitisation at one year of age (*Figure 12*). It may be explained by a very small sample size as only 14 babies from the three cohorts developed allergic sensitisation.

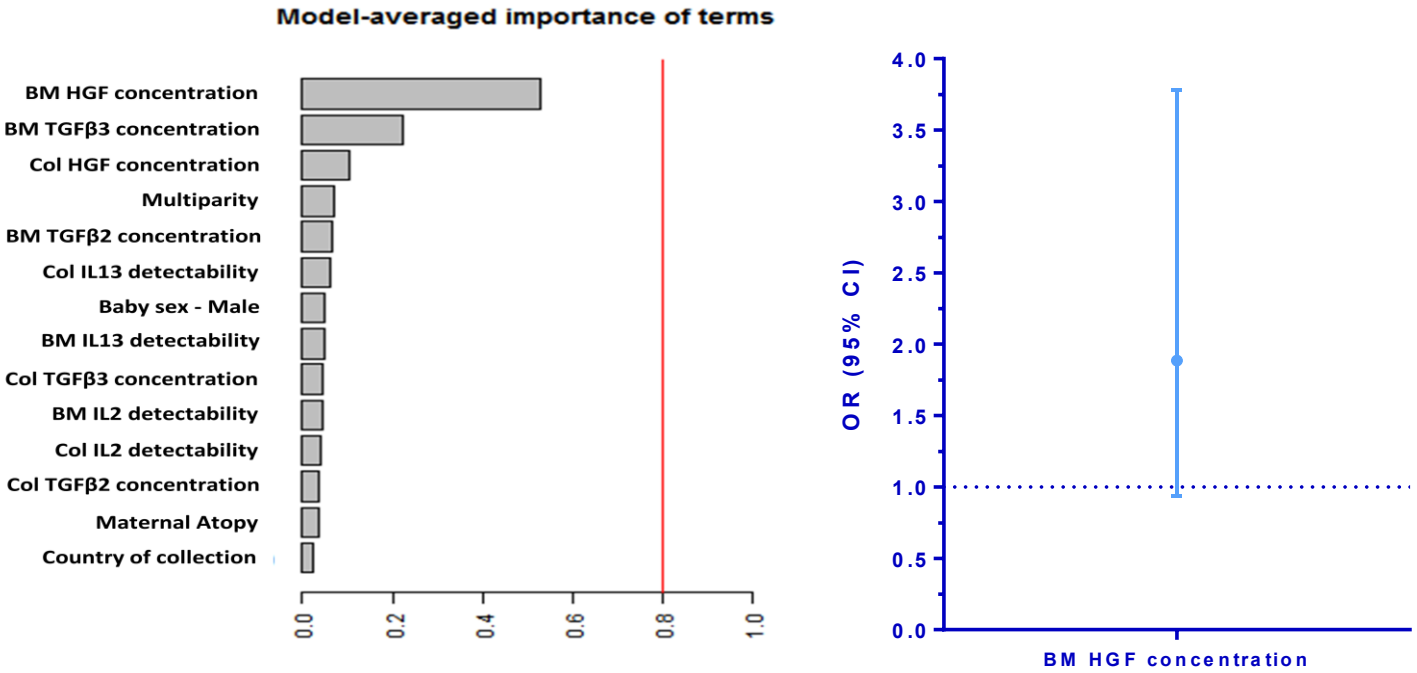
Figure 12 Importance of variables influence on infant allergic sensitisation recorded at 12 months.



5.4.7 Cough or wheeze reported at 12 months

The only factor shown to be important to be for cough and/or wheeze development at the age of 12 months was HGF concentration in breast milk (*Figure 13*). There is a non-significant trend for HGF to be associated with a higher risk of cough or wheeze development, OR 1,89 (95% CI 0,94 - 3,78).

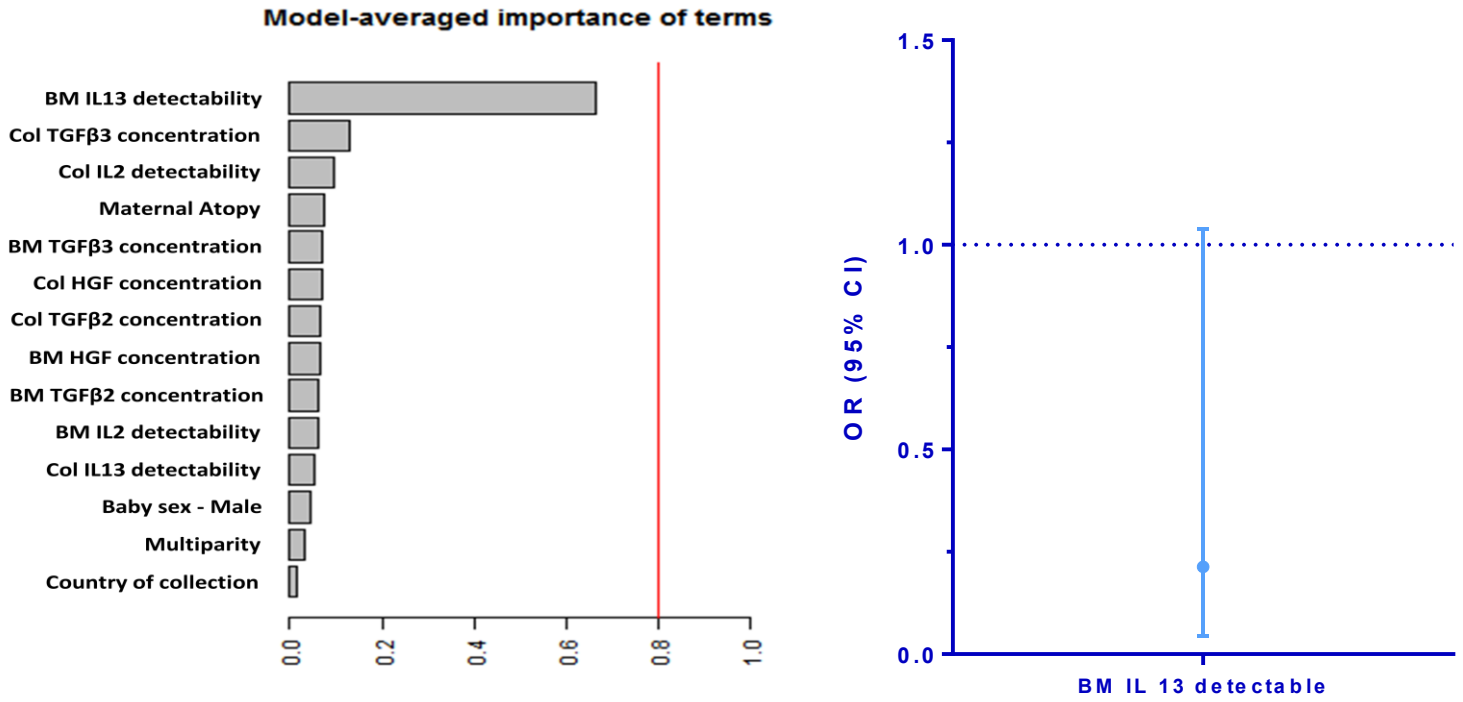
Figure 13 Importance of variables influence on cough/wheeze reported at 12 months and OR of cough/wheeze development due to the most important factors.



5.4.8 Reflux and/or vomiting reported at 12 months of age

The only factor to show importance for reflux and/or vomiting reported at one year of age was detectability of IL13 in breast milk (*Figure 14*). There is a non-significant trend for detectability of this cytokine to be associated with less reflux and/or vomiting OR 0,21 (95% CI 0,04 - 1,04).

Figure 14 Importance of variables influence on reflux/vomiting reported at 12 months and OR of reflux/vomiting development due to the most important factors.



5.5 Discussion

Mothers from the three geographical regions studied have different environment, diets, lifestyles making it difficult to take of account all factors which potentially may influence breast milk composition as well as health outcomes. I attempted to assess factors which from the common sense point of view and according to previous studies may have an impact of infants' health at first year of life. All the results were adjusted to the site of collection as a potential confounder.

5.5.1 Eczematous rash reported by the mother, atopy and allergy development

Many authors studied colostrum and breast milk composition influence on atopy and/or allergy development in the first years of life and existing data are very inconclusive and studies produced conflicting results.

The limitation of my study is that very few babies developed eczema, according to UK working party criteria and recurrent rash development reported by the mother is not the most accurate and precise criterion to use. It has been suggested that mothers tend to over-report food allergy in their children but childhood eczema can be accurately reported by caregiver (Silverberg et al., 2015).

My data suggest that the gender of the baby substantially increasing the risk of eczematous rash development, with girls at higher risk. It is in contrast with the known higher prevalence of eczema during the first year of life in boys. Differences in perception of eczema by women living in different countries and participating in our study could also play some role in reporting. Some of the previous studies have

shown that gender long term does not seem to play a significant role in risk of developing atopy, eczema (Schafer et al., 1999, Chonmaitree et al., 2014) and allergic rhinoconjunctivitis.

Jones et al. showed that low sCD14 levels in BM is associated with eczema development (Jones et al., 2002a) and Savilahti reported similar trends for colostrum (Savilahti et al., 2005). Later studies, however, failed to reproduce these results and did not report any protective effect of this soluble receptor on eczema (Snijders et al., 2006, Ismail et al., 2013).

Since it was impossible to conduct statistical analysis focused on true eczema in this study, due to a very small number of children with this condition the analysis focused on maternal reported eczema-like rash development and reported allergic events. My findings suggest that TGFβ2 in mature breast milk is associated with significant risk of eczematous rash reported by the age of 12 months. Some studies focused on growth factors analysis, supporting my data, found increased TGFβ1 and TGFβ2 in colostrum associated with the eczema onset in infants (Kalliomaki et al., 1999, Bottcher et al., 2008, Kuitunen et al., 2012). However, contrasting results of a few other studies do not allow final conclusions on the influence of TGFβ on eczema development (Bottcher et al., 2003, Snijders et al., 2006, Ismail et al., 2013, Orivuori et al., 2014).

Detectable IL13 in mature milk reduced the risk of parent reported eczematous rash development, whilst detectability of this cytokine in colostrum had a similar effect on food allergy/sensitivity/intollerance reported by the mothers at six months of age. This is the first study to report this association between IL13 in colostrum/breast milk and eczematous rash and/or food adverse events in children at 12 months of age.

Although maternal reported events is a subjective measure and risk of bias is increased, these data could be validated on a larger cohorts of high risk infants.

It is interesting to speculate on the likely mechanisms involved, if indeed it can be confirmed that TGFβ2 has an adverse effect on eczema development and IL13 is protective. While IL13 is a promoter of IgE production this and indeed all other Th1 and Th2 cytokines have been reported to be lower in infants who subsequently developed allergic problems. This may be a reflection of greater immunological immaturity in those destined to be allergic. Provision of IL13 in BM may militate against this trend. Furthermore, it is now understood that eczema is primarily not an allergic disease but a defect of skin epithelial barrier function where allergy is a secondary phenomenon occurring as a consequence of penetration of allergens through the skin. TGFβ is not only a regulatory cytokine reducing Th1 and 2 activity but also stimulates fibroblast generation of interstitial collagen. Thus increased levels could enhance the scarring effects of skin inflammation in an infant with an a skin epithelial barrier defect.

Most studies agree that levels of other breast milk immune active molecules, cytokines in particular, are not associated with atopy and/or allergy development in early life (Bottcher et al., 2003, Snijders et al., 2006, Kuitunen et al., 2012, Ismail et al., 2013). Only 14 infants developed allergic sensitisation by 1 year and it is therefore not possible to provide a statistical analysis on allergy alone.

5.5.2 Wheeze and asthma development

Breast milk has been shown to be beneficial for the baby, probably by providing oligosaccharides which facilitate colonization of a favourable micro-biome which protect against neonatal pneumonia (Duijts et al., 2009) but there is little evidence to prove a similar degree of protective effect against asthma. As discussed in detail in Introduction section of this thesis, Gdalevich and Mimouni provided a meta-analysis suggesting protective effects of exclusive breastfeeding in the first months of life on later asthma development (Gdalevich et al., 2001b). However, the difficulty in combining data is that the wheeze/asthma phenotype differs between studies. Most infant wheezers do not subsequently have persistent asthma.

Bronchiolitis in early childhood is known to be associated with asthma development later in life (Tregoning and Schwarze, 2010, Sigurs et al., 2005, Sigurs et al., 2010). Data from an Australian cohort showed low levels of IL10 to be associated with an increased risk of subsequent bronchiolitis (Bryan et al., 2007). Oddy and co-authors reported increased TGF-β1 levels in breast milk to have some protective effect against wheeze development in infancy (Oddy et al., 2003) but this conflicts with two other large cohort studies (Snijders et al., 2006, Orivuori et al., 2014). Based on the accumulated evidence now including my observations it is unlikely that levels of immune active molecules in human milk have significant influence on subsequent cough or wheeze development up to one year of age. My data did not show any significant influence of colostrum and/or BM immune active molecules association with parent reported cough or wheeze episodes at 12 months of age. However, there is a trend to report, although not significant OR 1,89 (95% CI 0,94 - 3,78), for an association between HGF concentration in BM and higher risk of cough or wheeze development. HGF is known for its ability to attenuate allergen-induced airway

hyper-responsiveness and reduce local IL5 and IL13 response in asthmatic mice (Ito et al., 2005). It is too premature to make any conclusions on why BM HGF may lead to an increased incidence of parental reported cough or wheeze but this finding requires further investigation and more studies focusing on breast milk immune composition and long-term health outcomes is needed.

5.5.3 Runny nose or cold and fever development

Breastfeeding provides protection against infectious diseases in early infancy and may reduce mortality rates due to common infections by half (Verhasselt, 2010), with this effect particularly evident in the developing world. These protective capabilities are evident for exclusive breastfeeding, while partial breastfeeding does not seem to provide the same level of protection (Ladomenou et al., 2010).

Hitherto there is little evidence on the specific breast milk immune modulators which influence infections rates in infancy and early childhood. Most authors suggested that outcomes may be explained by the "rich" immunological composition of breast milk (Hanson et al., 2002). Hasselbalch et al. reported that prolonged breastfeeding to 10 months of age was associated with increased thymic size (Hasselbalch et al., 1999). In another study breastfeeding amplified oral polio virus vaccination antibody responses (Pickering et al., 1998).

Maternal reported fever may not be a reliable indicator of infection, and fever during the first year of life may have other causes such as teething (Hamilton and John, 2013). My data suggest that mothers from Moscow tend to report both, fever and runny nose or cold episodes significantly rarer. It raises the question of difference in

perception of the same term by people residing in different countries and highlights importance of questionnaires validation in each population.

There was no association between breast milk composition and runny nose or cold reported at 6 months of age, however, when assessed at one year of age, HGF levels in BM were associated with reduced incidence of runny nose or cold reported at 12 months of age. HGF is well known for its ability to provide protection during inflammatory diseases, directly targeting macrophages or lymphocytes (Nakamura and Mizuno, 2010). HGF may well act in a similar fashion when transferred in a high amounts into the infant's gut. This finding shows that HGF as a part of BM composition may not only play a very important role during very first days of life, providing infant gut immunity development and maturation. It may also be capable to influence long term health outcomes. Maternal reported runny nose or cold as an outcome should be taken critically. Existing data suggests that a large proportion of infants suffer asymptomatic infections during first year of life (Chonmaitree et al., 2014). As our participants has not been tested for infections I realise that this is a limitation.

5.5.4 Reflux and/or vomiting development

Data on breast milk ability to influence reflux development in early infancy is very limited. It has been suggested that the protein content of human milk may influence acid gastro-oesophageal reflux incidence and fortification of human milk may lead to a more severe non acidic reflux which may be explained by high variablity of BM composition (Aceti et al., 2009). No studies have investigated breast milk immune

constituent influences on reflux development in infants. Given the association with food induced eosinophllic oesophagitis this may be a fruitful line of research.

From my study it appears that no factors are associated with reflux and/or vomiting incidence reported at the age of 12 months.

5.5.5 Conclusion

The aim of the study was to investigate whether key immune factors in breast milk have an impact on infant health outcomes. Unfortunately, sample size do not allow me to dichotomise colostrum into smaller groups, according to day of collection. This approach would provide stronger outcomes but not meaningful statistical results. Despite limitations, the results suggest that variability of breast milk composition has an effect on maternal reported eczematous rash, food allergy, sensitivity, intolerance, runny nose and cold and potentially even reflux and/or vomiting development in infancy.

The results support the concept that IL13 and HGF have some protective effects and TGFβ2 may act as a risk factor, which may explain the diversity of results from epidemiological studies on the health promoting effects of breastfeeding. These results provide additional information which can be used in non-communicable diseases prevention research. Intervention trials on larger cohorts from genetically diverse populations are needed in order to investigate potential target cytokines and/or growth factors, which may benefit infants' health both, short and long term. It is crucial to take into account a number of possible confounders which may influence human milk composition and health outcomes.

6 Colostrum immune composition and immunological outcomes assessment using Principal Component analysis (PCA)

6.1 Abstract

Background: Cytokines and growth factors in colostrum and breast milk (BM) are likely involved in facilitating infant gut immunity maturation. The complex immunological composition of human milk confers potential beneficial effects including protection against infections and atopy/allergy development. Environmental and maternal factors may influence the levels of immune active molecules in breast milk but there is still uncertainty which factors are responsible. I attempted to highlight specific groups which can be characterised by a particular compositional pattern which differed in outcome from the general population, using principal component analysis (PCA) of the data on colostrum and breast milk of women from three European countries. I also assessed correlations between the levels of immune active molecules in colostrum.

Objectives: To examine colostrum/breast milk composition for distinct patterns and cluster them into specific "lactotypes". To assess if any "lactotype" was associated with maternal or environmental exposures and/or immunological outcomes in the infants at 6 and 12 months of age.

Methods: A prospective cohort study of mother/infant pairs in London (N=105), Moscow (N=200) and Verona (N=80). Participants underwent allergy testing, and questionnaire interview. Colostrum samples (days 0-6) and Mature Breast Milk (4-6 weeks) were analysed in duplicate at Imperial College London using electrochemiluminescence (Meso Scale Discovery®) for level of TGF-β 1,2,3; HGF,

IL2, IL4, IL5, IL10, IL12, IL13, IFN-γ. Principal component analysis was used for data assessment.

Results: Colostrum composition could be divided into groups depending on the predominance of the immune active molecules profile. According to PCA analysis four "lactotypes", based from their coordinates on PCA, can be highlighted. They may be divided into two clusters, which I labelled A and B. Women having Cluster A "lactotype" are distinct from the cluster B "lactotype" and vice versa. *Coordinates of the clusters at PCA:* Cluster A1 (IL5 and IL10 group (r = Dim.1 from 0.503 to 0.627; Dim. 2 from 0.370 to 0.539)); A2 (IL12 and IL13 group (r = Dim.1 from 0.534 to 0.574; Dim. 2 from 0.712 to 0.718)) and Cluster B1 (IL2 and IL4 group (r = Dim.1 from 0.421 to 0.522; Dim. 2 -0.123)); and B2 (TGFβ 1,2,3 and HGF (r = Dim.1 from 0.626 to 0.722; Dim. 2 from-0.241 to -0.510)).

When exposures and health outcomes have been applied at PCA plot I found that mothers living in London significantly more often were associated with clusters B1 and B2 (Est. Dim.1=0.432, p<0.01; Est. Dim.2=-0.253, p=0.04). Assessing outcomes at 6 and 12 months: cough or wheeze reported (Est.=-0.380, p<0.01), eczematous rash (Est.=-0.261, p=0.04) or any immunological outcomes (Est.=-0.276, p=0.03) were more often associated with Clusters B1 and B2.

Conclusions: Breast milk has a very complex composition and novel approaches to its' assessment are needed. By applying PCA analysis to the data, it is possible to classify breast milk according to profile, differing from the general population. These "lactotypes" are characterised by predominance of a number of immune active molecules. The data suggests the presence of four "lactotype" groups: A1 (IL5 and IL10), A2 (IL12 and IL13), B1 (IL2 and IL4), B2 (TGFβ1,2,3 and HGF). The A1 and

A2 "lactotypes" are associated with reduced incidence of eczematous rash, cough or wheeze and any reported immunological outcomes (eczematous rash, cough or wheeze and positive atopic status of a baby combined together) at one year of age. Mothers from London tend to fall into cluster B "lactotype" groups significantly more often in comparison to women from Moscow and Verona.

6.2 Introduction

Breast milk is a complicated mixture of a large variety of components (D'Alessandro et al., 2010) and each may have an active role in the early stage of infant development. The standard approach in breast milk research is to study one or two components of breast milk but it is likely that no single immune active molecule but most probably a combination effect the immunological outcomes developing later in life.

In this study I assessed immune active molecules levels and exposures influencing breast milk composition using mixed models. This is a reliable method to highlight the most important exposures influencing breast milk composition, but it does not provide an insight into the complexity of the breast milk and does not highlight patterns or clusters of immune modulators which might affect outcomes in the infants.

As a last and final step of the data analysis I classified mothers breast milk composition into "lactotypes", in accordance with a particular physiological patterns, and analyse immunological outcomes attachment to these clustered "lactotypes".

The most suitable method to achieve this is principal component analysis (PCA) which allows pattern identification and clusters data and assesses if any trends are present within the set of data. This method has been previously used in breast milk research in one previous study (Johansson et al., 2011). However, the focus of the study was very different (PUFA BM composition), the cohort size was limited (22 people) and no cytokines and/or growth factors were taken into account.

6.3 Methods

The main purpose of principal component analysis is pattern identification, and reduces the dimensions of the dataset with a minimal loss of information available.

For the purpose of PCA I used growth factors and cytokine concentrations as continuous variables rather than just detectability as it is the only way to achieve meaningful and well powered data for this type of analysis. Categorical variables have been categorised and half of the threshold used when the concentration of cytokines were below the lowest level of detection.

The steps involved are listed below.

6.3.1 Missing values evaluation

In order to provide good quality information from PCA analysis it is preferable to analyse not less than 50% of the data. Data on immune active molecules which have more than 50% of missing values are excluded from the further analysis (*Table 19*).

Table 19 Variables and cytokines, growth factors missing data percentage

VARIABLE NAME	Missing	%	CYTOKINE NAME	Missing	%
Eczematous rash	58	14.65	*Colostrum HGF concentration*	25	6.31
Food Allergy/Sensitivity/Intolerance	59	14.90	*Colostrum IFNγ concentration*	54	13.64
Fever	58	14.65	*Colostrum IL10 concentration*	54	13.64
Runny nose or cold 6 mo	60	15.15	*Colostrum IL12 concentration*	54	13.64
Cough or wheeze	60	15.15	*Colostrum IL13 concentration*	54	13.64
Runny nose cold 12mo	123	31.06	*Colostrum IL2 concentration*	54	13.64
Stool consistency (Bristol chart)	181	45.71	*Colostrum IL4 concentration*	54	13.64
Baby atopy SPT	162	40.91	*Colostrum IL5 concentration*	54	13.64
Reflux/Vomiting 6 mo	120	30.30	*Colostrum TGFβ1 concentration*	116	29.29
Reflux Vomiting 12mo	117	29.55	*Colostrum TGFβ2 concentration*	116	29.29
Country of collection	0	0.00	*Colostrum TGFβ3 concentration*	116	29.29
Labour Type	3	0.76	*BM HGF concentration*	187	47.22
Sex of the baby	7	1.77	*BM IFNγ concentration*	206	52.02
Gestation	31	7.83	*BM IL10 concentration*	206	52.02
Exclusive BF	66	16.67	*BM IL12 concentration*	206	52.02
Age of the first food intake	117	29.55	*BM IL13 concentration*	206	52.02
Previous Deliveries 1	9	2.27	*BM IL2 concentration*	206	52.02
Maternal Atopy	109	27.53	*BM IL4 concentration*	206	52.02
			BM IL5 concentration	206	52.02
			BM TGFβ1 concentration	237	59.85
			BM TGFβ2 concentration	237	59.85
			BM TGFβ3 concentration	237	59.85

6.3.2 Missing values imputation

As a second step, imputation of missing values for all cytokines available has been performed. This is an absolute requirement as a preliminary step prior to performing a PCA.

Regularised iterative PCA algorithm have been used, in order to avoid over-fitting problems and this method consists of imputing missing values with initial values such as the mean of the variable and then performing PCA on the completed dataset. These steps of estimation of the parameters via PCA and imputation of the missing values are iterated until convergence.

6.3.3 PCA with no supplementary variables – standard PCA

This step is done in order to estimate the number of dimensions for PCA using different methods:

Scree-plot:

Table 20 Components for standard PCA

Number of Eigenvalue[a]	Eigenvalue[b]	Percentage of Variance[c]	Cumulative Percentage of Variance[d]
component 1	3.865	32.212	32.212
component 2	2.823	23.521	55.733
component 3	1.341	11.176	66.909
component 4	1.172	9.766	76.675
component 5	0.801	6.675	83.350
component 6	0.722	6.018	89.369
component 7	0.605	5.041	94.410
component 8	0.412	3.435	97.845
component 9	0.185	1.538	99.383
component 10	0.064	0.533	99.917
component 11	0.007	0.062	99.979
component 12	0.003	0.021	100.000

a. **Number of Eigenvalue** - The number of eigenvalues are always equal the number of variables that are used in the principal components analysis.

b. **Eigenvalue** - Eigenvalues are the variances of the principal components. As PCA is conducted on the correlation matrix, the variables are standardized, which means

that each variable has a variance of 1, and the total variance is equal to the number of variables used in the analysis, in the case of our study, 12.

c. **Percentage of Variance** - This is the proportion of the total variance that each factor accounts for. For example, 0.32212 = 3.865/12.

d. **Cumulative** - This is the sum of the percentage column. For example, 55.733 = 32.212+ 23.521.

Figure 15 Scree plot (Standard PCA)

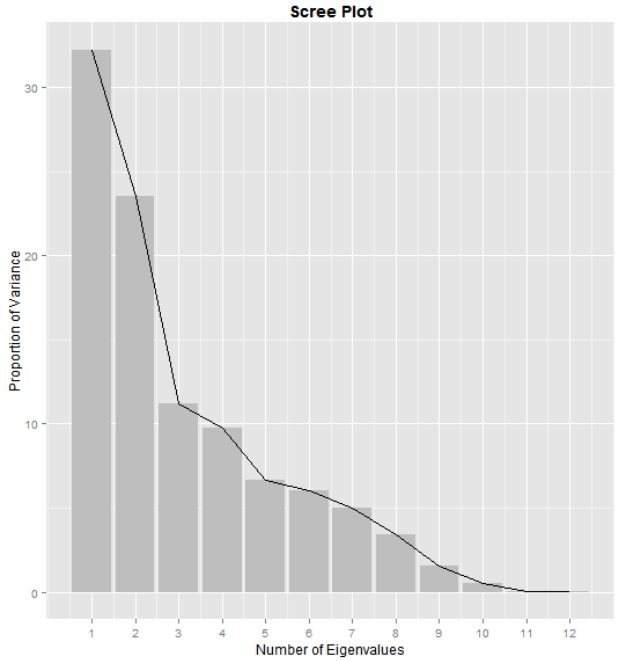


Figure 15 shows the proportion of variance against the component number. These values can be seen in the first and third column in the table above. *The line drops rapidly and from the third component* but subsequently the line progressively flattens, which means that each successive component is accounting for smaller and smaller amounts of the total variance.

Cross-validation: each cell of the data matrix is alternatively removed and predicted with a PCA model. The number of components which leads to the smallest mean

square error of prediction is retained. Specifically, the mean squared error of prediction (MSEP) is calculated for each number of components and 2 is the number of components retained for the PCA. *Three components could be used for PCA analysis but it would lead to a significant increase in difficulty of result interpretation.* Dimension 1 (PC1 on PCA plots) coordinates are more important than dimension 2 (PC2 on PCA plots) as they cover more data (32,21% vs. 23,52%).

The output of a completed dataset which is scaled to unit variance analysed in a previous step is used for an input of the PCA function.

Table 21 Immune active molecules correlations for both dimensions: Dim. 1 and Dim. 2 (Standard PCA with no exposures or outcomes)

Variables	Correlation Dim. 1	p.value	Variables	Correlation Dim. 2	p.value
Colostrum TGFβ1 concentration	0.868	<0.001	*Colostrum IL5 concentration*	0.854	<0.001
Colostrum TGFβ3 concentration	0.852	<0.001	*Colostrum IL13 concentration*	0.835	<0.001
Colostrum TGFβ2 concentration	0.777	<0.001	*Colostrum IL12 concentration*	0.835	<0.001
Colostrum IL2 concentration	0.756	<0.001	*Colostrum IL10 concentration*	0.798	<0.001
Colostrum IL4 concentration	0.702	<0.001	*Colostrum TGFβ3 concentration*	-0.100	0.047
BM HGF concentration	0.519	<0.001	*Colostrum TGFβ1 concentration*	-0.101	0.046
Colostrum HGF concentration	0.515	<0.001	*BM HGF concentration*	-0.137	0.006
Colostrum IFNγ concentration	0.364	<0.001			
Colostrum IL10 concentration	0.132	0.009			
Colostrum IL5 concentration	0.131	0.009			

This table shows which variables are highly correlated to each principal component. They provide not only the correlation coefficients, but also tests of significance of these variables. Numbers in columns Correlation Dim. 1 and 2 provide information on coordinates for each variable at a particular dimension.

Biplot graphics use points to represent the scores of the observations on the principal components, and it uses vectors to represent the coefficients of the variables (i.e. cytokines) on the principal components. The length of the vectors represent the importance of a particular variable within a particular dimension, but not concentration.

Points that are close together correspond to observations that have similar scores on the components displayed in the plot. These points also correspond to observations that have similar values on the variables.

Both the direction and length of the vectors can be interpreted. Vectors point away from the origin in some direction. A vector points in the direction which is most like the variable represented by the vector. Highly correlated variables point in the same direction; uncorrelated variables are at right angles to each other.

Coordinates of the observation can be approximated by projecting the point onto the variable vectors within the biplot. In order to do so, imagine the vector extension in both directions. The observations, whose points project furthest in the direction in which the vector points, are the observations that have the most of whatever the variable measures. Thus, vectors that point in the same direction correspond to variables that have similar response profiles, and can be interpreted as having similar meaning in the context set by the data.

6.3.4 PCA with the addition of supplementary individuals, supplementary quantitative variables and supplementary categorical variables (exposures and health outcomes).

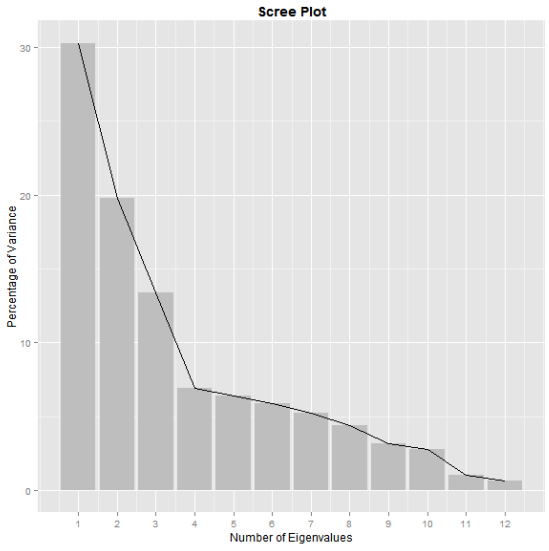
The number of dimensions for PCA has been estimated using different methods:

- Scree-plot

Table 22 Components (PCA with supplementary variables)

Nuber of Eigenvalue	Eigenvalue	Percentage of Variance	Cumulative Percentage of Variance
component 1	3.635	30.289	30.289
component 2	2.377	19.811	50.100
component 3	1.610	13.413	63.514
component 4	0.830	6.916	70.430
component 5	0.765	6.372	76.802
component 6	0.708	5.900	82.702
component 7	0.630	5.249	87.951
component 8	0.528	4.400	92.351
component 9	0.380	3.167	95.518
component 10	0.335	2.793	98.311
component 11	0.124	1.037	99.348
component 12	0.078	0.652	100.000

Figure 16 Scree plot (PCA with supplementary variables)



The line drops rapidly in a similar way to PCA without supplementary variables and from the fourth component line is almost flat.

As it has been done for Standard PCA analysis, cross-validation step has been performed: the MSEP is calculated for each number of components.

5) The last step is PCA performance and results interpretation. The output (completed dataset which is scaled to unit variance) of the previous step is as input of the PCA function.

The correlation coefficient between each continuous ***variable*** (e.g. HFG, IL5 etc.) and the ***dimension*** (PCA Dim. 1 or PCA Dim. 2) of the PCA is calculated for each dimension. The correlation coefficients significantly different from zero are sorted and returned by the software.

For the categorical variables, an ANOVA model with one factor is done for each dimension; the coordinates of the individuals are explained by the categorical variable. F-test is derived to assess if variable has an influence on the dimension and T-tests are done category by category. It can be seen if coordinates of the individuals of the sub-population defined by one particular category are significantly different from 0.

6.3.5 Correlation heat map

In order to evaluate how well immune active molecules correlate one with another I used Spearman rank's correlation as data is not normally distributed. Concentrations in pg/ml have been used for growth factors and detectability for cytokines.

6.4 Results

Table 23 and *Table 24* show particular variables and the axes to which they are linked. They illustrate which continuous variables (e.g. particular immune active molecule) are the most correlated to each component and which categorical variables and categories (e.g. country of collection, health outcomes) describe each axis the best. Variables and categories have been sorted by p-value and only significant ($p<0.05$) results are kept and presented in the tables.

6.4.1 Lactotypes

It is evident that immune active molecules can be grouped into four clusters according to their location on the plot (*Figure 17*) determined by correlation coefficient (coordinates, which can be found in *Table 23 and Table 24)* between each continuous variable (immune active molecules in colostrum) and the dimension of the PCA (PCA Dim. 1 or PCA Dim. 2). As PCA Dim. 1 covers more data than PCA Dim. 2, coordinates within this dimension are relatively more important. Growth factors are grouped together (r = Dim.1 from 0.626 to 0.722; Dim. 2 from-0.241 to -0.510), similar trends are seen for IL2 and IL4 (r = Dim.1 from 0.421 to 0.522; Dim. 2 -0.123), IL5 and IL10 (r = Dim.1 from 0.503 to 0.627; Dim. 2 from 0.370 to 0.539), IL12 and IL13 (r = Dim.1 from 0.534 to 0.574; Dim. 2 from 0.712 to 0.718) respectively.

6.4.2 Environmental factors and health outcomes

The first component (PCA Dim. 1) is mainly characterized by site of collection ($r=0.027$, $p<0.01$), runny nose or cold reported at 12 months ($r=0.019$, $p=0.02$), reflux

and/or vomiting reported at 12 months (r=0.021, p<0.01) and multiparity (r=0.017, p=0.04).

Absence of reflux and/or vomiting has a positive coordinate (Est.=0.319, p<0.01) on this axis, no reported runny nose or cold at 12 months of age also have a positive coordinate (Est.=0.273, p=0.04) same applies for the samples collected in London (Est.=0.432, p<0.01) whereas samples from Verona have a negative coordinate (Est.=-0.509, p<0.01). This gives the direction of the axis: individuals with high coordinates on the first component will tend not to have reflux and/or vomiting and runny nose or cold.

The second component (PCA Dim. 2) is characterized by cough or wheeze reported (r=0.021, p=0.02). Individuals with low coordinates on this axis will tend to have cough or wheeze reported (Est.=-0.380, p<0.01), eczematous rash (Est.=-0.261, *p=0.04) or any immunological outcomes (Est.=-0.276, p=0.03) (Figure 18Figure 19Figure 20).*

6.4.3 Correlation heat map

I assessed the correlation between different markers in colostrum and results are presented in (*Table 25*).

Table 23 Dimension 1 variables

Variables	Correlation Dim.1	p.value	Variables	R2 Dim.1	p.value
Colostrum TGFβ1 concentration	0.722	<0.001	*Reflux/Vomiting at 12 months of age*	0.021	0.004
Colostrum TGFβ3 concentration	0.699	<0.001	*Site of collection*	0.027	0.005
Colostrum TGFβ2 concentration	0.639	<0.001	*Runny nose or cold at 12 months of age*	0.019	0.024
Colostrum IL5 concentration	0.627	<0.001	*Multiparity*	0.017	0.037
Colostrum HGF concentration	0.626	<0.001			
Colostrum IL12 concentration	0.574	<0.001	**Category**	**Estimate Dim.1**	**p.value**
Colostrum IL13 concentration	0.534	<0.001	*No Reflux/Vomiting*	0.319	0.004
Colostrum IL2 concentration	0.522	<0.001	*Country of collection UK*	0.432	0.005
Colostrum IL10 concentration	0.503	<0.001	*No Runny nose or cold at 12 months of age*	0.273	0.040
Colostrum IL4 concentration	0.421	<0.001	*Country of collection Italy*	-0.509	0.001
BM HGF concentration	0.258	<0.001			
Colostrum IFNγ concentration	0.218	<0.001			

Table 24 Dimension 2 variables

Variables	Correlation Dim.2	p.value	Variables	R2 Dim.2	p.value
Colostrum IL13 concentration	0.718	<0.001	Cough or wheeze	0.021	0.018
Colostrum IL12 concentration	0.712	<0.001			
Colostrum IL5 concentration	0.539	<0.001	**Category**	**Estimate Dim.2**	**p.value**
Colostrum IL10 concentration	0.370	<0.001	*Eczematous rash reported*	-0.261	0.045
Exclusive breastfeeding	0.100	0.048	*Country of collection UK*	-0.253	0.042
Colostrum IL2 concentration	-0.123	0.014	*Cough or wheeze reported*	-0.380	0.004
Colostrum HGF concentration	-0.241	<0.001			
Colostrum TGFβ2 concentration	-0.388	<0.001			
BM HGF concentration	-0.440	<0.001			
Colostrum TGFβ3 concentration	-0.494	<0.001			
Colostrum TGFβ1 concentration	-0.510	<0.001			

Figure 17 PCA with supplementary variables plot

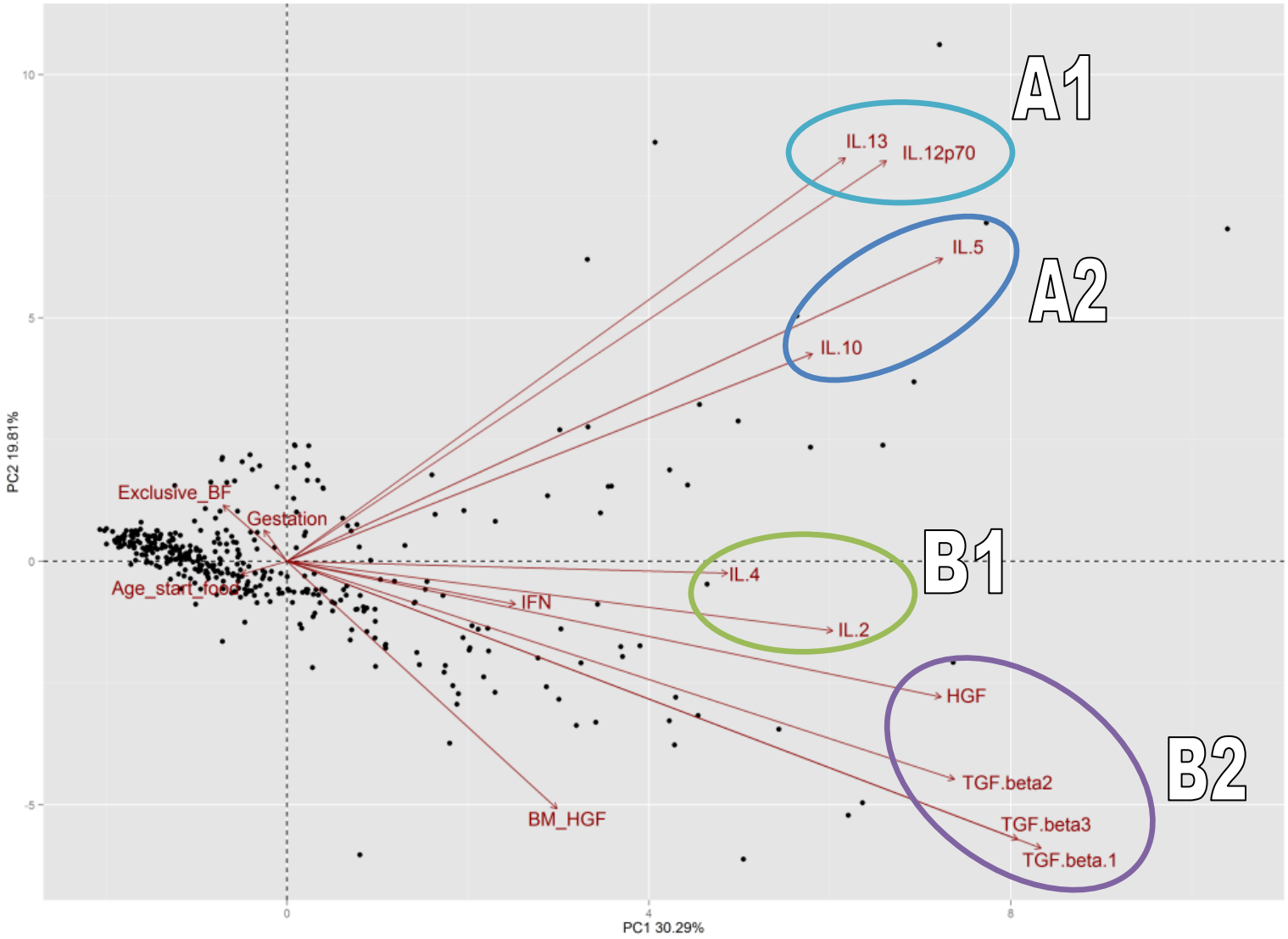


Figure 18 PCA with Immunological outcomes plot

Figure 19 PCA with Cough or wheeze outcome plot

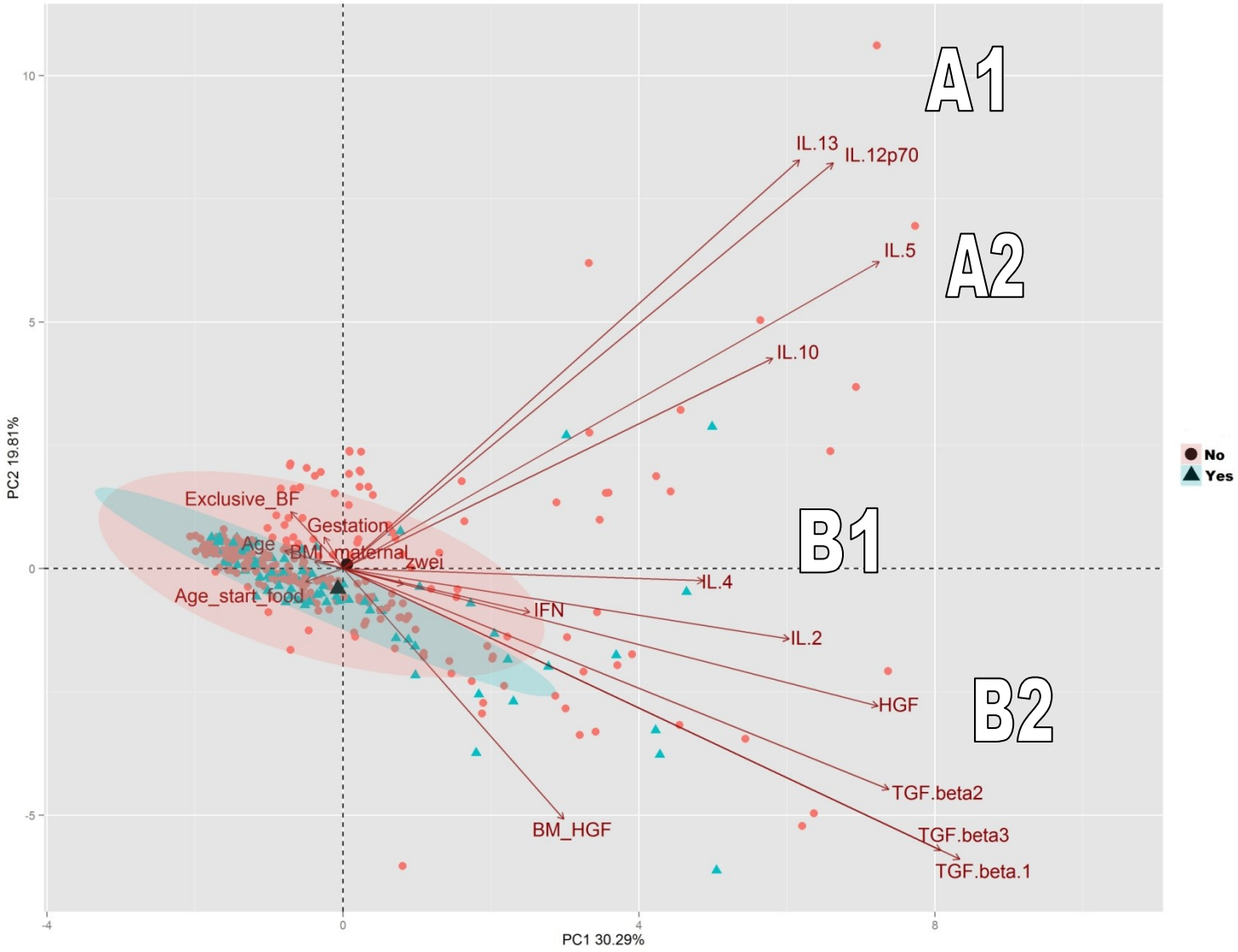


Figure 20 PCA with Eczematous rash reported outcome plot

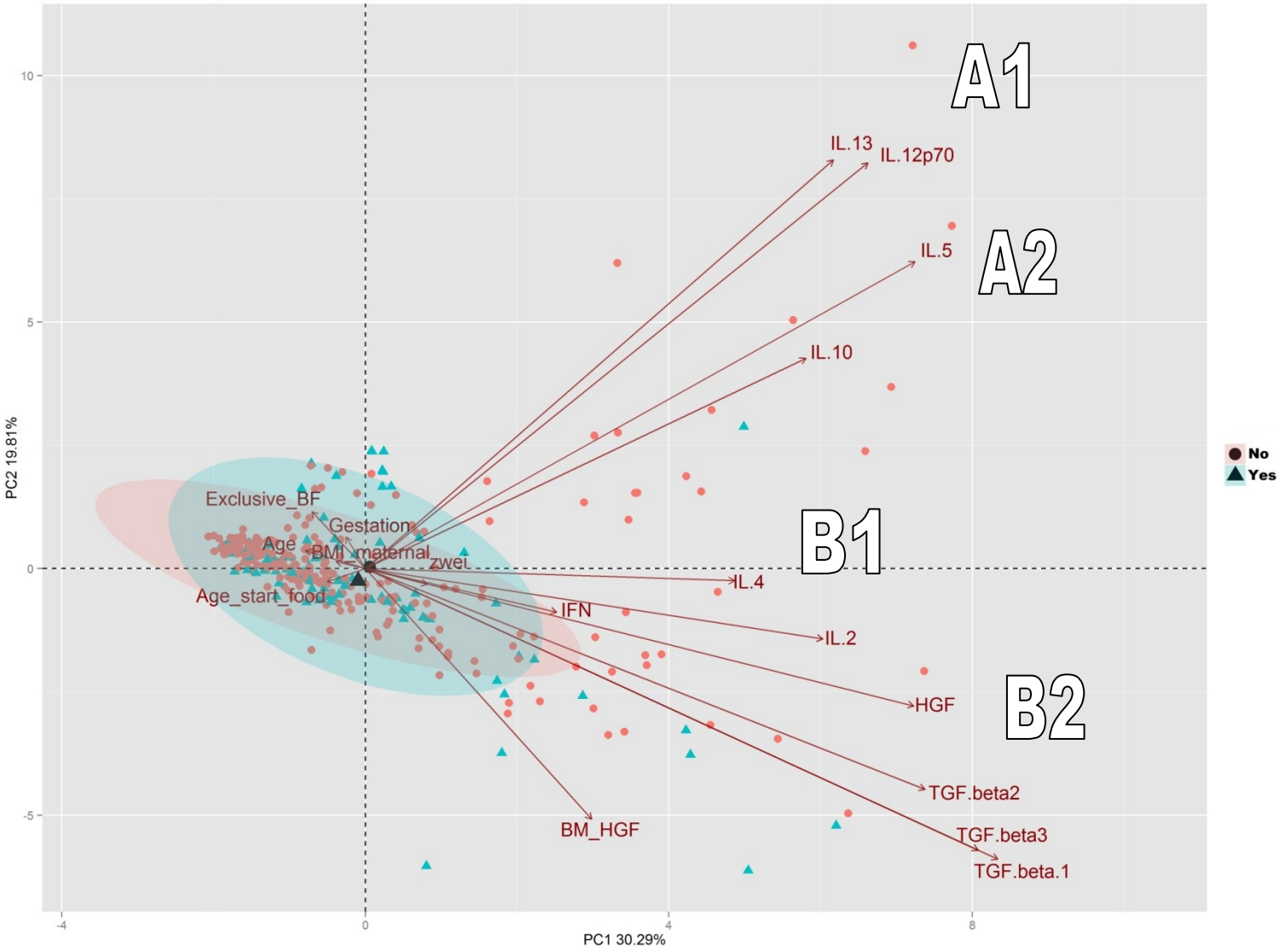


Table 25 Correlation between breast milk composition components

	HGF	IFN-γ	IL10	IL12	IL13	IL2	IL4	IL5	TGFβ1	TGFβ2	TGFβ3	Protein	Na	Colostrum collection time
HGF		0,31**	0,53**	0,19**	0,20**	0,29**	0,12*	0,35**	0,58**	0,64**	0,72**	0,70**	0,66**	-0,39**
IFN-γ	0,31**		0,31**	0,31**	0,32**	0,58**	0,30**	0,28**	0,19**	0,27**	0,22**	0,24**	0,25**	-0,18**
IL10	0,53**	0,31**		0,33**	0,38**	0,30**	0,19**	0,35**	0,33**	0,44**	0,46**	0,40**	0,47**	-0,25**
IL12	0,19**	0,31**	0,33**		0,58**	0,23**	0,56**	0,41**	0,09 NS	0,12*	0,11 NS	0,09 NS	0,15*	-0,09 NS
IL13	0,20**	0,32**	0,38**	0,58**		0,20**	0,25**	0,44**	0,17**	0,23**	0,18**	0,09 NS	0,14*	-0,15*
IL2	0,29**	0,58**	0,30**	0,23**	0,20**		0,30**	0,38**	0,18**	0,25**	0,24**	0,21**	0,22**	-0,11 NS
IL4	0,12*	0,30**	0,19**	0,56**	0,25**	0,30**		0,17**	-0,02 NS	0,05 NS	0,02 NS	0,07 NS	0,13*	-0,04 NS
IL5	0,35**	0,28**	0,35**	0,41**	0,44**	0,38**	0,17**		0,22**	0,21**	0,30**	0,27**	0,26**	-0,18**
TGFβ1	0,59**	0,19**	0,33**	0,09 NS	0,17**	0,18**	-0,02 NS	0,22**		0,79**	0,77**	0,39**	0,44**	-0,21**
TGFβ2	0,64**	0,27**	0,44**	0,12*	0,23**	0,25**	0,05 NS	0,21**	0,79**		0,72**	0,44**	0,53**	-0,16*
TGFβ3	0,72**	0,22**	0,46**	0,11 NS	0,18**	0,24**	0,02 NS	0,30**	0,77**	0,72**		0,56**	0,58**	-0,35**
Protein	0,70**	0,24**	0,40**	0,09 NS	0,09 NS	0,21**	0,07 NS	0,27**	0,39**	0,44**	0,56**		0,72**	-0,42**
Na	0,66**	0,25**	0,47**	0,15*	0,14*	0,22**	0,13*	0,26**	0,44**	0,53**	0,58**	0,72**		-0,38**
Colostrum collection time	-0,39**	-0,18**	-0,25**	-0,09 NS	-0,15*	-0,11 NS	-0,04 NS	-0,18**	-0,21**	-0,16*	-0,35**	-0,42**	-0,38**	

* p value <0.05; ** p value <0.01; NS - No statistical significance

6.5 Discussion

Breast milk research comprises a number of studies focused on immune active composition with investigators usually focusing on a particular pathway of interest. In this study I attempted to assess data on the colostrum immunological composition using PCA. It is likely that a combination of factors rather than a single immune active molecule have a higher probability to play a major role in gut immunity maturation and immune modulation during first months of life. Whether women can be classified according to a particular breast milk immune composition pattern, remains unknown. This is the first attempt, to define breast milk "lactotypes".

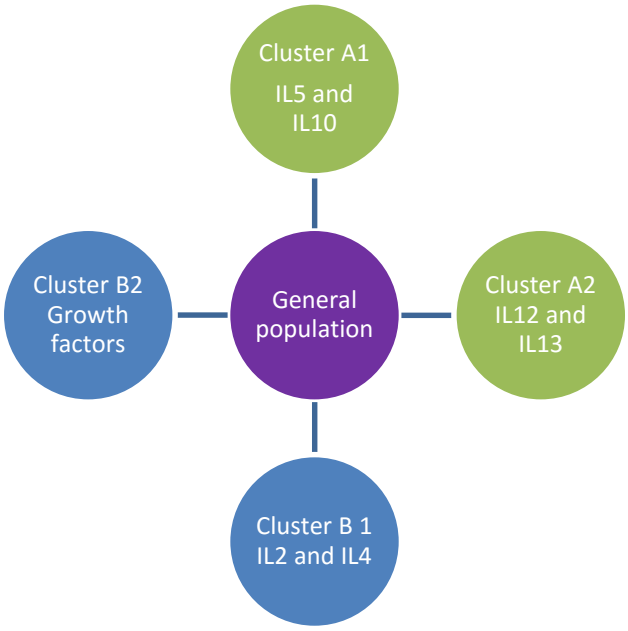
Although it is of an undoubted importance to study and analyse single factors of a particular interest, cluster analysis is also required. Among the large number of studies done in the field of breast milk research few have used PCA or similar cluster analysis approaches. Cabrera-Rubio et al. studied human milk microbiome, applying PCA analysis (Cabrera-Rubio et al., 2012) and Johansson and co-authors assessed PUFA's levels association with allergy in mothers, providing an intriguing insight into this topic (Johansson et al., 2011).

6.5.1 Lactotypes

In this work I attempted to highlight groups of mothers which can be characterised by a certain immune profile of their breast milk. As it can be seen from the PCA plot (*Figure 18*) mothers can be clustered according to the trends seen in their breast milk composition and following groups can be visualised (*Figure 22*):

- General population group
- IL5 and IL10 group (Cluster A1)
- IL12 and IL13 group (Cluster A2)
- IL2 and IL4 group (Cluster B1)
- Growth factors group (Cluster B2)

Figure 22 Lactotypes concept



It is very important to understand that if woman has high levels of one cytokine in her breast milk it does not mean that other immune active molecules in her breast milk will be present in lower concentrations. At the same time, women from Cluster A groups are not able to share high levels of immune active molecules of Cluster B groups.

It is difficult to explain how some of the immune active molecules cluster in a particular way. IL5 andIL10 are clustered together, although IL10 is known to inhibit production of IL-5 by Th2 cells (Del Prete et al., 1993). Although IL12 and IL13 have opposite vectors of action they grouped together in colostrum. It is known that IL13 is a potent enhancer of IL12 production (D'Andrea et al., 1995).

Two other clusters are more expected as IL2 and IL4 are members of a same cytokine family and may synergize in a certain circumstances (Steinke and Borish, 2001). All growth factors are grouped together which is also explainable, as TGFβ 1,2 and 3 are members of the same family and HGF activity is closely linked with TGFβ activity (Nakamura and Mizuno, 2010).

The results provide a new angle on breast milk composition and suggest that it can be grouped according to predominance of immune active molecules. Future research should be focused on attempts to clarify the groups depending on their breast milk profile and assess any environmental and maternal factors, as well as short and long term health outcomes associated with these groups.

6.5.2 Correlation heat map

Knowledge on correlations can provide complimentary information to PCA analysis. As it can be seen from the *Table 25* most of the immune active molecules in colostrum

correlate one with another, although the strength of these correlations varies. All immune active molecules are negatively correlated with the time of collection, as previously discussed.

Growth factors are moderately to strongly correlated, and there is a similar pattern on PCA plot. It is expected, for TGFβ 1,2 and 3 as they are different isoforms of the same protein. HGF is also strongly correlated with TGFβ, most probably associated with complimentary biological activity. Growth factors do not correlate or have a very weak correlation with cytokines apart from IL10. Weak to moderate correlation between TGFβ and IL10 can be explained by their cooperation in T reg. responses (Jutel et al., 2003).

Another significant and moderate correlation to be noted is IL4 with IL12. It is known that IL4 can synergise with IL12 (Bream et al., 2003) and this moderate correlation allows us to assume that they are involved in similar teamwork within colostrum. IL12 is also moderately correlated with IL13 and to a lesser extent with IL5. Future research on cytokine correlations in conjunction with PCA models will allow assessment of patterns predominant for the general population and highlight specific groups differing from the reference range.

6.5.3 Immunological outcomes associated with "lactotypes"

Data analysis using PCA facilitates evaluation of health outcomes associations with "lactotypes". *Figure 19* shows that infants with adverse immunological outcomes reported at one year of age are often located in the PCA Dim.2 negative coordinate and associated with the cluster B2 "growth factors group", especially colostrum HGF levels. The same applies to eczematous rash (*Figure 21*) and cough or wheeze (*Figure 20*) when assessed individually. At the same time it seems that colostrum with profile

clusters A1 and A2 have a more prominent protective effect on cough or wheeze development.

These results correlate well with the data from Chapter 3, where using mixed models higher HGF was associated with more maternal reported eczematous rash and episodes of food allergy/sensitivity/intolerance.

Most of the studies showed some protective effect of TGFβ on immunological outcomes development (Oddy and Rosales, 2010), but data is far from conclusive as study design and outcomes assessed differ one from another. It may be explained by a certain difference in TGFβ isoforms function as it has been shown that TGFβ3 is different from TGFβ1 and 2 when reviewed as a protein structure, with a particular difference present in the structure of the active domain (Laverty et al., 2009). When mammary gland involution has been studied TGFβ3 has been found to be up-regulated by milk stasis, and not by circulating concentration, and induces apoptosis in mammary gland epithelium (Nguyen and Pollard, 2000). TGFβ1 and 2 do not seem to possess similar capabilities. Differences in TGFβ isoforms function have been later reported in cutaneous scarring experiments (Ferguson and O'Kane, 2004) in animal models (Whitby and Ferguson, 1991a) and in human, with TGFβ3 isoform associated with improvement in scarring (Ferguson et al., 2009).

Potential action in breast milk leading to an increased risk of eczematous rash development as well as cough or wheeze may be similar to HGF function in skin shown in mice models (Kurz et al., 2002). HGF induced migration of Langerhans cells from mouse epidermis in skin biopsy assays.

Interestingly, samples collected in London has a tendency to fall into clusters B1 and B2 much more often than samples collected in Moscow and Verona. It is not clear what are the reasons leading to this tendency. Clusters B1 and B2 are associated with increased potential allergy outcomes, but it remains to be established whether this is a cause and effect relationship. It could equally be an epi-phenomenon as allergy overall is more common in UK. The gene/environment interactions which contribute to increased risk of allergy could in parallel also change BM cytokine profiles without influencing allergy outcomes. A within population intervention study will be required to clarify this.

6.6 Conclusion

The results do not give a clear picture of breast milk composition but provide a different approach to breast milk analysis. Human milk has a very complex composition, full of wide range of immunological markers and many other important components. In this study I attempted to use a different approach to data analysis focusing on a combination of factors, rather than single factors. Lactating women can be divided into groups, according to specific individual characteristics of their milk. Future studies should address this issue on larger numbers to identify lactotypes associated with different immunological outcomes.

Additional approaches to cluster analysis should be considered for future research in attempts to get the best quality of statistical analysis and potentially more meaningful results.

7 Final summary

Allergic diseases such as asthma, eczema, hay-fever and food allergy are the commonest chronic diseases of childhood in many countries, and there is evidence that early life events, such as variations in breastfeeding patterns, maternal diet, environmental and microbial exposures may be important in their development. There are still a number of hurdles to overcome before we come to a clear understanding on how to translate these associations into clinical practice, because association is not synonymous with cause and effect.. There is some evidence that probiotic (Prescott et al., 2008, Boyle et al., 2011, Rautava et al., 2002) administration to pregnant and lactating women or high fish intake (Urwin et al., 2012) alters breast milk immune composition. Although the specific changes identified are not always correlated with clinical outcomes, maternal supplementation during pregnancy and lactation to enhance breast milk quality may have a beneficial influence on health outcomes, and modulation of breast milk composition is the most likely mechanism (Dotterud et al., 2010).

The possibility that interventions which modify maternal immunity can impact infant immune responses by changing breast-milk composition is supported by associations between breast milk composition and allergic outcomes (Jones et al., 2002a, Savilahti et al., 2005). Variations in breast milk immune composition (and the infant's response to breast milk immune constituents) may also explain some of the conflicting results of studies evaluating whether prolonged exclusive breast-feeding can prevent allergic disease (Hong et al., 2011, Kramer and Kakuma, 2012). There are over 250 proteins in human breast milk, including a wide variety of cytokines, inflammatory mediators, signaling molecules, and soluble receptors (D'Alessandro et al., 2010). Yet there is only

a limited literature on the relationship between maternal diet, breast milk immune constituents and allergy development.

In this prospective birth cohort study mothers were recruited from three cities, in countries, with differing climate, genetics, and diet. Environmental and/or maternal exposures were assessed in relation to colostrum and BM immune composition and in turn on health outcomes at 6 and 12 months of age. The sequence of investigations is described in *Figure 23* and *Figure 24.*

Figure 23 Study process

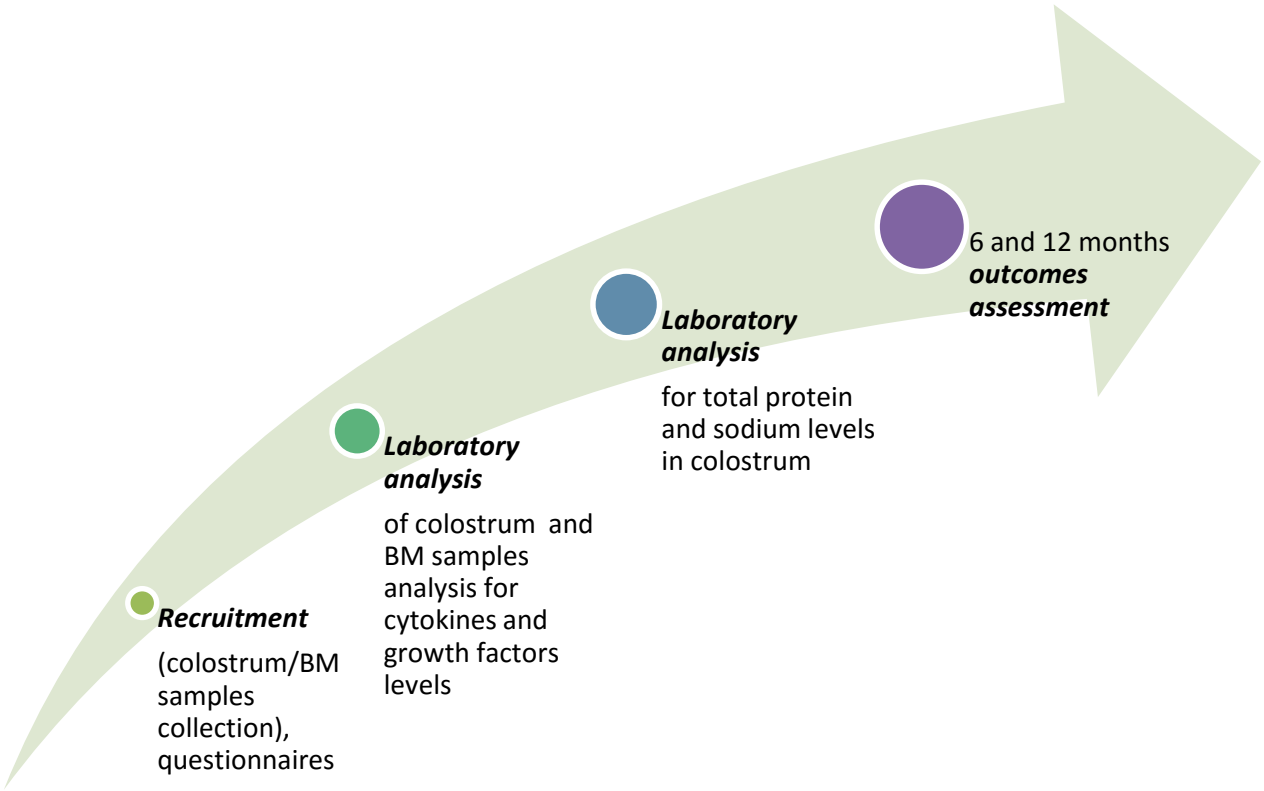
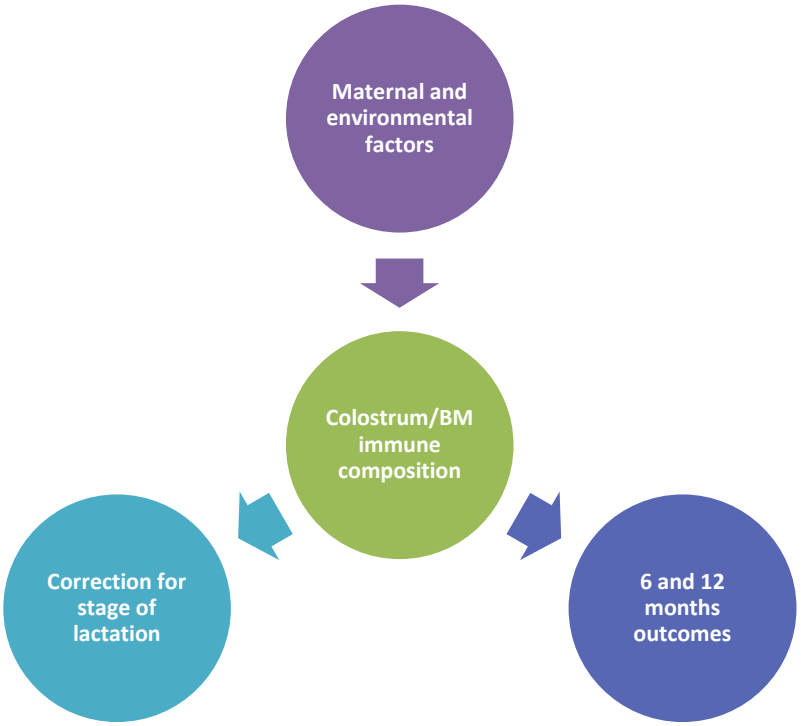


Figure 24 Study logics



Published studies suggest that a number of exposures can influence colostrum and BM composition, and my data supports this concept. Among them, the most influential factor leading to a significant decline of immune active molecules in colostrum is time of collection. This finding is in agreement with many studies reporting similar patterns (Striker et al., 2004, Urwin et al., 2012). This factor is crucial and should be taken into account in interpreting results from studies. It is known that levels of cytokines in colostrum are higher than in BM as it has been previously reported (Ustundag et al., 2005, Rigotti et al., 2006, Peroni et al., 2010) and confirmed by my data.

It is unclear whether higher concentrations in colostrum are due to low volume with consistent absolute levels or due to differences in active transport of the immune modulators into colostrum. I attempted to correct for the stage of lactation, to normalise immune active molecules decline over time. Standardisation using the levels of total protein and sodium were potential correction molecules for the stage of lactation. However, using ratios of immune modulator to protein or sodium failed to account for

the stage of lactation. However, using this approach the absolute and relative declines of immune active molecules over a period of time varied particularly in relation to TGFβ isotype. As it is assumed that TGFβ has biological relevance and is active in the infant gut (Ogawa et al., 2004), these results suggest that infants may need relatively higher amounts of TGFβ 1 and 2 in the first week of life. TGFβ3 on the other hand, in relation to total protein, had a different pattern with increasing ratio over time. This may be explained by TGFβ3 being significantly different from TGFβ1 and 2 in its detailed tertiary structure of the active domain despite homology in amino acid sequence (Laverty et al., 2009). Existing data (Laverty et al., 2009) provides some evidence to suggest that TGFβ3 isoform may be distinct in its' functions to other TGFβ isoforms. These differences have been observed in cutaneous scarring experiments (Ferguson and O'Kane, 2004). Mammalian embryos heal with no scarring and full skin recovery (Whitby and Ferguson, 1991b) in association with expression of high levels of TGFβ3 and low levels of TGFβ1 and 2 (Whitby and Ferguson, 1991a). Similar TGFβ3 function have been shown in human with this isoform associated with improvement in scarring (Ferguson et al., 2009). TGFβ3 have been shown to be up-regulated by milk stasis, and not by circulating concentration, and induces apoptosis in mammary gland epithelium during involution, in contrast to TGFβ1 and 2 (Nguyen and Pollard, 2000), which may explain variations in TGFβ isoforms decline over time.

Another exposure influencing colostrum/breast milk constituents is country of collection. Significant differences were apparent for TGFβ1,2 and 3 levels being highest in milk of mothers residing in London and HGF concentration lowest in colostrum/BM of mothers from Verona. The explanation for this phenomenon is not due to variations in mode of delivery, parity, maternal age, maternal diet, maternal atopy, smoking pet or mould exposure between sites. Other studies also found a significant difference of BM

immunological profiles between lactating mothers residing in different countries (Amoudruz et al., 2009, Holmlund et al., 2010, Peroni et al., 2010), but there is no clear understanding what caused the differences. A possible explanation is that bacterial exposure in the three countries is different which, in turn could lead to changes in BM immune composition. It may be also explained by differences in maternal diet, which was only assessed relatively superficially and should be improved in future research.

Further work is needed to understand the reasons for variations between sites in human milk composition. There is a trend for parity to influence colostrum/BM immune composition, with higher HGF and TGFβ3 levels in primiparous mothers. Similar patterns have been found in a few other studies (Walter et al., 2009, Amoudruz et al., 2009, Groer and Shelton, 2009). If parity is associated with differences in human milk composition it may be an additional mechanism to explain decreased allergy risk with an increase of birth order, but more work on a larger sample size is needed.

An additional approach to assessing the impact of human milk constituents on outcomes is to consider the composition as a soup or “lacto-type” and apply Principle Component Analysis (PCA) to analyse the data, in order to identify any existing patterns. There are clusters of immune active molecules in some colostrum samples which differ from the general population. These lactotypes, are grouped into two clusters A and B, depending on their coordinates in the PCA plot and each cluster has 2 groups within them. The final "lactotype" groups are: A1 (IL5 and IL10), A2 (IL12 and IL13), B1 (IL2 and IL4), B2 (TGFβ1,2,3 and HGF). Samples from London belong to cluster B "lactotypes" significantly more often. These results suggest that this approach applied on a larger sample size will generate hypotheses around the impact on health outcomes of different lactotypes. Mixed models analysis suggests that increased TGFβ2 levels in breast milk leads to a higher incidence of maternal reported recurrent

eczematous rash the age of 12 months. The PCA analysis identifies that lactotype B2, is associated with more adverse immunological outcomes in general and eczematous rash and cough/wheeze reported at 12 months of age. My results support the concept that IL13 may have some protective effects on both, eczematous rash development and food allergy/sensitivity/intollerance reported events and TGFβ2 may act as a risk factor, assessing health outcomes at one year of age. This could be a possible explanation for the diversity of results from epidemiological studies on the health promoting effects of breastfeeding.

Overall, the data, provides intriguing insight on colostrum and mature milk immunology, highlighting significant differences and rapid changes during the very first days of life. Differences between the countries, although not fully explainable at this point, show that women living in different geographical locations may have distinct breast milk immunological profile. In some respects, results add to the confusing picture of the impact of breast milk immune constituents in relation to maternal and environmental influence and immunological outcomes in the infants. In view of the large number of potentially immune-active constituents in breast milk, investigation of only a limited range of constituents may well produce conflicting results. Future studies comparing breast milk composition between populations should consider strict harmonisation of sampling, storage and analysis protocols especially time of sampling, between sites, as this thesis has shown time of sampling to be a strong influence on breast milk immune composition. It would also be helpful to evaluate breast milk immune composition within a group of lactating women studied at multiple time points prospectively - such studies would reduce variations caused by differences between populations and between sampling methods, although variation in storage time of milk samples could not be controlled in this way. My research highlights the importance of time of colostrum collection and underlines the need to define colostrum based on its time of collection,

and its difference from transitional and/or mature milk. Data on health outcomes, combined with PCA results show the potential importance of breast milk composition in the first year of life and points to its potential to influence short term health outcomes.

Future research needs to account for differences environmental exposures (Holmlund et al., 2010, Peroni et al., 2010, Tomicic et al., 2010) and use modern systematic methodologies to characterize variations in breast milk composition in relation to well-defined clinical and immune outcomes during childhood (Hunt et al., 2011). Statistical approaches using cluster analysis should be implemented, in order to define patterns of immune active molecules, PUFA's, microbiome composition (Lacto-types). Understanding the relationship between breast milk composition and development of non communicable diseases, and particularly allergy, may allow us to establish a new paradigm in allergy prevention research – namely modulation of breast milk composition via maternal dietary and other interventions, in order to promote healthy infant immune development.

REFERENCES

ABROL, P. 2014. ***Comparison of growth factor and immune component concentrations from women in Burundi and Italy.*** **BSc in Reproductive Science, Imperial College London.**

ACETI, A., CORVAGLIA, L., PAOLETTI, V., MARIANI, E., ANCORA, G., GALLETTI, S. & FALDELLA, G. 2009. Protein content and fortification of human milk influence gastroesophageal reflux in preterm infants. ***J Pediatr Gastroenterol Nutr,*** **49, 613-8.**

ADMYRE, C., BOHLE, B., JOHANSSON, S. M., FOCKE-TEJKL, M., VALENTA, R., SCHEYNIUS, A. & GABRIELSSON, S. 2007a. B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. ***J Allergy Clin Immunol,*** **120, 1418-24.**

ADMYRE, C., JOHANSSON, S. M., QAZI, K. R., FILEN, J. J., LAHESMAA, R., NORMAN, M., NEVE, E. P., SCHEYNIUS, A. & GABRIELSSON, S. 2007b. Exosomes with immune modulatory features are present in human breast milk. ***J Immunol,*** **179, 1969-78.**

AGARWAL, S., KARMAUS, W., DAVIS, S. & GANGUR, V. 2011. Immune markers in breast milk and fetal and maternal body fluids: a systematic review of perinatal concentrations. ***J Hum Lact,*** **27, 171-86.**

AGOSTONI, C., MARANGONI, F., GRANDI, F., LAMMARDO, A. M., GIOVANNINI, M., RIVA, E. & GALLI, C. 2003. Earlier smoking habits are associated with higher serum lipids and lower milk fat and polyunsaturated fatty acid content in the first 6 months of lactation. ***Eur J Clin Nutr,*** **57, 1466-72.**

AKOBENG, A. K., RAMANAN, A. V., BUCHAN, I. & HELLER, R. F. 2006. Effect of breast feeding on risk of coeliac disease: a systematic review and meta-analysis of observational studies. ***Arch Dis Child,*** **91, 39-43.**

AMERICAN ACADEMY OF PEDIATRICS 2012. Breastfeeding and the use of human milk. ***Pediatrics,*** **129, e827-41.**

AMERICAN ACADEMY OF PEDIATRICS COMMITTEE ON NUTRITION 2004. ***Pediatric Nutrition Handbook*****.**

AMOUDRUZ, P., HOLMLUND, U., SCHOLLIN, J., SVERREMARK-EKSTROM, E. & MONTGOMERY, S. M. 2009. Maternal country of birth and previous pregnancies are associated with breast milk characteristics. ***Pediatr Allergy Immunol,*** **20, 19-29.**

ANAND, B. S., ROMERO, J. J., SANDUJA, S. K. & LICHTENBERGER, L. M. 1999. Phospholipid association reduces the gastric mucosal toxicity of aspirin in human subjects. ***Am J Gastroenterol,*** **94, 1818-22.**

ANDO, T., HATSUSHIKA, K., WAKO, M., OHBA, T., KOYAMA, K., OHNUMA, Y., KATOH, R., OGAWA, H., OKUMURA, K., LUO, J., WYSS-CORAY, T. & NAKAO, A. 2007. Orally administered TGF-beta is biologically active in the intestinal mucosa and enhances oral tolerance. ***J Allergy Clin Immunol,*** **120, 916-23.**

ATHANASSAKIS, I., BLEACKLEY, R. C., PAETKAU, V., GUILBERT, L., BARR, P. J. & WEGMANN, T. G. 1987. The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells. ***J Immunol,*** **138, 37-44.**

ATKINSON, W., HARRIS, J., MILLS, P., MOFFAT, S., WHITE, C., LYNCH, O., JONES, M., CULLINAN, P. & NEWMAN TAYLOR, A. J. 1999. Domestic aeroallergen exposures among infants in an English town. ***Eur Respir J,*** **13, 583-9.**

BACHOUR, P., YAFAWI, R., JABER, F., CHOUEIRI, E. & ABDEL-RAZZAK, Z. 2012. Effects of smoking, mother's age, body mass index, and parity number on lipid, protein, and secretory immunoglobulin A concentrations of human milk. ***Breastfeed Med,*** **7, 179-88.**

BACHRACH, V. R., SCHWARZ, E. & BACHRACH, L. R. 2003. Breastfeeding and the risk of hospitalization for respiratory disease in infancy: a meta-analysis. ***Arch Pediatr Adolesc Med,*** **157, 237-43.**

BALLARD, O. & MORROW, A. L. 2013. Human milk composition: nutrients and bioactive factors. ***Pediatr Clin North Am,*** **60, 49-74.**

BAUER, J. & GERSS, J. 2011. Longitudinal analysis of macronutrients and minerals in human milk produced by mothers of preterm infants. ***Clin Nutr,*** **30, 215-20.**

BECKER, N., DE SANJOSE, S., NIETERS, A., MAYNADIE, M., FORETOVA, L., COCCO, P. L., STAINES, A., ALVARO, T., VORNANEN, M., BRENNAN, P. & BOFFETTA, P. 2007. Birth order, allergies and lymphoma risk: results of the European collaborative research project Epilymph. ***Leuk Res,*** **31, 1365-72.**

BERGMANN, R. L., SCHULZ, J., GUNTHER, S., DUDENHAUSEN, J. W., BERGMANN, K. E., BAUER, C. P., DORSCH, W., SCHMIDT, E., LUCK, W., LAU, S. & ET AL. 1995. Determinants of cord-blood IgE concentrations in 6401 German neonates. ***Allergy,*** **50, 65-71.**

BERKMAN, N., JOHN, M., ROESEMS, G., JOSE, P. J., BARNES, P. J. & CHUNG, K. F. 1995. Inhibition of macrophage inflammatory protein-1 alpha expression by IL-10.

Differential sensitivities in human blood monocytes and alveolar macrophages. *J Immunol,* 155, 4412-8.

BERKMAN, N., ROBICHAUD, A., KRISHNAN, V. L., ROESEMS, G., ROBBINS, R., JOSE, P. J., BARNES, P. J. & CHUNG, K. F. 1996. Expression of RANTES in human airway epithelial cells: effect of corticosteroids and interleukin-4, -10 and -13. *Immunology,* 87, 599-603.

BEZKOROVAINY, A. 2001. Probiotics: determinants of survival and growth in the gut. *Am J Clin Nutr,* 73, 399S-405S.

BILLINGTON, W. D. 1992. The normal fetomaternal immune relationship. *Baillieres Clin Obstet Gynaecol,* 6, 417-38.

BOCCI, V., VON BREMEN, K., CORRADESCHI, F., FRANCHI, F., LUZZI, E. & PAULESU, L. 1993. Presence of interferon-gamma and interleukin-6 in colostrum of normal women. *Lymphokine Cytokine Res,* 12, 21-4.

BODE, L. 2009. Human milk oligosaccharides: prebiotics and beyond. *Nutr Rev,* 67 Suppl 2, S183-91.

BOGEN, S. A., FOGELMAN, I. & ABBAS, A. K. 1993. Analysis of IL-2, IL-4, and IFN-gamma-producing cells in situ during immune responses to protein antigens. *J Immunol,* 150, 4197-205.

BORSUTZKY, S., CAZAC, B. B., ROES, J. & GUZMAN, C. A. 2004. TGF-beta receptor signaling is critical for mucosal IgA responses. *J Immunol,* 173, 3305-9.

BOTTCHER, M. F., ABRAHAMSSON, T. R., FREDRIKSSON, M., JAKOBSSON, T. & BJORKSTEN, B. 2008. Low breast milk TGF-beta2 is induced by Lactobacillus reuteri supplementation and associates with reduced risk of sensitization during infancy. *Pediatr Allergy Immunol,* 19, 497-504.

BOTTCHER, M. F., JENMALM, M. C. & BJORKSTEN, B. 2003. Cytokine, chemokine and secretory IgA levels in human milk in relation to atopic disease and IgA production in infants. *Pediatr Allergy Immunol,* 14, 35-41.

BOTTCHER, M. F., JENMALM, M. C., GAROFALO, R. P. & BJORKSTEN, B. 2000. Cytokines in breast milk from allergic and nonallergic mothers. *Pediatr Res,* 47, 157-62.

BOYLE, R. J., ISMAIL, I. H., KIVIVUORI, S., LICCIARDI, P. V., ROBINS-BROWNE, R. M., MAH, L. J., AXELRAD, C., MOORE, S., DONATH, S., CARLIN, J. B., LAHTINEN, S. J. & TANG, M. L. 2011. Lactobacillus GG treatment during pregnancy for the prevention of eczema: a randomized controlled trial. *Allergy,* 66, 509-16.

BRANDTZAEG, P. 2010. The mucosal immune system and its integration with the mammary glands. *J Pediatr,* 156, S8-15.

BREAM, J. H., CURIEL, R. E., YU, C. R., EGWUAGU, C. E., GRUSBY, M. J., AUNE, T. M. & YOUNG, H. A. 2003. IL-4 synergistically enhances both IL-2- and IL-12-induced IFN-gamma expression in murine NK cells. *Blood,* 102, 207-14.

BREW, B. K., ALLEN, C. W., TOELLE, B. G. & MARKS, G. B. 2011. Systematic review and meta-analysis investigating breast feeding and childhood wheezing illness. *Paediatr Perinat Epidemiol,* 25, 507-18.

BRYAN, D. L., FORSYTH, K. D., GIBSON, R. A. & HAWKES, J. S. 2006. Interleukin-2 in human milk: a potential modulator of lymphocyte development in the breastfed infant. *Cytokine,* 33, 289-93.

BRYAN, D. L., HART, P. H., FORSYTH, K. D. & GIBSON, R. A. 2007. Immunomodulatory constituents of human milk change in response to infant bronchiolitis. *Pediatr Allergy Immunol,* 18, 495-502.

BRYAN, D. L., HAWKES, J. S. & GIBSON, R. A. 1999. Interleukin-12 in human milk. *Pediatr Res,* 45, 858-9.

BURNHAM, K. & DR, A. 2004. Multimodel Inference: Understanding AIC and BIC in Model Selection. *Sociological Methods and Research,* 33, 261-304.

CABRERA-RUBIO, R., COLLADO, M. C., LAITINEN, K., SALMINEN, S., ISOLAURI, E. & MIRA, A. 2012. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr,* 96, 544-51.

CARLSON, S. E., MONTALTO, M. B., PONDER, D. L., WERKMAN, S. H. & KORONES, S. B. 1998. Lower incidence of necrotizing enterocolitis in infants fed a preterm formula with egg phospholipids. *Pediatr Res,* 44, 491-8.

CASTELLOTE, C., CASILLAS, R., RAMIREZ-SANTANA, C., PEREZ-CANO, F. J., CASTELL, M., MORETONES, M. G., LOPEZ-SABATER, M. C. & FRANCH, A. 2011. Premature delivery influences the immunological composition of colostrum and transitional and mature human milk. *J Nutr,* 141, 1181-7.

CERUTTI, A., ZAN, H., SCHAFFER, A., BERGSAGEL, L., HARINDRANATH, N., MAX, E. E. & CASALI, P. 1998. CD40 ligand and appropriate cytokines induce switching to IgG, IgA, and IgE and coordinated germinal center and plasmacytoid phenotypic differentiation in a human monoclonal IgM+IgD+ B cell line. *J Immunol,* 160, 2145-57.

CHIEN, P. F. & HOWIE, P. W. 2001. Breast milk and the risk of opportunistic infection in infancy in industrialized and non-industrialized settings. *Adv Nutr Res,* 10, 69-104.

CHONMAITREE, T., ALVAREZ-FERNANDEZ, P., JENNINGS, K., TRUJILLO, R., MAROM, T., LOEFFELHOLZ, M. J., MILLER, A. L., MCCORMICK, D. P., PATEL, J. A. & PYLES, R. B. 2014. Symptomatic and Asymptomatic Respiratory Viral Infections in the First Year of Life: Association With Acute Otitis Media Development. ***Clin Infect Dis*****.**

CHOWANADISAI, W. & LONNERDAL, B. 2002. Alpha(1)-antitrypsin and antichymotrypsin in human milk: origin, concentrations, and stability. ***Am J Clin Nutr,*** **76, 828-33.**

CHRIST, M., MCCARTNEY-FRANCIS, N. L., KULKARNI, A. B., WARD, J. M., MIZEL, D. E., MACKALL, C. L., GRESS, R. E., HINES, K. L., TIAN, H., KARLSSON, S. & ET AL. 1994. Immune dysregulation in TGF-beta 1-deficient mice. ***J Immunol,*** **153, 1936-46.**

CHUNG, K. F., PATEL, H. J., FADLON, E. J., ROUSELL, J., HADDAD, E. B., JOSE, P. J., MITCHELL, J. & BELVISI, M. 1999. Induction of eotaxin expression and release from human airway smooth muscle cells by IL-1beta and TNFalpha: effects of IL-10 and corticosteroids. ***Br J Pharmacol,*** **127, 1145-50.**

COCKRILL, T., DEL JUNCO, D. J., ARNETT, F. C., ASSASSI, S., TAN, F. K., MCNEARNEY, T., FISCHBACH, M., PERRY, M. & MAYES, M. D. 2010. Separate influences of birth order and gravidity/parity on the development of systemic sclerosis. ***Arthritis Care Res (Hoboken),*** **62, 418-24.**

COFFMAN, R. L., OHARA, J., BOND, M. W., CARTY, J., ZLOTNIK, A. & PAUL, W. E. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. ***J Immunol,*** **136, 4538-41.**

COPPA, G. V., GABRIELLI, O., PIERANI, P., CATASSI, C., CARLUCCI, A. & GIORGI, P. L. 1993. Changes in carbohydrate composition in human milk over 4 months of lactation. ***Pediatrics,*** **91, 637-41.**

CULLINAN, P. 2006. Childhood allergies, birth order and family size. ***Thorax,*** **61, 3-5.**

CUMMINS, A. G. & THOMPSON, F. M. 2002. Effect of breast milk and weaning on epithelial growth of the small intestine in humans. ***Gut,*** **51, 748-54.**

D'ALESSANDRO, A., SCALONI, A. & ZOLLA, L. 2010. Human milk proteins: an interactomics and updated functional overview. ***J Proteome Res,*** **9, 3339-73.**

D'ANDREA, A., MA, X., ASTE-AMEZAGA, M., PAGANIN, C. & TRINCHIERI, G. 1995. Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production. ***J Exp Med,*** **181, 537-46.**

D'SOUZA, W. N. & LEFRANCOIS, L. 2003. IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. ***J Immunol,*** **171, 5727-35.**

DA SILVA, J. A. & SPECTOR, T. D. 1992. The role of pregnancy in the course and aetiology of rheumatoid arthritis. ***Clin Rheumatol,*** **11, 189-94.**

DABBAGH, K., TAKEYAMA, K., LEE, H. M., UEKI, I. F., LAUSIER, J. A. & NADEL, J. A. 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. ***J Immunol,*** **162, 6233-7.**

DARRAGH, A. & LÖNNERDAL, B. 2011. Human Milk. ***Encyclopedia of Dairy Sciences (Second Edition).*** **Elsevier.**

DE ROOS, B. 2008. Proteomic analysis of human plasma and blood cells in nutritional studies: development of biomarkers to aid disease prevention. ***Expert Rev Proteomics,*** **5, 819-26.**

DE ROOS, B. & MCARDLE, H. J. 2008. Proteomics as a tool for the modelling of biological processes and biomarker development in nutrition research. ***Br J Nutr,*** **99 Suppl 3, S66-71.**

DEL PRETE, G., DE CARLI, M., ALMERIGOGNA, F., GIUDIZI, M. G., BIAGIOTTI, R. & ROMAGNANI, S. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. ***J Immunol,*** **150, 353-60.**

DENNLER, S., GOUMANS, M. J. & TEN DIJKE, P. 2002. Transforming growth factor beta signal transduction. ***J Leukoc Biol,*** **71, 731-40.**

DIBIASIE, A. 2006. Evidence-based review of retinopathy of prematurity prevention in VLBW and ELBW infants. ***Neonatal Netw,*** **25, 393-403.**

DOGARU, C. M., NYFFENEGGER, D., PESCATORE, A. M., SPYCHER, B. D. & KUEHNI, C. E. 2014. Breastfeeding and childhood asthma: systematic review and meta-analysis. ***Am J Epidemiol,*** **179, 1153-67.**

DONOVAN, S. M. 2009. Human milk oligosaccharides - the plot thickens. ***Br J Nutr,*** **101, 1267-9.**

DONOVAN, S. M. & ODLE, J. 1994. Growth factors in milk as mediators of infant development. ***Annu Rev Nutr,*** **14, 147-67.**

DOTTERUD, C. K., STORRO, O., JOHNSEN, R. & OIEN, T. 2010. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. ***Br J Dermatol,*** **163, 616-23.**

DOUCET, C., BROUTY-BOYE, D., POTTIN-CLEMENCEAU, C., JASMIN, C., CANONICA, G. W. & AZZARONE, B. 1998. IL-4 and IL-13 specifically increase

adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int Immunol,* 10, 1421-33.

DUCHEN, K., CASAS, R., FAGERAS-BOTTCHER, M., YU, G. & BJORKSTEN, B. 2000. Human milk polyunsaturated long-chain fatty acids and secretory immunoglobulin A antibodies and early childhood allergy. *Pediatr Allergy Immunol,* 11, 29-39.

DUIJTS, L., RAMADHANI, M. K. & MOLL, H. A. 2009. Breastfeeding protects against infectious diseases during infancy in industrialized countries. A systematic review. *Matern Child Nutr,* 5, 199-210.

DUNSTAN, J. A., MORI, T. A., BARDEN, A., BEILIN, L. J., TAYLOR, A. L., HOLT, P. G. & PRESCOTT, S. L. 2003. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. *J Allergy Clin Immunol,* 112, 1178-84.

DURHAM, S. R. & TILL, S. J. 1998. Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol,* 102, 157-64.

DVORAK, B., HALPERN, M. D., HOLUBEC, H., WILLIAMS, C. S., MCWILLIAM, D. L., DOMINGUEZ, J. A., STEPANKOVA, R., PAYNE, C. M. & MCCUSKEY, R. S. 2002. Epidermal growth factor reduces the development of necrotizing enterocolitis in a neonatal rat model. *Am J Physiol Gastrointest Liver Physiol,* 282, G156-64.

EGLINTON, B. A., ROBERTON, D. M. & CUMMINS, A. G. 1994. Phenotype of T cells, their soluble receptor levels, and cytokine profile of human breast milk. *Immunol Cell Biol,* 72, 306-13.

EHLERS, S. & SMITH, K. A. 1991. Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. *J Exp Med,* 173, 25-36.

ELLIOTT, L., HENDERSON, J., NORTHSTONE, K., CHIU, G. Y., DUNSON, D. & LONDON, S. J. 2008. Prospective study of breast-feeding in relation to wheeze, atopy, and bronchial hyperresponsiveness in the Avon Longitudinal Study of Parents and Children (ALSPAC). *J Allergy Clin Immunol,* 122, 49-54, 54 e1-3.

ENKE, U., SEYFARTH, L., SCHLEUSSNER, E. & MARKERT, U. R. 2008. Impact of PUFA on early immune and fetal development. *Br J Nutr,* 100, 1158-68.

ERMIS, B., YILDIRIM, A., ORS, R., TASTEKIN, A., OZKAN, B. & AKCAY, F. 2005. Influence of smoking on serum and milk malondialdehyde, superoxide dismutase, glutathione peroxidase, and antioxidant potential levels in mothers at the postpartum seventh day. *Biol Trace Elem Res,* 105, 27-36.

FEACHEM, R. G. & KOBLINSKY, M. A. 1984. Interventions for the control of diarrhoeal diseases among young children: promotion of breast-feeding. ***Bull World Health Organ,*** **62, 271-91.**

FEHNIGER, T. A. & CALIGIURI, M. A. 2001. Interleukin 15: biology and relevance to human disease. ***Blood,*** **97, 14-32.**

FEKETEA, G. 2014. ***Investigation of the relationship between serum and breast milk immune factors, and their change over time.*** **MSc in Allergy, Imperial College London.**

FERGUSON, M. W., DUNCAN, J., BOND, J., BUSH, J., DURANI, P., SO, K., TAYLOR, L., CHANTREY, J., MASON, T., JAMES, G., LAVERTY, H., OCCLESTON, N. L., SATTAR, A., LUDLOW, A. & O'KANE, S. 2009. Prophylactic administration of avotermin for improvement of skin scarring: three double-blind, placebo-controlled, phase I/II studies. ***Lancet,*** **373, 1264-74.**

FERGUSON, M. W. & O'KANE, S. 2004. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. ***Philos Trans R Soc Lond B Biol Sci,*** **359, 839-50.**

FILIPP, D., ALIZADEH-KHIAVI, K., RICHARDSON, C., PALMA, A., PAREDES, N., TAKEUCHI, O., AKIRA, S. & JULIUS, M. 2001. Soluble CD14 enriched in colostrum and milk induces B cell growth and differentiation. ***Proc Natl Acad Sci U S A,*** **98, 603-8.**

FOISY, M., BOYLE, R. J., CHALMERS, J. R., SIMPSON, E. L. & WILLIAMS, H. C. 2011. Overview of Reviews The prevention of eczema in infants and children: an overview of Cochrane and non-Cochrane reviews. ***Evid Based Child Health,*** **6, 1322-1339.**

FOMON, S. 2001. Infant feeding in the 20th century: formula and beikost. ***J Nutr,*** **131, 409S-20S.**

GAROFALO, R. 2010. Cytokines in human milk. ***J Pediatr,*** **156, S36-40.**

GAROFALO, R., CHHEDA, S., MEI, F., PALKOWETZ, K. H., RUDLOFF, H. E., SCHMALSTIEG, F. C., RASSIN, D. K. & GOLDMAN, A. S. 1995. Interleukin-10 in human milk. ***Pediatr Res,*** **37, 444-9.**

GAROFALO, R. P. & GOLDMAN, A. S. 1998. Cytokines, chemokines, and colony-stimulating factors in human milk: the 1997 update. ***Biol Neonate,*** **74, 134-42.**

GASCAN, H., GAUCHAT, J. F., RONCAROLO, M. G., YSSEL, H., SPITS, H. & DE VRIES, J. E. 1991. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4+ T cell clones. ***J Exp Med,*** **173, 747-50.**

GAZZINELLI, R. T., WYSOCKA, M., HAYASHI, S., DENKERS, E. Y., HIENY, S., CASPAR, P., TRINCHIERI, G. & SHER, A. 1994. Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with Toxoplasma gondii. *J Immunol,* 153, 2533-43.

GDALEVICH, M., MIMOUNI, D., DAVID, M. & MIMOUNI, M. 2001a. Breast-feeding and the onset of atopic dermatitis in childhood: a systematic review and meta-analysis of prospective studies. *J Am Acad Dermatol,* 45, 520-7.

GDALEVICH, M., MIMOUNI, D. & MIMOUNI, M. 2001b. Breast-feeding and the risk of bronchial asthma in childhood: a systematic review with meta-analysis of prospective studies. *J Pediatr,* 139, 261-6.

GENE DATABASE. 2008. *HGF hepatocyte growth factor (hepapoietin A; scatter factor) [Homo sapiens (human)],* [Online]. http://www.ncbi.nlm.nih.gov/gene/3082. [Accessed 04 November 2013].

GERAGHTY, S. R., DAVIDSON, B. S., WARNER, B. B., SAPSFORD, A. L., BALLARD, J. L., LIST, B. A., AKERS, R. & MORROW, A. L. 2005. The development of a research human milk bank. *J Hum Lact,* 21, 59-66.

GIWERCMAN, C., HALKJAER, L. B., JENSEN, S. M., BONNELYKKE, K., LAURITZEN, L. & BISGAARD, H. 2010. Increased risk of eczema but reduced risk of early wheezy disorder from exclusive breast-feeding in high-risk infants. *J Allergy Clin Immunol,* 125, 866-71.

GLEAVE, M. E., HSIEH, J. T., WU, H. C., HONG, S. J., ZHAU, H. E., GUTHRIE, P. D. & CHUNG, L. W. 1993. Epidermal growth factor receptor-mediated autocrine and paracrine stimulation of human transitional cell carcinoma. *Cancer Res,* 53, 5300-7.

GREER, F. R. & APPLE, R. D. 1991. Physicians, formula companies, and advertising. A historical perspective. *Am J Dis Child,* 145, 282-6.

GROER, M. W. & SHELTON, M. M. 2009. Exercise is associated with elevated proinflammatory cytokines in human milk. *J Obstet Gynecol Neonatal Nurs,* 38, 35-41.

GRUBER, C., VAN STUIJVENBERG, M., MOSCA, F., MORO, G., CHIRICO, G., BRAEGGER, C. P., RIEDLER, J., BOEHM, G. & WAHN, U. 2010. Reduced occurrence of early atopic dermatitis because of immunoactive prebiotics among low-atopy-risk infants. *J Allergy Clin Immunol,* 126, 791-7.

GRULEE, C. & SANFORD, H. 1936. The influence of breast and artificial feeding on infantile eczema. *Journal of Pediatrics,* 9, 223-5.

GRULICH, A. E., VAJDIC, C. M., KALDOR, J. M., HUGHES, A. M., KRICKER, A., FRITSCHI, L., TURNER, J. J., MILLIKEN, S., BENKE, G. & ARMSTRONG, B. K. 2005. Birth order, atopy, and risk of non-Hodgkin lymphoma. *J Natl Cancer Inst,* 97, 587-94.

HAMILTON, J. L. & JOHN, S. P. 2013. Evaluation of fever in infants and young children. *Am Fam Physician,* 87, 254-60.

HANSON, L. A., KOROTKOVA, M., HAVERSEN, L., MATTSBY-BALTZER, I., HAHN-ZORIC, M., SILFVERDAL, S. A., STRANDVIK, B. & TELEMO, E. 2002. Breast-feeding, a complex support system for the offspring. *Pediatr Int,* 44, 347-52.

HASSAN, J. & REEN, D. J. 1996. Reduced primary antigen-specific T-cell precursor frequencies in neonates is associated with deficient interleukin-2 production. *Immunology,* 87, 604-8.

HASSELBALCH, H., ENGELMANN, M. D., ERSBOLL, A. K., JEPPESEN, D. L. & FLEISCHER-MICHAELSEN, K. 1999. Breast-feeding influences thymic size in late infancy. *Eur J Pediatr,* 158, 964-7.

HAWKES, J. S., BRYAN, D. L., NEUMANN, M. A., MAKRIDES, M. & GIBSON, R. A. 2001. Transforming growth factor beta in human milk does not change in response to modest intakes of docosahexaenoic acid. *Lipids,* 36, 1179-81.

HERBERTS, C., MELGERT, B., VAN DER LAAN, J. W. & FAAS, M. 2010. New adjuvanted vaccines in pregnancy: what is known about their safety? *Expert Rev Vaccines,* 9, 1411-22.

HOLLOWAY, J. A., THORNTON, C. A., DIAPER, N. D., HOWE, D. T. & WARNER, J. O. 2009. Phenotypic analysis of circulating dendritic cells during the second half of human gestation. *Pediatr Allergy Immunol,* 20, 119-25.

HOLMLUND, U., AMOUDRUZ, P., JOHANSSON, M. A., HAILESELASSIE, Y., ONGOIBA, A., KAYENTAO, K., TRAORE, B., DOUMBO, S., SCHOLLIN, J., DOUMBO, O., MONTGOMERY, S. M. & SVERREMARK-EKSTROM, E. 2010. Maternal country of origin, breast milk characteristics and potential influences on immunity in offspring. *Clin Exp Immunol,* 162, 500-9.

HONG, X., WANG, G., LIU, X., KUMAR, R., TSAI, H. J., ARGUELLES, L., HAO, K., PEARSON, C., ORTIZ, K., BONZAGNI, A., APOLLON, S., FU, L., CARUSO, D., PONGRACIC, J. A., SCHLEIMER, R., HOLT, P. G., BAUCHNER, H. & WANG, X. 2011. Gene polymorphisms, breast-feeding, and development of food sensitization in early childhood. *J Allergy Clin Immunol,* 128, 374-81 e2.

HOPPU, U., ISOLAURI, E., LAAKSO, P., MATOMAKI, J. & LAITINEN, K. 2012. Probiotics and dietary counselling targeting maternal dietary fat intake modifies breast milk fatty acids and cytokines. ***Eur J Nutr,*** **51, 211-9.**

HORNELL, A., LAGSTROM, H., LANDE, B. & THORSDOTTIR, I. 2013. Breastfeeding, introduction of other foods and effects on health: a systematic literature review for the 5th Nordic Nutrition Recommendations. ***Food Nutr Res,*** **57.**

HORTA B, B. R., MARTINES J, VICTORA C. . 2007. ***Evidence of the Long-Term Effects of Breastfeeding: Systematic Reviews and Meta-Analysis*****.**

HORTA, B. & VICTORA, C. 2013. Long-term effects of breastfeeding: a systematic review. ***Systematic reviews and meta-analyses.*** **WHO.**

HSIEH, C. S., MACATONIA, S. E., TRIPP, C. S., WOLF, S. F., O'GARRA, A. & MURPHY, K. M. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. ***Science,*** **260, 547-9.**

HUANG, X. H., CHEN, L., GAO, W., ZHANG, W., CHEN, S. J., XU, L. B. & ZHANG, S. Q. 2008. Specific IgG activity of bovine immune milk against diarrhea bacteria and its protective effects on pathogen-infected intestinal damages. ***Vaccine,*** **26, 5973-80.**

HUNT, K. M., FOSTER, J. A., FORNEY, L. J., SCHUTTE, U. M., BECK, D. L., ABDO, Z., FOX, L. K., WILLIAMS, J. E., MCGUIRE, M. K. & MCGUIRE, M. A. 2011. Characterization of the diversity and temporal stability of bacterial communities in human milk. ***PLoS One,*** **6, e21313.**

HURLEY, W. L. & THEIL, P. K. 2011. Perspectives on immunoglobulins in colostrum and milk. ***Nutrients,*** **3, 442-74.**

ICHIKAWA, M., SUGITA, M., TAKAHASHI, M., SATOMI, M., TAKESHITA, T., ARAKI, T. & TAKAHASHI, H. 2003. Breast milk macrophages spontaneously produce granulocyte-macrophage colony-stimulating factor and differentiate into dendritic cells in the presence of exogenous interleukin-4 alone. ***Immunology,*** **108, 189-95.**

IMHOFF, B., MORSE, D., SHIFERAW, B., HAWKINS, M., VUGIA, D., LANCE-PARKER, S., HADLER, J., MEDUS, C., KENNEDY, M., MOORE, M. R. & VAN GILDER, T. 2004. Burden of self-reported acute diarrheal illness in FoodNet surveillance areas, 1998-1999. ***Clin Infect Dis,*** **38 Suppl 3, S219-26.**

INDUMATHI, S., DHANASEKARAN, M., RAJKUMAR, J. S. & SUDARSANAM, D. 2013. Exploring the stem cell and non-stem cell constituents of human breast milk. ***Cytotechnology,*** **65, 385-93.**

IP, S., CHUNG, M., RAMAN, G., CHEW, P., MAGULA, N., DEVINE, D., TRIKALINOS, T. & LAU, J. 2007. Breastfeeding and maternal and infant health outcomes in developed countries. *Evid Rep Technol Assess (Full Rep)*, 1-186.

ISLAM, S. K., AHMED, L., KHAN, M. N., HUQUE, S., BEGUM, A. & YUNUS, A. B. 2006. Immune components (IgA, IgM, IgG, immune cells) of colostrum of Bangladeshi mothers. *Pediatr Int,* 48, 543-8.

ISMAIL, I. H., LICCIARDI, P. V., OPPEDISANO, F., BOYLE, R. J. & TANG, M. L. 2013. Relationship between breast milk sCD14, TGF-beta1 and total IgA in the first month and development of eczema during infancy. *Pediatr Allergy Immunol,* 24, 352-60.

ITO, W., KANEHIRO, A., MATSUMOTO, K., HIRANO, A., ONO, K., MARUYAMA, H., KATAOKA, M., NAKAMURA, T., GELFAND, E. W. & TANIMOTO, M. 2005. Hepatocyte growth factor attenuates airway hyperresponsiveness, inflammation, and remodeling. *Am J Respir Cell Mol Biol,* 32, 268-80.

ITO, W., TAKEDA, M., TANABE, M., KIHARA, J., KATO, H., CHIBA, T., YAMAGUCHI, K., UEKI, S., KANEHIRO, A., KAYABA, H. & CHIHARA, J. 2008. Anti-allergic inflammatory effects of hepatocyte growth factor. *Int Arch Allergy Immunol,* 146 Suppl 1, 82-7.

JARVINEN, K. M. & SUOMALAINEN, H. 2002. Leucocytes in human milk and lymphocyte subsets in cow's milk-allergic infants. *Pediatr Allergy Immunol,* 13, 243-54.

JOHANSSON, S., WOLD, A. E. & SANDBERG, A. S. 2011. Low breast milk levels of long-chain n-3 fatty acids in allergic women, despite frequent fish intake. *Clin Exp Allergy,* 41, 505-15.

JONES, C. A., HOLLOWAY, J. A., POPPLEWELL, E. J., DIAPER, N. D., HOLLOWAY, J. W., VANCE, G. H., WARNER, J. A. & WARNER, J. O. 2002a. Reduced soluble CD14 levels in amniotic fluid and breast milk are associated with the subsequent development of atopy, eczema, or both. *J Allergy Clin Immunol,* 109, 858-66.

JONES, C. A., HOLLOWAY, J. A. & WARNER, J. O. 2002b. Phenotype of fetal monocytes and B lymphocytes during the third trimester of pregnancy. *J Reprod Immunol,* 56, 45-60.

JONES, C. A., VANCE, G. H., POWER, L. L., PENDER, S. L., MACDONALD, T. T. & WARNER, J. O. 2001. Costimulatory molecules in the developing human gastrointestinal tract: a pathway for fetal allergen priming. *J Allergy Clin Immunol,* 108, 235-41.

JUTEL, M., AKDIS, M., BUDAK, F., AEBISCHER-CASAULTA, C., WRZYSZCZ, M., BLASER, K. & AKDIS, C. A. 2003. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol,* 33, 1205-14.

KALLIOMAKI, M., OUWEHAND, A., ARVILOMMI, H., KERO, P. & ISOLAURI, E. 1999. Transforming growth factor-beta in breast milk: a potential regulator of atopic disease at an early age. *J Allergy Clin Immunol,* 104, 1251-7.

KANKAANPAA, P., NURMELA, K., ERKKILA, A., KALLIOMAKI, M., HOLMBERG-MARTTILA, D., SALMINEN, S. & ISOLAURI, E. 2001. Polyunsaturated fatty acids in maternal diet, breast milk, and serum lipid fatty acids of infants in relation to atopy. *Allergy,* 56, 633-8.

KARMAUS, W., ARSHAD, H. & MATTES, J. 2001. Does the sibling effect have its origin in utero? Investigating birth order, cord blood immunoglobulin E concentration, and allergic sensitization at age 4 years. *Am J Epidemiol,* 154, 909-15.

KAY, A. B. 2001. Allergy and allergic diseases. First of two parts. *N Engl J Med,* 344, 30-7.

KERMORGANT, S., WALKER, F., HORMI, K., DESSIRIER, V., LEWIN, M. J. & LEHY, T. 1997. Developmental expression and functionality of hepatocyte growth factor and c-Met in human fetal digestive tissues. *Gastroenterology,* 112, 1635-47.

KIELIAN, T. L. & BLECHA, F. 1995. CD14 and other recognition molecules for lipopolysaccharide: a review. *Immunopharmacology,* 29, 187-205.

KIM, J. H., KIM, K. H., WOO, H. Y. & SHIM, J. Y. 2008. Maternal cytokine production during pregnancy and the development of childhood wheezing and allergic disease in offspring three years of age. *J Asthma,* 45, 948-52.

KIVINEN, A., TARPILA, S., KIVILUOTO, T., MUSTONEN, H. & KIVILAAKSO, E. 1995. Milk and egg phospholipids act as protective surfactants against luminal acid in Necturus gastric mucosa. *Aliment Pharmacol Ther,* 9, 685-91.

KNEEPKENS, C. M. & BRAND, P. L. 2010. Clinical practice : Breastfeeding and the prevention of allergy. *Eur J Pediatr.*

KOBATA, R., TSUKAHARA, H., OHSHIMA, Y., OHTA, N., TOKURIKI, S., TAMURA, S. & MAYUMI, M. 2008. High levels of growth factors in human breast milk. *Early Hum Dev,* 84, 67-9.

KOBAYASHI, M., FITZ, L., RYAN, M., HEWICK, R. M., CLARK, S. C., CHAN, S., LOUDON, R., SHERMAN, F., PERUSSIA, B. & TRINCHIERI, G. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a

cytokine with multiple biologic effects on human lymphocytes. *J Exp Med,* 170, 827-45.

KONDO, N., SUDA, Y., NAKAO, A., OH-OKA, K., SUZUKI, K., ISHIMARU, K., SATO, M., TANAKA, T., NAGAI, A. & YAMAGATA, Z. 2011. Maternal psychosocial factors determining the concentrations of transforming growth factor-beta in breast milk. *Pediatr Allergy Immunol,* 22, 853-61.

KOPF, M., BAUMANN, H., FREER, G., FREUDENBERG, M., LAMERS, M., KISHIMOTO, T., ZINKERNAGEL, R., BLUETHMANN, H. & KOHLER, G. 1994. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature,* 368, 339-42.

KRAMER, M. S. 1988. Does breast feeding help protect against atopic disease? Biology, methodology, and a golden jubilee of controversy. *J Pediatr,* 112, 181-90.

KRAMER, M. S., CHALMERS, B., HODNETT, E. D., SEVKOVSKAYA, Z., DZIKOVICH, I., SHAPIRO, S., COLLET, J. P., VANILOVICH, I., MEZEN, I., DUCRUET, T., SHISHKO, G., ZUBOVICH, V., MKNUIK, D., GLUCHANINA, E., DOMBROVSKIY, V., USTINOVITCH, A., KOT, T., BOGDANOVICH, N., OVCHINIKOVA, L. & HELSING, E. 2001. Promotion of Breastfeeding Intervention Trial (PROBIT): a randomized trial in the Republic of Belarus. *JAMA,* 285, 413-20.

KRAMER, M. S. & KAKUMA, R. 2012. Optimal duration of exclusive breastfeeding. *Cochrane Database Syst Rev,* 8, CD003517.

KUITUNEN, M., KUKKONEN, A. K. & SAVILAHTI, E. 2012. Impact of maternal allergy and use of probiotics during pregnancy on breast milk cytokines and food antibodies and development of allergy in children until 5 years. *Int Arch Allergy Immunol,* 159, 162-70.

KULKARNI, A. B., HUH, C. G., BECKER, D., GEISER, A., LYGHT, M., FLANDERS, K. C., ROBERTS, A. B., SPORN, M. B., WARD, J. M. & KARLSSON, S. 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A,* 90, 770-4.

KULSKI, J. K. & HARTMANN, P. E. 1981. Changes in human milk composition during the initiation of lactation. *Aust J Exp Biol Med Sci,* 59, 101-14.

KURZ, S. M., DIEBOLD, S. S., HIERONYMUS, T., GUST, T. C., BARTUNEK, P., SACHS, M., BIRCHMEIER, W. & ZENKE, M. 2002. The impact of c-met/scatter factor receptor on dendritic cell migration. *Eur J Immunol,* 32, 1832-8.

KVERKA, M., BURIANOVA, J., LODINOVA-ZADNIKOVA, R., KOCOURKOVA, I., CINOVA, J., TUCKOVA, L. & TLASKALOVA-HOGENOVA, H. 2007. Cytokine profiling in human colostrum and milk by protein array. *Clin Chem,* 53, 955-62.

LABETA, M. O., DURIEUX, J. J., FERNANDEZ, N., HERRMANN, R. & FERRARA, P. 1993. Release from a human monocyte-like cell line of two different soluble forms of the lipopolysaccharide receptor, CD14. *Eur J Immunol,* 23, 2144-51.

LACK, G., NELSON, H. S., AMRAN, D., OSHIBA, A., JUNG, T., BRADLEY, K. L., GICLAS, P. C. & GELFAND, E. W. 1997. Rush immunotherapy results in allergen-specific alterations in lymphocyte function and interferon-gamma production in CD4+ T cells. *J Allergy Clin Immunol,* 99, 530-8.

LADOMENOU, F., MOSCHANDREAS, J., KAFATOS, A., TSELENTIS, Y. & GALANAKIS, E. 2010. Protective effect of exclusive breastfeeding against infections during infancy: a prospective study. *Arch Dis Child,* 95, 1004-8.

LAGADARI, M., BLOIS, S., MARGNI, R. & MIRANDA, S. 2004. Analysis of macrophage presence in murine placenta: influence of age and parity status. *Am J Reprod Immunol,* 51, 49-55.

LAIHO, K., LAMPI, A. M., HAMALAINEN, M., MOILANEN, E., PIIRONEN, V., ARVOLA, T., SYRJANEN, S. & ISOLAURI, E. 2003. Breast milk fatty acids, eicosanoids, and cytokines in mothers with and without allergic disease. *Pediatr Res,* 53, 642-7.

LANDMANN, R., MULLER, B. & ZIMMERLI, W. 2000. CD14, new aspects of ligand and signal diversity. *Microbes Infect,* 2, 295-304.

LARCHE, M., ROBINSON, D. S. & KAY, A. B. 2003. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol,* 111, 450-63; quiz 464.

LAUBEREAU, B., BROCKOW, I., ZIRNGIBL, A., KOLETZKO, S., GRUEBL, A., VON BERG, A., FILIPIAK-PITTROFF, B., BERDEL, D., BAUER, C. P., REINHARDT, D., HEINRICH, J. & WICHMANN, H. E. 2004. Effect of breast-feeding on the development of atopic dermatitis during the first 3 years of life--results from the GINI-birth cohort study. *J Pediatr,* 144, 602-7.

LAURITZEN, L., HALKJAER, L. B., MIKKELSEN, T. B., OLSEN, S. F., MICHAELSEN, K. F., LOLAND, L. & BISGAARD, H. 2006. Fatty acid composition of human milk in atopic Danish mothers. *Am J Clin Nutr,* 84, 190-6.

LAVERTY, H. G., WAKEFIELD, L. M., OCCLESTON, N. L., O'KANE, S. & FERGUSON, M. W. 2009. TGF-beta3 and cancer: a review. *Cytokine Growth Factor Rev,* 20, 305-17.

LAWRENCE, R. M. & PANE, C. A. 2007. Human breast milk: current concepts of immunology and infectious diseases. ***Curr Probl Pediatr Adolesc Health Care,*** **37, 7-36.**

LEE, S. Y., KANG, M. J., KWON, J. W., PARK, K. S. & HONG, S. J. 2013. Breastfeeding Might Have Protective Effects on Atopy in Children With the CD14C-159T CT/CC Genotype. ***Allergy Asthma Immunol Res,*** **5, 239-41.**

LINNAMAA, P., NIEMINEN, K., KOULU, L., TUOMASJUKKA, S., KALLIO, H., YANG, B., TAHVONEN, R. & SAVOLAINEN, J. 2013. Black currant seed oil supplementation of mothers enhances IFN-gamma and suppresses IL-4 production in breast milk. ***Pediatr Allergy Immunol,*** **24, 562-6.**

LONNERDAL, B. 2003. Nutritional and physiologic significance of human milk proteins. ***Am J Clin Nutr,*** **77, 1537S-1543S.**

LOTVALL, J. & VALADI, H. 2007. Cell to cell signalling via exosomes through esRNA. ***Cell Adh Migr,*** **1, 156-8.**

LOUI, A., EILERS, E., STRAUSS, E., POHL-SCHICKINGER, A., OBLADEN, M. & KOEHNE, P. 2012. Vascular Endothelial Growth Factor (VEGF) and soluble VEGF receptor 1 (sFlt-1) levels in early and mature human milk from mothers of preterm versus term infants. ***J Hum Lact,*** **28, 522-8.**

LOWE, A. J., THIEN, F. C., STONEY, R. M., BENNETT, C. M., HOSKING, C. S., HILL, D. J., CARLIN, J. B., ABRAMSON, M. J. & DHARMAGE, S. C. 2008. Associations between fatty acids in colostrum and breast milk and risk of allergic disease. ***Clin Exp Allergy,*** **38, 1745-51.**

LUO, Z. C., AN, N., XU, H. R., LARANTE, A., AUDIBERT, F. & FRASER, W. D. 2007. The effects and mechanisms of primiparity on the risk of pre-eclampsia: a systematic review. ***Paediatr Perinat Epidemiol,*** **21 Suppl 1, 36-45.**

MALISZEWSKI, C. R. 1991. CD14 and immune response to lipopolysaccharide. ***Science,*** **252, 1321-2.**

MANETTI, R., PARRONCHI, P., GIUDIZI, M. G., PICCINNI, M. P., MAGGI, E., TRINCHIERI, G. & ROMAGNANI, S. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. ***J Exp Med,*** **177, 1199-204.**

MAREK, A., ZAGIERSKI, M., LIBEREK, A., ALEKSANDROWICZ, E., KORZON, M., KRZYKOWSKI, G., KAMINSKA, B. & SZLAGATYS-SIDORKIEWICZ, A. 2009. TGF-beta(1), IL-10 and IL-4 in colostrum of allergic and nonallergic mothers. ***Acta Biochim Pol,*** **56, 411-4.**

MARTIN, H. M., HANCOCK, J. T., SALISBURY, V. & HARRISON, R. 2004. Role of xanthine oxidoreductase as an antimicrobial agent. ***Infect Immun,*** **72, 4933-9.**

MARZI, M., VIGANO, A., TRABATTONI, D., VILLA, M. L., SALVAGGIO, A., CLERICI, E. & CLERICI, M. 1996. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. ***Clin Exp Immunol,*** **106, 127-33.**

MASTROIANNI, C. M., D'ETTORRE, G., FORCINA, G. & VULLO, V. 2004. Teaching tired T cells to fight HIV: time to test IL-15 for immunotherapy? ***Trends Immunol,*** **25, 121-5.**

MCKENZIE, A. N., CULPEPPER, J. A., DE WAAL MALEFYT, R., BRIERE, F., PUNNONEN, J., AVERSA, G., SATO, A., DANG, W., COCKS, B. G., MENON, S. & ET AL. 1993. Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. ***Proc Natl Acad Sci U S A,*** **90, 3735-9.**

MENNELLA, J. A., YOURSHAW, L. M. & MORGAN, L. K. 2007. Breastfeeding and smoking: short-term effects on infant feeding and sleep. ***Pediatrics,*** **120, 497-502.**

MINTY, A., CHALON, P., DEROCQ, J. M., DUMONT, X., GUILLEMOT, J. C., KAGHAD, M., LABIT, C., LEPLATOIS, P., LIAUZUN, P., MILOUX, B. & ET AL. 1993. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. ***Nature,*** **362, 248-50.**

MIX, E., GOERTSCHES, R. & ZETT, U. K. 2006. Immunoglobulins--basic considerations. ***J Neurol,*** **253 Suppl 5, V9-17.**

MORROW, A. L., RUIZ-PALACIOS, G. M., ALTAYE, M., JIANG, X., GUERRERO, M. L., MEINZEN-DERR, J. K., FARKAS, T., CHATURVEDI, P., PICKERING, L. K. & NEWBURG, D. S. 2004. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. ***J Pediatr,*** **145, 297-303.**

MORROW, A. L., RUIZ-PALACIOS, G. M., JIANG, X. & NEWBURG, D. S. 2005. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. ***J Nutr,*** **135, 1304-7.**

MUNBLIT, D., BOYLE, R. J. & WARNER, J. O. 2014. Factors affecting Breast Milk composition, and potential consequences for development of the allergic phenotype. ***Clin Exp Allergy*****.**

NAKAMURA, T. & MIZUNO, S. 2010. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. ***Proc Jpn Acad Ser B Phys Biol Sci,*** **86, 588-610.**

NAKAO, A. 2010. The role and potential use of oral transforming growth factor-beta in the prevention of infant allergy. *Clin Exp Allergy,* 40, 725-30.

NARULA, P., MITTAL, S. K., GUPTA, S. & SAHA, K. 1982. Cellular and humoral factors of human milk in relation to nutritional status in lactating mothers. *Indian J Med Res,* 76, 415-23.

NEWBURG, D. S. 1997. Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? *J Nutr,* 127, 980S-984S.

NEWBURG, D. S. 1999. Human milk glycoconjugates that inhibit pathogens. *Curr Med Chem,* 6, 117-27.

NEWBURG, D. S., RUIZ-PALACIOS, G. M., ALTAYE, M., CHATURVEDI, P., MEINZEN-DERR, J., GUERRERO MDE, L. & MORROW, A. L. 2004. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology,* 14, 253-63.

NGUYEN, A. V. & POLLARD, J. W. 2000. Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development,* 127, 3107-18.

NGUYEN, T. V., YUAN, L., AZEVEDO, M. S., JEONG, K. I., GONZALEZ, A. M. & SAIF, L. J. 2007. Transfer of maternal cytokines to suckling piglets: in vivo and in vitro models with implications for immunomodulation of neonatal immunity. *Vet Immunol Immunopathol,* 117, 236-48.

NOMMSEN, L. A., LOVELADY, C. A., HEINIG, M. J., LONNERDAL, B. & DEWEY, K. G. 1991. Determinants of energy, protein, lipid, and lactose concentrations in human milk during the first 12 mo of lactation: the DARLING Study. *Am J Clin Nutr,* 53, 457-65.

ODDY, W. H., HALONEN, M., MARTINEZ, F. D., LOHMAN, I. C., STERN, D. A., KURZIUS-SPENCER, M., GUERRA, S. & WRIGHT, A. L. 2003. TGF-beta in human milk is associated with wheeze in infancy. *J Allergy Clin Immunol,* 112, 723-8.

ODDY, W. H. & ROSALES, F. 2010. A systematic review of the importance of milk TGF-beta on immunological outcomes in the infant and young child. *Pediatr Allergy Immunol,* 21, 47-59.

OGAWA, J., SASAHARA, A., YOSHIDA, T., SIRA, M. M., FUTATANI, T., KANEGANE, H. & MIYAWAKI, T. 2004. Role of transforming growth factor-beta in breast milk for initiation of IgA production in newborn infants. *Early Hum Dev,* 77, 67-75.

OKUNISHI, K., DOHI, M., NAKAGOME, K., TANAKA, R., MIZUNO, S., MATSUMOTO, K., MIYAZAKI, J., NAKAMURA, T. & YAMAMOTO, K. 2005. A novel role of hepatocyte growth factor as an immune regulator through suppressing dendritic cell function. ***J Immunol,*** **175, 4745-53.**

OKUNISHI, K., SASAKI, O., OKASORA, T., NAKAGOME, K., IMAMURA, M., HARADA, H., MATSUMOTO, T., TANAKA, R., YAMAMOTO, K., TABATA, Y. & DOHI, M. 2009. Intratracheal delivery of hepatocyte growth factor directly attenuates allergic airway inflammation in mice. ***Int Arch Allergy Immunol,*** **149 Suppl 1, 14-20.**

ORISS, T. B., MCCARTHY, S. A., MOREL, B. F., CAMPANA, M. A. & MOREL, P. A. 1997. Crossregulation between T helper cell (Th)1 and Th2: inhibition of Th2 proliferation by IFN-gamma involves interference with IL-1. ***J Immunol,*** **158, 3666-72.**

ORIVUORI, L., LOSS, G., RODUIT, C., DALPHIN, J. C., DEPNER, M., GENUNEIT, J., LAUENER, R., PEKKANEN, J., PFEFFERLE, P., RIEDLER, J., ROPONEN, M., WEBER, J., VON MUTIUS, E., BRAUN-FAHRLANDER, C. & VAARALA, O. 2014. Soluble immunoglobulin A in breast milk is inversely associated with atopic dermatitis at early age: the PASTURE cohort study. ***Clin Exp Allergy,*** **44, 102-12.**

ORLANDO, S. 1995. The immunologic significance of breast milk. ***J Obstet Gynecol Neonatal Nurs,*** **24, 678-83.**

OSBORN, M. L. 1979. The rent breasts: a brief history of wet-nursing. ***Midwife Health Visit Community Nurse,*** **15, 302-6.**

OSHIDA, K., SHIMIZU, T., TAKASE, M., TAMURA, Y. & YAMASHIRO, Y. 2003. Effects of dietary sphingomyelin on central nervous system myelination in developing rats. ***Pediatr Res,*** **53, 589-93.**

OUWEHAND, A. C., ISOLAURI, E., HE, F., HASHIMOTO, H., BENNO, Y. & SALMINEN, S. 2001. Differences in Bifidobacterium flora composition in allergic and healthy infants. ***J Allergy Clin Immunol,*** **108, 144-5.**

PANG, W. W. & HARTMANN, P. E. 2007. Initiation of human lactation: secretory differentiation and secretory activation. ***J Mammary Gland Biol Neoplasia,*** **12, 211-21.**

PARASHAR, U. D., HUMMELMAN, E. G., BRESEE, J. S., MILLER, M. A. & GLASS, R. I. 2003. Global illness and deaths caused by rotavirus disease in children. ***Emerg Infect Dis,*** **9, 565-72.**

PATKI, S., KADAM, S., CHANDRA, V. & BHONDE, R. 2010. Human breast milk is a rich source of multipotent mesenchymal stem cells. *Hum Cell,* 23, 35-40.

PAWANKAR, R., OKUDA, M., YSSEL, H., OKUMURA, K. & RA, C. 1997. Nasal mast cells in perennial allergic rhinitics exhibit increased expression of the Fc epsilonRI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J Clin Invest,* 99, 1492-9.

PENTTILA, I. A., FLESCH, I. E., MCCUE, A. L., POWELL, B. C., ZHOU, F. H., READ, L. C. & ZOLA, H. 2003. Maternal milk regulation of cell infiltration and interleukin 18 in the intestine of suckling rat pups. *Gut,* 52, 1579-86.

PERONI, D. G., PESCOLLDERUNGG, L., PIACENTINI, G. L., RIGOTTI, E., MASELLI, M., WATSCHINGER, K., PIAZZA, M., PIGOZZI, R. & BONER, A. L. 2010. Immune regulatory cytokines in the milk of lactating women from farming and urban environments. *Pediatr Allergy Immunol,* 21, 977-82.

PICKERING, L. K., GRANOFF, D. M., ERICKSON, J. R., MASOR, M. L., CORDLE, C. T., SCHALLER, J. P., WINSHIP, T. R., PAULE, C. L. & HILTY, M. D. 1998. Modulation of the immune system by human milk and infant formula containing nucleotides. *Pediatrics,* 101, 242-9.

PIEMONTESE, P., GIANNI, M. L., BRAEGGER, C. P., CHIRICO, G., GRUBER, C., RIEDLER, J., ARSLANOGLU, S., VAN STUIJVENBERG, M., BOEHM, G., JELINEK, J. & ROGGERO, P. 2011. Tolerance and safety evaluation in a large cohort of healthy infants fed an innovative prebiotic formula: a randomized controlled trial. *PLoS One,* 6, e28010.

PISTOIA, V. 1997. Production of cytokines by human B cells in health and disease. *Immunol Today,* 18, 343-50.

PLAYFORD, R. J., MACDONALD, C. E. & JOHNSON, W. S. 2000. Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *Am J Clin Nutr,* 72, 5-14.

POOLE, J. A. & CLAMAN, H. N. 2004. Immunology of pregnancy. Implications for the mother. *Clin Rev Allergy Immunol,* 26, 161-70.

POWER, L. L., POPPLEWELL, E. J., HOLLOWAY, J. A., DIAPER, N. D., WARNER, J. O. & JONES, C. A. 2002. Immunoregulatory molecules during pregnancy and at birth. *J Reprod Immunol,* 56, 19-28.

PRESCOTT, S. L. & DUNSTAN, J. A. 2007. Prenatal fatty acid status and immune development: the pathways and the evidence. *Lipids,* 42, 801-10.

PRESCOTT, S. L., WICKENS, K., WESTCOTT, L., JUNG, W., CURRIE, H., BLACK, P. N., STANLEY, T. V., MITCHELL, E. A., FITZHARRIS, P., SIEBERS, R., WU, L. & CRANE, J. 2008. Supplementation with Lactobacillus rhamnosus or Bifidobacterium lactis probiotics in pregnancy increases cord blood interferon-gamma and breast milk transforming growth factor-beta and immunoglobin A detection. *Clin Exp Allergy,* 38, 1606-14.

PROKESOVA, L., LODINOVA-ZADNIKOVA, R., ZIZKA, J., KOCOURKOVA, I., NOVOTNA, O., PETRASKOVA, P. & STERZL, I. 2006. Cytokine levels in healthy and allergic mothers and their children during the first year of life. *Pediatr Allergy Immunol,* 17, 175-83.

PUGIN, J., HEUMANN, I. D., TOMASZ, A., KRAVCHENKO, V. V., AKAMATSU, Y., NISHIJIMA, M., GLAUSER, M. P., TOBIAS, P. S. & ULEVITCH, R. J. 1994. CD14 is a pattern recognition receptor. *Immunity,* 1, 509-16.

RADBILL, S. X. 1981. Infant feeding through the ages. *Clin Pediatr (Phila),* 20, 613-21.

RAUTAVA, S., KALLIOMAKI, M. & ISOLAURI, E. 2002. Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. *J Allergy Clin Immunol,* 109, 119-21.

REICHARDT, P., MULLER, D., POSSELT, U., VORBERG, B., DIEZ, U., SCHLINK, U., REUTER, W. & BORTE, M. 2004. Fatty acids in colostrum from mothers of children at high risk of atopy in relation to clinical and laboratory signs of allergy in the first year of life. *Allergy,* 59, 394-400.

RIGOTTI, E., PIACENTINI, G. L., RESS, M., PIGOZZI, R., BONER, A. L. & PERONI, D. G. 2006. Transforming growth factor-beta and interleukin-10 in breast milk and development of atopic diseases in infants. *Clin Exp Allergy,* 36, 614-8.

ROBERTS, C. W., WALKER, W. & ALEXANDER, J. 2001. Sex-associated hormones and immunity to protozoan parasites. *Clin Microbiol Rev,* 14, 476-88.

ROTH, I., CORRY, D. B., LOCKSLEY, R. M., ABRAMS, J. S., LITTON, M. J. & FISHER, S. J. 1996. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. *J Exp Med,* 184, 539-48.

RUDLOFF, S., NIEHUES, T., RUTSCH, M., KUNZ, C. & SCHROTEN, H. 1999. Inflammation markers and cytokines in breast milk of atopic and nonatopic women. *Allergy,* 54, 206-11.

SAARINEN, K. M., VAARALA, O., KLEMETTI, P. & SAVILAHTI, E. 1999. Transforming growth factor-beta1 in mothers' colostrum and immune responses to

cows' milk proteins in infants with cows' milk allergy. *J Allergy Clin Immunol,* 104, 1093-8.

SABAT, R., GRUTZ, G., WARSZAWSKA, K., KIRSCH, S., WITTE, E., WOLK, K. & GEGINAT, J. 2010. Biology of interleukin-10. *Cytokine Growth Factor Rev,* 21, 331-44.

SAITO, S. & SAKAI, M. 2003. Th1/Th2 balance in preeclampsia. *J Reprod Immunol,* 59, 161-73.

SAITO, S., SAKAI, M., SASAKI, Y., TANEBE, K., TSUDA, H. & MICHIMATA, T. 1999. Quantitative analysis of peripheral blood Th0, Th1, Th2 and the Th1:Th2 cell ratio during normal human pregnancy and preeclampsia. *Clin Exp Immunol,* 117, 550-5.

SAKAGUCHI, S., YAMAGUCHI, T., NOMURA, T. & ONO, M. 2008. Regulatory T cells and immune tolerance. *Cell,* 133, 775-87.

SAVILAHTI, E., SILTANEN, M., KAJOSAARI, M., VAARALA, O. & SAARINEN, K. M. 2005. IgA antibodies, TGF-beta1 and -beta2, and soluble CD14 in the colostrum and development of atopy by age 4. *Pediatr Res,* 58, 1300-5.

SCHAFER, T., HEINRICH, J., WJST, M., KRAUSE, C., ADAM, H., RING, J. & WICHMANN, H. E. 1999. Indoor risk factors for atopic eczema in school children from East Germany. *Environ Res,* 81, 151-8.

SCHARTON-KERSTEN, T. M., WYNN, T. A., DENKERS, E. Y., BALA, S., GRUNVALD, E., HIENY, S., GAZZINELLI, R. T. & SHER, A. 1996. In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. *J Immunol,* 157, 4045-54.

SCHOENBECK, S., MCKENZIE, D. T. & KAGNOFF, M. F. 1989. Interleukin 5 is a differentiation factor for IgA B cells. *Eur J Immunol,* 19, 965-9.

SHETH, S. 2014. *Soluble CD14 levels in Colostrum of mothers from Russia, Italy and England.* BSc, Imperial College London.

SHULL, M. M., ORMSBY, I., KIER, A. B., PAWLOWSKI, S., DIEBOLD, R. J., YIN, M., ALLEN, R., SIDMAN, C., PROETZEL, G., CALVIN, D. & ET AL. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature,* 359, 693-9.

SIDOR, K., JARMOLOWSKA, B., KACZMARSKI, M., KOSTYRA, E., IWAN, M. & KOSTYRA, H. 2008. Content of beta-casomorphins in milk of women with a history of allergy. *Pediatr Allergy Immunol,* 19, 587-91.

SIGURS, N., ALJASSIM, F., KJELLMAN, B., ROBINSON, P. D., SIGURBERGSSON, F., BJARNASON, R. & GUSTAFSSON, P. M. 2010. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. ***Thorax,*** **65, 1045-52.**

SIGURS, N., GUSTAFSSON, P. M., BJARNASON, R., LUNDBERG, F., SCHMIDT, S., SIGURBERGSSON, F. & KJELLMAN, B. 2005. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. ***Am J Respir Crit Care Med,*** **171, 137-41.**

SILVERBERG, J. I., PATEL, N., IMMANENI, S., RUSNIAK, B., SILVERBERG, N. B., DEBASHIS, R., FEWKES, N. & SIMPSON, E. L. 2015. Assessment of atopic dermatitis using self- and caregiver- report: a multicenter validation study. ***Br J Dermatol.***

SIMISTER, N. E. & STORY, C. M. 1997. Human placental Fc receptors and the transmission of antibodies from mother to fetus. ***J Reprod Immunol,*** **37, 1-23.**

SKOKOS, D., BOTROS, H. G., DEMEURE, C., MORIN, J., PERONET, R., BIRKENMEIER, G., BOUDALY, S. & MECHERI, S. 2003. Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. ***J Immunol,*** **170, 3037-45.**

SMITH, C. W. & GOLDMAN, A. S. 1968. The cells of human colostrum. I. In vitro studies of morphology and functions. ***Pediatr Res,*** **2, 103-9.**

SNIJDERS, B. E., DAMOISEAUX, J. G., PENDERS, J., KUMMELING, I., STELMA, F. F., VAN REE, R., VAN DEN BRANDT, P. A. & THIJS, C. 2006. Cytokines and soluble CD14 in breast milk in relation with atopic manifestations in mother and infant (KOALA Study). ***Clin Exp Allergy,*** **36, 1609-15.**

SOKOL, C. L., BARTON, G. M., FARR, A. G. & MEDZHITOV, R. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. ***Nat Immunol,*** **9, 310-8.**

SOTO-RAMIREZ, N., KARMAUS, W., YOUSEFI, M., ZHANG, H., LIU, J. & GANGUR, V. 2012. Maternal immune markers in serum during gestation and in breast milk and the risk of asthma-like symptoms at ages 6 and 12 months: a longitudinal study. ***Allergy Asthma Clin Immunol,*** **8, 11.**

SOZANSKA, B., BLASZCZYK, M., PEARCE, N. & CULLINAN, P. 2014. Atopy and allergic respiratory disease in rural Poland before and after accession to the European Union. ***J Allergy Clin Immunol,*** **133, 1347-53.**

SRIVASTAVA, M. D., LIPPES, J. & SRIVASTAVA, B. I. 1999. Hepatocyte growth factor in human milk and reproductive tract fluids. ***Am J Reprod Immunol,*** **42, 347-54.**

STEINKE, J. W. & BORISH, L. 2001. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. ***Respir Res,*** **2, 66-70.**

STEVENS, E. E., PATRICK, T. E. & PICKLER, R. 2009. A History of Infant Feeding. ***J Perinat Educ,*** **18, 32-39.**

STONEY, R. M., WOODS, R. K., HOSKING, C. S., HILL, D. J., ABRAMSON, M. J. & THIEN, F. C. 2004. Maternal breast milk long-chain n-3 fatty acids are associated with increased risk of atopy in breastfed infants. ***Clin Exp Allergy,*** **34, 194-200.**

STRACHAN, D. P. 1989. Hay fever, hygiene, and household size. ***BMJ,*** **299, 1259-60.**

STRACHAN, D. P. 2000. Family size, infection and atopy: the first decade of the "hygiene hypothesis". ***Thorax,*** **55 Suppl 1, S2-10.**

STRIKER, G. A., CASANOVA, L. D. & NAGAO, A. T. 2004. [Influence of type of delivery on A, G and M immunoglobulin concentration in maternal colostrum]. ***J Pediatr (Rio J),*** **80, 123-8.**

STROBEL, S. 2002. Oral tolerance, systemic immunoregulation, and autoimmunity. ***Ann N Y Acad Sci,*** **958, 47-58.**

SZLAGATYS-SIDORKIEWICZ, A., WOS, E., ALEKSANDROWICZ, E., LUCZAK, G., ZAGIERSKI, M., MARTYSIAK-ZUROWSKA, D., MAREK, K. & KAMINSKA, B. 2013. Cytokine profile of mature milk from smoking and nonsmoking mothers. ***J Pediatr Gastroenterol Nutr,*** **56, 382-4.**

TAKAHATA, Y., TAKADA, H., NOMURA, A., OHSHIMA, K., NAKAYAMA, H., TSUDA, T., NAKANO, H. & HARA, T. 2001. Interleukin-18 in human milk. ***Pediatr Res,*** **50, 268-72.**

TAKATSU, K. 1998. Interleukin 5 and B cell differentiation. ***Cytokine Growth Factor Rev,*** **9, 25-35.**

TAKATSU, K. & NAKAJIMA, H. 2008. IL-5 and eosinophilia. ***Curr Opin Immunol,*** **20, 288-94.**

THIJS, C., MULLER, A., RIST, L., KUMMELING, I., SNIJDERS, B. E., HUBER, M., VAN REE, R., SIMOES-WUST, A. P., DAGNELIE, P. C. & VAN DEN BRANDT, P. A. 2011. Fatty acids in breast milk and development of atopic eczema and allergic sensitisation in infancy. ***Allergy,*** **66, 58-67.**

THORNTON, C. A., CAPRISTO, C. C., POWER, L. L., HOLLOWAY, J., POPPLEWELL, E. J., DIAPER, N. D. & WARNER, J. O. 2003a. The effect of labor on neonatal T-cell phenotype and function. ***Pediatr Res,*** **54, 120-4.**

THORNTON, C. A., HOLLOWAY, J. A., POPPLEWELL, E. J., SHUTE, J. K., BOUGHTON, J. & WARNER, J. O. 2003b. Fetal exposure to intact immunoglobulin E occurs via the gastrointestinal tract. *Clin Exp Allergy,* 33, 306-11.

THORNTON, C. A., HOLLOWAY, J. A., SHUTE, J. K., HOLLOWAY, J. W., DIAPER, N. D. & WARNER, J. O. 2009. Human mid-gestation amniotic fluid contains interleukin-16 bioactivity. *Immunology,* 126, 543-51.

THORNTON, C. A., HOLLOWAY, J. A. & WARNER, J. O. 2002. Expression of CD21 and CD23 during human fetal development. *Pediatr Res,* 52, 245-50.

THURL, S., HENKER, J., SIEGEL, M., TOVAR, K. & SAWATZKI, G. 1997. Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. *Glycoconj J,* 14, 795-9.

TOMICIC, S., JOHANSSON, G., VOOR, T., BJORKSTEN, B., BOTTCHER, M. F. & JENMALM, M. C. 2010. Breast milk cytokine and IgA composition differ in Estonian and Swedish mothers-relationship to microbial pressure and infant allergy. *Pediatr Res,* 68, 330-4.

TREGONING, J. S. & SCHWARZE, J. 2010. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev,* 23, 74-98.

TRINCHIERI, G. 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol,* 70, 83-243.

TRINCHIERI, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol,* 3, 133-46.

TRUNDLEY, A. & MOFFETT, A. 2004. Human uterine leukocytes and pregnancy. *Tissue Antigens,* 63, 1-12.

URWIN, H. J., MILES, E. A., NOAKES, P. S., KREMMYDA, L. S., VLACHAVA, M., DIAPER, N. D., PEREZ-CANO, F. J., GODFREY, K. M., CALDER, P. C. & YAQOOB, P. 2012. Salmon consumption during pregnancy alters fatty acid composition and secretory IgA concentration in human breast milk. *J Nutr,* 142, 1603-10.

USTUNDAG, B., YILMAZ, E., DOGAN, Y., AKARSU, S., CANATAN, H., HALIFEOGLU, I., CIKIM, G. & AYGUN, A. D. 2005. Levels of cytokines (IL-1beta, IL-2, IL-6, IL-8, TNF-alpha) and trace elements (Zn, Cu) in breast milk from mothers of preterm and term infants. *Mediators Inflamm,* 2005, 331-6.

VAN ODIJK, J., KULL, I., BORRES, M. P., BRANDTZAEG, P., EDBERG, U., HANSON, L. A., HOST, A., KUITUNEN, M., OLSEN, S. F., SKERFVING, S., SUNDELL, J. & WILLE, S. 2003. Breastfeeding and allergic disease: a multidisciplinary review of

the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. ***Allergy,*** **58, 833-43.**

VAN VLASSELAER, P., PUNNONEN, J. & DE VRIES, J. E. 1992. Transforming growth factor-beta directs IgA switching in human B cells. ***J Immunol,*** **148, 2062-7.**

VEENSTRA VAN NIEUWENHOVEN, A. L., BOUMAN, A., MOES, H., HEINEMAN, M. J., DE LEIJ, L. F., SANTEMA, J. & FAAS, M. M. 2003. Endotoxin-induced cytokine production of monocytes of third-trimester pregnant women compared with women in the follicular phase of the menstrual cycle. ***Am J Obstet Gynecol,*** **188, 1073-7.**

VEIKKOLA, T. & ALITALO, K. 1999. VEGFs, receptors and angiogenesis. ***Semin Cancer Biol,*** **9, 211-20.**

VERCELLI, D. 2008. Discovering susceptibility genes for asthma and allergy. ***Nat Rev Immunol,*** **8, 169-82.**

VERHASSELT, V. 2010. Neonatal tolerance under breastfeeding influence: the presence of allergen and transforming growth factor-beta in breast milk protects the progeny from allergic asthma. ***J Pediatr,*** **156, S16-20.**

VON BERG, A., FILIPIAK-PITTROFF, B., KRAMER, U., LINK, E., BOLLRATH, C., BROCKOW, I., KOLETZKO, S., GRUBL, A., HEINRICH, J., WICHMANN, H. E., BAUER, C. P., REINHARDT, D. & BERDEL, D. 2008. Preventive effect of hydrolyzed infant formulas persists until age 6 years: long-term results from the German Infant Nutritional Intervention Study (GINI). ***J Allergy Clin Immunol,*** **121, 1442-7.**

VRIEZINGA, S. L., AURICCHIO, R., BRAVI, E., CASTILLEJO, G., CHMIELEWSKA, A., CRESPO ESCOBAR, P., KOLACEK, S., KOLETZKO, S., KORPONAY-SZABO, I. R., MUMMERT, E., POLANCO, I., PUTTER, H., RIBES-KONINCKX, C., SHAMIR, R., SZAJEWSKA, H., WERKSTETTER, K., GRECO, L., GYIMESI, J., HARTMAN, C., HOGEN ESCH, C., HOPMAN, E., IVARSSON, A., KOLTAI, T., KONING, F., MARTINEZ-OJINAGA, E., TE MARVELDE, C., PAVIC, A., ROMANOS, J., STOOPMAN, E., VILLANACCI, V., WIJMENGA, C., TRONCONE, R. & MEARIN, M. L. 2014. Randomized feeding intervention in infants at high risk for celiac disease. ***N Engl J Med,*** **371, 1304-15.**

WALTER, J., GHOSH, M. K., KUHN, L., SEMRAU, K., SINKALA, M., KANKASA, C., THEA, D. M. & ALDROVANDI, G. M. 2009. High concentrations of interleukin 15 in breast milk are associated with protection against postnatal HIV transmission. ***J Infect Dis,*** **200, 1498-502.**

WANG, Y., SELDEN, C., FARNAUD, S., CALNAN, D. & HODGSON, H. J. 1994. Hepatocyte growth factor (HGF/SF) is expressed in human epithelial cells during embryonic development; studies by in situ hybridisation and northern blot analysis. *J Anat,* 185 (Pt 3), 543-51.

WARD, R. E., NINONUEVO, M., MILLS, D. A., LEBRILLA, C. B. & GERMAN, J. B. 2006. In vitro fermentation of breast milk oligosaccharides by Bifidobacterium infantis and Lactobacillus gasseri. *Appl Environ Microbiol,* 72, 4497-9.

WEGIENKA, G., HAVSTAD, S., BOBBITT, K. R., WOODCROFT, K. J., ZORATTI, E. M., OWNBY, D. R. & COLE JOHNSON, C. 2011. Within-woman change in regulatory T cells from pregnancy to the postpartum period. *J Reprod Immunol,* 88, 58-65.

WEGMANN, T. G., LIN, H., GUILBERT, L. & MOSMANN, T. R. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today,* 14, 353-6.

WEINBERG, F. 1993. Infant feeding through the ages. *Can Fam Physician,* 39, 2016-20.

WHITBY, D. J. & FERGUSON, M. W. 1991a. The extracellular matrix of lip wounds in fetal, neonatal and adult mice. *Development,* 112, 651-68.

WHITBY, D. J. & FERGUSON, M. W. 1991b. Immunohistochemical localization of growth factors in fetal wound healing. *Dev Biol,* 147, 207-15.

WHO 2001. Global Strategy for Infant and Young Child Feeding, The Optimal Duration of Exclusive Breastfeeding. Geneva: World Health Organization.

WHO 2009a. *Infant and young child feeding,* Geneva, World Health Organization.

WHO 2009b. WHO Global Data Bank on Infant and Young Child Feeding. Geneva: World Health Organization.

WHO/UNICEF 2003. Global strategy for infant and young child feeding. Geneva: World Health Organisation.

WICKES, I. G. 1953a. A history of infant feeding. I. Primitive peoples; ancient works; Renaissance writers. *Arch Dis Child,* 28, 151-8.

WICKES, J. G. 1953b. A history of infant feeding. IV. Nineteenth century continued. *Arch Dis Child,* 28, 416-22.

WYNN, T. A. 2003. IL-13 effector functions. *Annu Rev Immunol,* 21, 425-56.

XING, Z., GAULDIE, J., COX, G., BAUMANN, H., JORDANA, M., LEI, X. F. & ACHONG, M. K. 1998. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest,* 101, 311-20.

YAGI, Y., WATANABE, E., WATARI, E., SHINYA, E., SATOMI, M., TAKESHITA, T. & TAKAHASHI, H. 2010. Inhibition of DC-SIGN-mediated transmission of human immunodeficiency virus type 1 by Toll-like receptor 3 signalling in breast milk macrophages. *Immunology,* 130, 597-607.

YAMADA, Y., SAITO, S. & MORIKAWA, H. 1998. Hepatocyte growth factor in human breast milk. *Am J Reprod Immunol,* 40, 112-20.

YANG, Y. W., TSAI, C. L. & LU, C. Y. 2009. Exclusive breastfeeding and incident atopic dermatitis in childhood: a systematic review and meta-analysis of prospective cohort studies. *Br J Dermatol,* 161, 373-83.

YASWEN, L., KULKARNI, A. B., FREDRICKSON, T., MITTLEMAN, B., SCHIFFMAN, R., PAYNE, S., LONGENECKER, G., MOZES, E. & KARLSSON, S. 1996. Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. *Blood,* 87, 1439-45.

YILMAZ, H. L., SAYGILI-YILMAZ, E. S. & GUNESACAR, R. 2007. Interleukin-10 and -12 in human milk at 3 stages of lactation: a longitudinal study. *Adv Ther,* 24, 603-10.

ZANARDO, V., NICOLUSSI, S., CAVALLIN, S., TREVISANUTO, D., BARBATO, A., FAGGIAN, D., FAVARO, F. & PLEBANI, M. 2005. Effect of maternal smoking on breast milk interleukin-1alpha, beta-endorphin, and leptin concentrations and leptin concentrations. *Environ Health Perspect,* 113, 1410-3.

ZANIERI, L., GALVAN, P., CHECCHINI, L., CINCINELLI, A., LEPRI, L., DONZELLI, G. P. & DEL BUBBA, M. 2007. Polycyclic aromatic hydrocarbons (PAHs) in human milk from Italian women: influence of cigarette smoking and residential area. *Chemosphere,* 67, 1265-74.

ZHOU, Y., DAMSKY, C. H. & FISHER, S. J. 1997. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest,* 99, 2152-64.

ZIZKA, J., KVERKA, M., NOVOTNA, O., STANKOVA, I., LODINOVA-ZADNIKOVA, R., KOCOURKOVA, I., STERZL, I. & PROKESOVA, L. 2007. Perinatal period cytokines related to increased risk of future allergy development. *Folia Microbiol (Praha),* 52, 549-55.

8 Appendices

8.1 Appendix A

This questionnaire asks about your health. Your answers will help us to understand how mothers' own health and background might affect their pregnancies.

All the answers you give are confidential.

We would be grateful if you would help us by answering as many of these questions as possible but if there is any question you do not want to answer that is fine. Just leave it blank.

Thank you very much for your help!

Participant's Number ☐☐☐☐

Consent Form Signed: St. Mary's ☐ Durham Hospital ☐

Date of Enrolment __ __/__ __/__ __ __ __

Your Name.....D.O.B__ __/__ __/__ __
Surname First Name

Father's Name.....D.O.B__ __/__ __/__ __
Surname First Name

Your Address.....
.....
.....

Your phone.....
Home Work Mobile

Your email.....@.....

Inclusion Criteria:

Healthy term infant (full-term newborn) and his/her mother ☐

Exclusion Criteria:

Premature infant (<37 weeks) ☐

Mother taking immunosuppressive agents during lactation ☐

Major birth defects ☐

Admission to NICU ☐

Intrauterine growth restriction (weight below 2nd centile) ☐

Significant maternal or infant illness, such that participation will place an unreasonable burden on the mother or child eg maternal postnatal depression ☐

Do you have any Allergic Disease? :

Q1.Asthma	1 YES ☐	0 NO ☐
Q2.Allergic Rhinitis	1 YES ☐	0 NO ☐
Q3.Eczema	1 YES ☐	0 NO ☐
Q4.Food Allergy	1 YES ☐	0 NO ☐
Q5.Other	1 YES ☐	0 NO ☐

Details.....

Father's Allergic Disease:

Q6.Asthma	1 YES ☐	0 NO ☐
Q7.Allergic Rhinitis	1 YES ☐	0 NO ☐
Q8. Eczema	1 YES ☐	0 NO ☐
Q9. Food Allergy	1 YES ☐	0 NO ☐
Q10. Other	1 YES ☐	0 NO ☐

Details.....

Any Sibling's Allergic Disease:

Q11. Asthma	1 YES ☐	0 NO ☐
Q12.Allergic Rhinitis	1 YES ☐	0 NO ☐
Q13.Eczema	1 YES ☐	0 NO ☐
Q14.Food Allergy	1 YES ☐	0 NO ☐
Q15. Other	1 YES ☐	0 NO ☐

Details.....

Meets Inclusion [& Not Exclusion] Criteria? 1 YES ☐ **0** NO ☐

Obstetric History

Q16. No. previous pregnancies **0**☐ **1**☐ **2**☐ **3**☐ **4**☐ **5**☐ **>5**☐

Q17. No. previous live deliveries **0**☐ **1**☐ **2**☐ **3**☐ **4**☐ **5**☐ **>5**☐

Q18. Delivery type

Normal **0** ☐

Caesarian with labor **1** ☐

Elective caesarian **2** ☐

Q19. Date of delivery __ __/__ __/__ __ __ __

Obstetrician _____

Child Obstetric History

Q20.Gender ☐**1** Male ☐**2** Female

Q21.Gestation at birth __ __ Weeks __ Days **9** N/A ☐

Q22.Birth weight __ __ __ __ Grams **9** N/A ☐

Q23.Head circumference at birth __ __ cm **9** N/A ☐

Q23a. Antibiotics during this pregnancy **0**☐ **1**☐ **2**☐ **3**☐ **4**☐ **>4**☐

Details.....

.....

.....

Q24. Other medications during this pregnancy **1**YES ☐ **0** NO ☐

Medication 1 *Gestation* *Indication*

.....

Medication 2

.....

Medication 3

.....

Medication 4

.....

Medication 5

.....

Q25. Infections during this pregnancy **0** ☐ **1** ☐ **2** ☐ **3** ☐ **4** ☐ **>4** ☐

Infection 1 *Gestation* *Duration*

.....

Infection 2

.....

Infection 3

.....

Infection 4

.....

Q26. Other complications during this pregnancy **1**YES ☐ **0** NO ☐

Complication 1 *Gestation*

.....

Complication 2 *Gestation*

.....

Complication 3 *Gestation*

.....

Complication 4 *Gestation*

.....

Complication 5 *Gestation*

.....

Q27. Any previous medical problems? **1**YES ☐ **0** NO ☐

Details.....

.....

.....

Q28. **Your Level of Education**

School to 16 **0** ☐
School to 18 **1** ☐
Further Education **2** ☐

Q29. Mother's Usual Occupation.....

Q30. Currently Working? **1**YES ☐ **0** NO☐

Details.....

Q31. **Partner's Level of Education**

School to 16 **0** ☐
School to 18 **1** ☐
Further Education **2** ☐

Q32. Partner's Usual Occupation.....

Q33. Currently Working? **1**YES ☐ **0** NO☐

Details.....

Tobacco Smoke Exposure

Q34. Any household member smoking currently: **1**Yes ☐ **0**No ☐

Q35. Do you exposed to second-hand cigarette smoke?

1Yes ☐ **0**No ☐

Q36. Your cigarettes per day

0 Nil ☐
1 1-10 ☐
2 11-20 ☐
3 21-30 ☐
4 31-40 ☐
5 >40 ☐

Q37. Other household member cigarettes per day

0Nil ☐
1 1-10 ☐
2 11-20 ☐
3 21-30 ☐
4 31-40 ☐
5 >40 ☐

Q38. Do you drink alcohol?

1Yes ☐ **0**No ☐

Household Pets

Q39.Dog **0**No ☐ **1**Outside ☐ **2**In and Out ☐ **3**Inside ☐

Q40.Cat **0**No ☐ **1**Outside ☐ **2**In and Out ☐ **3**Inside ☐

Q41.Other Pet(1) **0**No ☐ **1**Outside ☐ **2**In and Out ☐ **3**Inside ☐
Details.....

Q42.Other Pet(2) **0**No ☐ **1**Outside ☐ **2**In and Out ☐ **3**Inside ☐
Details.....

Q43.Other Pet(3) **0**No ☐ **1**Outside ☐ **2**In and Out ☐ **3**Inside ☐
Details.....

Q44.Regular Dog Contact Outside the Home **1**YES ☐ **0**NO☐
Q45.Regular Cat Contact Outside the Home **1**YES ☐ **0**NO☐
Q46.Other regular Animal Contact Outside the Home **1**YES ☐ **0**NO☐
Details.....

Q47.Farm Residence

0No ☐
1Arable ☐
2Mixed ☐
3Livestock ☐

Q48.Is there any noticeable mold or mildew in your house/flat??
1YES☐ **0**NO☐

Colostrum sample:

Time Taken ☐☐:☐☐ N/A

Time Frozen ☐☐:☐☐ N/A

S4. Time to Freeze (hours) ☐☐ ☐☐ 9 N/A

How old is baby at the time of colostrum's collection (hours) _____

Meconium sample:

Time Taken ☐☐:☐☐ N/A

Time Frozen ☐☐:☐☐ N/A

S4. Time to Freeze (hours) ☐☐ ☐☐ 9 N/A

How old is baby at the time of meconium's collection (hours) _____

Hair sample taken: 1YES ☐ 0NO ☐

Time Taken ☐☐ :☐☐

Skin Prick Test Results [positive = ≥3mm wheal at 15 minutes]

Skin Prick Test Completed? **1** Yes ☐ **0** No ☐ **9** N/A ☐

Skin Prick Test Problem?

0 Nil ☐
1 No clear skin ☐
2 Antihistamine taken ☐
3 Dermatographism ☐
4 Other ☐

Details.....

Skin Prick Test Site: **1**Back ☐ **2**Forearm ☐ **9**N/A ☐

Q110.Derp1	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Q111.Cat	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Q112.Ryegrass	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Q113.Cow's Milk	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Q114.Egg	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Q115.Peanut	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Q116. Other(1)	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Name.....				
Q117. Other(2)	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Name.....				
Q118. Other(3)	____.____mm	**0** Neg ☐	**1** Pos ☐	**9** N/A ☐
Name.....				
Q119. Neg Control	____.____mm			
Q120. Histamine	____.____mm	____mg/ml		
Q121. Atopy? (≥1 +ve SPT from 6)		**0** No ☐	**1** Yes ☐	**9** N/A ☐

8.2 Appendix B

Participant's Number | | | | |

Lactation

Did you ever breast fed your baby?

Yes ☐ No ☐

If yes,

How long did you breast fed your baby for (exclusively)?

Months _____ Weeks _____ Days _____

Still breastfeeding exclusively ☐

Still breastfeeding ☐

Stoped breastfeeding ☐

How old was your baby when you completely stopped breastfeeding and expressing milk?

Months _____ Weeks _____ Days _____

If you did breastfeed what were the reasons for stopping?

How old was your baby when he/she first had formula (even on a single occasion)?

Months _____ Weeks _____ Days _____

Medication

Q. Did you take any medications during this breastfeeding

Yes ☐ No ☐

If yes,

Medication *How old was your baby* *Indication*

.....

.....

.....

Eczema signs

Has your child had itchy rash?

Yes ☐ No ☐

If yes,

Was it recurrent? Yes ☐ No ☐

Did it last more than two weeks? Yes ☐ No ☐

If yes,

In which 2 weeks periods did your child have the rash?

Where was the rash located?

What kind of treatment did your child receive?

Was it Eczema (Atopic Dermatitis)?
Yes ☐ No ☐

If yes,

Has a doctor told you that your child had Eczema (Atopic Dermatitis)?

Yes ☐ No ☐

Food Allergy

Has your baby ever had problems caused by food, such as an allergic reaction, sensitivity, or intolerance?

Yes ☐ No ☐

If yes,

Did your baby have a reaction the first time he or she ate the food?

Yes ☐ No ☐ Not Sure ☐

Were the problems caused by:

Food your baby ate (including infant formula) ☐

Food your baby was exposed to through breast milk because of something you at ☐

How old was your baby the first time he or she had a problem with food? (Include food your baby reacted to through breast milk)

1 month or less ☐ 2 months ☐ ☐3 months ☐ ☐4 months ☐ ☐ 5 months ☐ ☐ 6 months ☐

Did you take your baby to a medical doctor because of these problems with food?
Yes ☐ ☐ No ☐ ☐

If yes,

If your baby was tested or examined for food allergy, what method was used?

Parents' description of symptoms ☐

A skin prick test ☐

A blood test such as RAST, or CAP-RAST ☐

An esophageal or intestinal study ☐

Food elimination (withdrawal of the specific food to see if symptoms disappeared) ☐

Food challenge (introduction of a specific food to see if symptoms reappeared) ☐

Other(**SPECIFY**) _____ ☐

What symptoms of a problem with food has your baby had?

Congestion ☐

Gassiness or stomach cramps ☐

Runny nose ☐

Vomiting ☐

Asthma or wheezing ☐

Diarrhea ☐

Trouble breathing ☐

Constipation ☐

Coughing ☐

Colic ☐

Swollen eyes and or lips ☐

Irritability ☐

Hives or welts ☐

Sleeplessness ☐

Flushing ☐

Blood in stool ☐

Skin rash or eczema ☐

Loss of consciousness ☐

How have these symptoms been treated?

Treated in a doctor's office or emergency room ☐

Treated by emergency medical technician ☐

Admitted to a hospital ☐

Given adrenalin, such as with an EpiPen ☐

Given Piriton or other anti-histamine ☐

Prescribed an EpiPen or other adrenailn ☐

None of the above ☐

Please indicate which foods caused a problem for your baby in column A, including food your baby reacted to through breast milk. In column B, indicate the foods that your baby has been diagnosed as allergic to. (If your baby has had a problem with a food and has been diagnosed as allergic to the food, mark both columns for that food.)

Food	*A*	*B*
Cow's milk or other dairy products (including infant formula made with cow milk)	☐	☐
Soy milk or other soy food (including infant formula made with soy)	☐	☐
Peanuts, peanut butter, or peanut oil	☐	☐
Eggs	☐	☐
Nuts (such as, almonds, pecans, walnuts)	☐	☐
Sesame seed, tahini, or sesame seed oil	☐	☐
Fish, shellfish, or other seafood	☐	☐
Beef, chicken or turkey	☐	☐
Wheat, gluten, or wheat starch	☐	☐
Other grain or cereal (such as oats, barely)	☐	☐
Fruit or fruit juice	☐	☐
Vegetable	☐	☐
Other food **(SPECIFY)** _____	☐	☐

Other Health issues
Which of the following problems did your baby have during the past 6 months?

Food	*Yes*	*How many times?*	*At what age?*
Fever	☐		
Runny nose or cold	☐		
Diarrhea	☐		
Respiratory Syncytial Virus (RSV)	☐		
Vomiting	☐		
Cough or wheeze	☐		
Ear infection	☐		
Asthma	☐		
Colic	☐		
Food allergy	☐		
Fussy or irritable	☐		
Reflux	☐		
None of these	☐		

Has your baby been hospitalized for any reason or has your baby been taken to a hospital for any outpatient procedure or surgery in the past 6 months?

Yes ☐ No ☐

If yes specify,

.....

.....

Did your baby receive any medicines in the past 6 months? (including vitamins or minerals.)

Yes ☐ No ☐

If yes,

Medication *How old was your baby* *Indication*

.....

.....

.....

Immunisation

.....

.....

8.3 Appendix C

One Year Visit MecMilk Questionnaire ver. 1

Center

Participant's Number ☐☐☐☐

Date of assessment ☐☐ ☐☐

Interviewer

.....

Baby's Weight	Mother's Weight	Baby's Head Circumference
☐☐.☐☐	☐☐.☐☐	☐☐.☐☐
Baby's Length	**Mother's Height**	
☐☐.☐☐	☐☐☐.☐	

Lactation

Did you ever breast fed your baby?

Yes ☐ No ☐

If yes,

How old was your baby when you completely stopped breastfeeding and expressing milk?

before 1 month ☐

2-3 months ☐

3-4 months ☐

4-5 months ☐

5-6 months ☐

6-7 months ☐

7-8 months ☐

One Year Visit MecMilk Questionnaire ver. 1

8-9 months ☐

9-10 months ☐

10-11 months ☐

11-12 months ☐

more than 12 months ☐

still breastfeeding ☐

Medication

Did you take any medications during this breastfeeding (including probiotics, vitamins or minerals.)

Yes ☐ No ☐

If yes,

Medication *How old was your baby* *Indication*

.....

.....

.....

When did you introduce any food into your baby diet?

before 1 month ☐

2-3 months ☐

3-4 months ☐

4-5 months ☐

5-6 months ☐

6-7 months ☐

7-8 months ☐

8-9 months ☐

9-10 months ☐

10-11 months ☐

One Year Visit MecMilk Questionnaire ver. 1

11-12 months ☐

more than 12 months ☐

not applicable ☐

Did you or anyone else give formula milk or cow's/goat's milk to your baby?

Yes ☐ No ☐ If yes, at what age you first gave it?

before 1 month ☐

2-3 months ☐

3-4 months ☐

4-5 months ☐

5-6 months ☐

6-7 months ☐

7-8 months ☐

8-9 months ☐

9-10 months ☐

10-11 months ☐

11-12 months ☐

more than 12 months ☐

not applicable ☐

How old was your baby when you introduced following foods into his/her diet?

Egg

before 1 month ☐

2-3 months ☐

3-4 months ☐

4-5 months ☐

5-6 months ☐

6-7 months ☐

7-8 months ☐

One Year Visit MecMilk Questionnaire ver. 1

8-9 months ☐

9-10 months ☐

10-11 months ☐

11-12 months ☐

more than 12 months ☐

not applicable ☐

Peanut

before 1 month ☐

2-3 months ☐

3-4 months ☐

4-5 months ☐

5-6 months ☐

6-7 months ☐

7-8 months ☐

8-9 months ☐

9-10 months ☐

10-11 months ☐

11-12 months ☐

more than 12 months ☐

not applicable ☐

Eczema signs

Has your baby ever had a history of scratching or rubbing plus 2 or more of the following occuring between birth and 1 year

1. a history of flexural involvement

Yes ☐ No ☐

2. a history of a generally dry skin

Yes ☐ No ☐

One Year Visit MecMilk Questionnaire ver. 1

3. a history of allergic disease in the child or first-degree relative

Yes ☐ No ☐

For the research team member

Is visible flexural dermatitis present?

Yes ☐ No ☐

How old was your baby when s/he had these symptoms for the first time?

before 1 month ☐

2-3 months ☐

3-4 months ☐

4-5 months ☐

5-6 months ☐

6-7 months ☐

7-8 months ☐

8-9 months ☐

9-10 months ☐

10-11 months ☐

11-12 months ☐

more than 12 months ☐

not applicable ☐

Food Allergy

Has your baby ever had problems caused by food, such as an allergic reaction, sensitivity, or intolerance?

Yes ☐ No ☐

If yes,
Did your baby have a reaction the first time he or she ate the food?

Yes ☐ No ☐ Not Sure ☐

One Year Visit MecMilk Questionnaire ver. 1

Were the problems caused by:
Food your baby ate (including infant formula) ☐

Food your baby was exposed to through breast milk because of something you at ☐

How old was your baby the first time he or she had a problem with food? (Include food your baby reacted to through breast milk)

before 1 month ☐

2-3 months ☐

3-4 months ☐

4-5 months ☐

5-6 months ☐

6-7 months ☐

7-8 months ☐

8-9 months ☐

9-10 months ☐

10-11 months ☐

11-12 months ☐

more than 12 months ☐

not applicable ☐

Other Health issues
Which of the following problems did your baby have during the past 12 months?

Food	*Yes*	*How many times?*	*At what age?*
Fever	☐		
Runny nose or cold	☐		
Diarrhea	☐		
Respiratory Syncytial Virus (RSV)	☐		
Vomiting	☐		
Cough or wheeze	☐		
Ear infection	☐		
Asthma	☐		
Colic	☐		
Food allergy	☐		
Fussy or irritable	☐		
Reflux	☐		
None of these	☐		

One Year Visit MecMilk Questionnaire ver. 1

Did your child regularly vomit in the first 2-6 months

Yes ☐ No ☐

How many stools a day your child has at the moment

☐☐.☐

What is your child usual stool consistency (Bristol Stool Chart)

1 ☐

2 ☐

3 ☐

4 ☐

5 ☐

6 ☐

7 ☐

Has your baby been hospitalized for any reason or has your baby been taken to a hospital for any outpatient procedure or surgery in the past 6 months?

Yes ☐ No ☐

If yes specify,

.....

.....

Have you ever given your child vitamin supplements?

Yes ☐ No ☐

Have you ever given you child multivitamins?

Yes ☐ No ☐

One Year Visit MecMilk Questionnaire ver. 1

At what age did you give multivitamins (tick all that apply)?

- before 1 month ☐
- 2-3 months ☐
- 3-4 months ☐
- 4-5 months ☐
- 5-6 months ☐
- 6-7 months ☐
- 7-8 months ☐
- 8-9 months ☐
- 9-10 months ☐
- 10-11 months ☐
- 11-12 months ☐
- more than 12 months ☐
- not applicable ☐

If yes, how often did you give multivitamins?

- Every day ☐
- Most days of the week ☐
- Infrequently ☐
- NA ☐

Which brand did you give?

_____ ☐

- Don't know ☐
- NA ☐

Have you ever given your child vitamin D supplements apart from, or in addition to multivitamins? For example cod liver oil, fish oil?

Yes ☐ No ☐ NA ☐

One Year Visit MecMilk Questionnaire ver. 1

At what age did you give vitamin D (tick all that apply)?

- before 1 month ☐
- 2-3 months ☐
- 3-4 months ☐
- 4-5 months ☐
- 5-6 months ☐
- 6-7 months ☐
- 7-8 months ☐
- 8-9 months ☐
- 9-10 months ☐
- 10-11 months ☐
- 11-12 months ☐
- more than 12 months ☐
- not applicable ☐

If yes, how often did you give vitamin D?

- Every day ☐
- Most days of the week ☐
- Infrequently ☐
- NA ☐

Which brand or form of vitamin D did you give?

- _____ ☐
- Don't know ☐
- NA ☐

One Year Visit MecMilk Questionnaire ver. 1

Did your baby receive any medicines in the past year?

Yes ☐ No ☐

If yes,

Medication	*How old was your baby*	*Indication*
.....	.....	.....
.....	.....	.....
.....	.....	.....

Immunisation up to date?

Yes ☐ No ☐

One Year Visit MecMilk Questionnaire ver. 1

Baby's Skin Prick Test Results [positive = ≥3mm wheal at 15 minutes]

Skin Prick Test Completed? Yes ☐ No ☐ N/A ☐

Skin Prick Test Problem?

0 No ☐
1 No clear skin ☐
2 Antihistamine taken ☐
3 Dermatographism ☐
4 Other ☐

Details.....

Skin Prick Test Site: 0 Back ☐ 1 Forearm ☐ 9 N/A ☐

House Dust Mite	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Alternaria	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Grass Pollen	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Birch Pollen	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Cat	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Fresh Egg	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Fresh Milk	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Codfish	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Peanut	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Histamine	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Negative control	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐

Atopy? 0 No ☐ 1 Yes ☐ 9 N/A ☐

One Year Visit MecMilk Questionnaire ver. 1

Mother's Skin Prick Test Results [positive = ≥3mm wheal at 15 minutes]

Skin Prick Test Completed? Yes ☐ No ☐ N/A ☐

Skin Prick Test Problem?

0 No ☐
1 No clear skin ☐
2 Antihistamine taken ☐
3 Dermatographism ☐
4 Other ☐

Details.....

Skin Prick Test Site: 0 Back ☐ 1 Forearm ☐ 9 N/A ☐

House Dust Mite	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Grass Pollen	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Ryegrass	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Birch Pollen	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Cat	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Egg	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Milk	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Hazelnut	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Peanut	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Other_____	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Other_____	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Histamine	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐
Negative control	____.____mm	0 Neg ☐	1 Pos ☐	9 N/A ☐

Atopy? 0 No ☐ 1 Yes ☐ 9 N/A ☐

8.4 Appendix D

8.4.1 Covariates – Crosstable and groups definitions

Age (years):

Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
19.00	28.00	31.00	31.98	36.00	50.00

Colostrum collection time (hours):

Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
2.0	39.0	51.0	54.2	68.0	144.0

Country of collection:

UK	Russia	Italy
101 (25.4%)	221 (55.5%)	76 (19.1%)

Maternal Atopy:

No Atopy	Atopic
224 (77.8%)	64 (22.2%)

Previous Deliveries:

Primiparous	Multiparous
175 (45%)	214 (55%)

Labour Type: Vaginal delivery vs. Caesarean section:

Vaginal	C/Section
319 (80.8%)	76 (19.2%)

Antenatal Infections:

No	Yes
282 (72.9%)	105 (27.1%)

Exposed to tobacco smoke:

No	Yes
204 (51.9%)	189 (48.1%)

Pets at home or having regular contact:

No	***Yes***
218 (54.8%)	*180 (45.2%)*

Mould/Mildew at home:

No	***Yes***
301 (77.4%)	*88 (22.6%)*

Probiotics Prebiotics consumption during pregnancy:

<Once a week	***≥Once a week***
259 (66.8%)	*129 (33.2%)*

Fish consumption during pregnancy two categories:

<Once a week	***≥Once a week***
170 (43.3%)	*223 (56.7%)*

Fruits consumption during pregnancy:

<Daily	***Daily***
97 (24.7%)	296 (75.3%)

8.4.2 Data on maternal and environmental exposures influencing Growth factors level in colostrum and breast milk (Final Mixed models)

HGF concentration

	Value	Std.Error	p-value
(Intercept)	8.81	0.20	<0.001
Breast Milk vs. Colostrum	-1.35	0.28	<0.001
Country of collection Russia	-0.05	0.14	0.70
Country of collection Italy	-0.63	0.15	<0.001
Multiparous vs. Primiparous	-0.17	0.09	0.06
Later colostrum collection time	-0.01	0.00	<0.001
C/Section vs. Vaginal	0.04	0.13	0.73
Smoking vs. No smoking	0.12	0.10	0.20
Fruits daily consumption vs. less than daily	-0.22	0.13	0.10
Probiotics/Prebiotics Once a week or more	0.14	0.10	0.16
For BM - fruits daily consumption vs. less than daily	0.14	0.20	0.48
For BM - Country of collection Russia	-0.07	0.24	0.76
For BM - Country of collection Italy	0.93	0.28	0.001

Country	Time	mean	SE	LCL	UCL	Country	Time	difference	SE	*p.value
UK	*Colostrum*	7.978	0.108	7.766	8.189	*UK - Russia*	*Colostrum*	-0.010	0.132	0.997
Russia	*Colostrum*	7.988	0.095	7.801	8.175	*UK - Italy*	*Colostrum*	0.599	0.152	<0.001
Italy	*Colostrum*	7.379	0.119	7.146	7.611	*Russia - Italy*	*Colostrum*	0.609	0.141	<0.001
UK	*BM*	6.767	0.194	6.387	7.146	*UK - Russia*	*BM*	0.112	0.211	0.856
Russia	*BM*	6.655	0.109	6.442	6.868	*UK - Italy*	*BM*	-0.311	0.251	0.428
Italy	*BM*	7.078	0.175	6.736	7.420	*Russia - Italy*	*BM*	-0.423	0.194	0.074

Deliveries	Time	mean	SE	LCL	UCL	Deliveries	Time	difference	SE	*p.value
Primiparous	*Colostrum*	7.869	0.083	7.706	8.031	*Primiparous - Multiparous*	*Colostrum*	0.174	0.090	0.050
Multiparous	*Colostrum*	7.694	0.083	7.532	7.856	*Primiparous - Multiparous*	*BM*	0.174	0.090	0.050
Primiparous	*BM*	6.920	0.112	6.701	7.140					
Multiparous	*BM*	6.746	0.112	6.527	6.965					

TGF-β1 concentration

	Estimate	Std.Error	p-value
(Intercept)	6.71	0.21	<0.001
Breast Milk vs. Colostrum	-0.93	0.14	<0.001
Age influence	0.01	0.01	0.10
Country of collection Russia	-0.20	0.09	0.02
Country of collection Italy	-0.28	0.09	0.003
Later colostrum collection time	0.00	0.00	0.01
Fish ≥ Once a week vs. < Once a week	-0.15	0.07	0.04
C/Section vs. Vaginal	-0.05	0.08	0.57
Probiotics/Prebiotics ≥ Once a week vs. < Once a week	0.09	0.06	0.15
For BM : Fish ≥ Once a week vs. < Once a week	0.18	0.13	0.17
For BM : Country of collection Russia	0.59	0.15	<0.001
For BM : Country of collection Italy	0.18	0.17	0.30

Country	Time	mean	SE	LCL	UCL	Country	Time	difference	SE	*p.value
UK	*Colostrum*	6.801	0.066	6.672	6.931	*UK - Russia*	*Colostrum*	0.197	0.086	0.059
Russia	*Colostrum*	6.605	0.069	6.470	6.740	*UK - Italy*	*Colostrum*	0.276	0.094	0.010
Italy	*Colostrum*	6.526	0.075	6.379	6.673	*Russia - Italy*	*Colostrum*	0.079	0.098	0.701
UK	*BM*	5.962	0.112	5.743	6.181	*UK - Russia*	*BM*	-0.396	0.126	0.005
Russia	*BM*	6.357	0.077	6.207	6.508	*UK - Italy*	*BM*	0.099	0.151	0.790
Italy	*BM*	5.863	0.113	5.642	6.084	*Russia - Italy*	*BM*	0.495	0.128	<0.001
Fish	Time	mean	SE	LCL	UCL	Fish	Time	difference	SE	*p.value
< Once a week	*Colostrum*	6.720	0.062	6.598	6.842	*< Once a week - ≥ Once a week*	*Colostrum*	0.152	0.072	0.034
≥ Once a week	*Colostrum*	6.568	0.053	6.465	6.671	*< Once a week - ≥ Once a week*	*BM*	-0.024	0.106	0.824
< Once a week	*BM*	6.049	0.094	5.864	6.234					
≥ Once a week	*BM*	6.072	0.072	5.932	6.213					

TGF-β2 concentration

	Estimate	Std.Error	p-value
(Intercept)	11.30	0.17	<0.001
Breast Milk vs. Colostrum	-1.12	0.16	<0.001
Country of collection Russia	-0.33	0.12	0.01
Country of collection Italy	-0.39	0.13	0.002
Later colostrum collection time	0.00	0.00	0.12
C/Section vs. Vaginal	0.02	0.14	0.86
Fish ≥ Once a week vs. <Once a week	-0.17	0.12	0.14
Multiparous vs. Primiparous	-0.10	0.10	0.31
For BM : Fish ≥ Once a week vs. <Once a week	0.26	0.20	0.19

Country	Time	mean	SE	LCL	UCL	Country	Time	difference	SE	*p.value
UK	*Colostrum*	11.002	0.100	10.806	11.199	*UK - Russia*	*Colostrum*	0.328	0.119	0.016
Russia	*Colostrum*	10.675	0.100	10.480	10.870	*UK - Italy*	*Colostrum*	0.393	0.128	0.006
Italy	*Colostrum*	10.610	0.107	10.400	10.819	*Russia - Italy*	*Colostrum*	0.065	0.118	0.845
UK	*BM*	10.013	0.126	9.766	10.260	*UK - Russia*	*BM*	0.328	0.119	0.016
Russia	*BM*	9.686	0.114	9.462	9.909	*UK - Italy*	*BM*	0.393	0.128	0.006
Italy	*BM*	9.621	0.130	9.365	9.876	*Russia - Italy*	*BM*	0.065	0.118	0.845

TGF-β3 concentration

	Estimate	Std.Error	*p.value
(Intercept)	8.10	0.34	<0.001
Breast Milk vs. Colostrum	-2.03	0.13	<0.001
Age	0.01	0.01	0.18
Country of collection Russia	-0.34	0.12	0.004
Country.of.collection Italy	-0.74	0.13	<0.001
Multiparous vs. Primiparous	-0.20	0.11	0.07
C/Section vs. Vaginal	-0.12	0.13	0.39
Later colostrum collection time	-0.01	0.00	<0.001
For BM : Multiparous vs. Primiparous	0.29	0.17	0.09

Country	Time	mean	SE	LCL	UCL	Country	Time	difference	SE	*p.value
UK	*Colostrum*	7.760	0.093	7.577	7.942	*UK - Russia*	*Colostrum*	0.342	0.118	0.010
Russia	*Colostrum*	7.418	0.098	7.226	7.609	*UK - Italy*	*Colostrum*	0.741	0.130	<0.001
Italy	*Colostrum*	7.019	0.111	6.802	7.237	*Russia - Italy*	*Colostrum*	0.398	0.134	0.008
UK	*BM*	5.877	0.115	5.652	6.102	*UK - Russia*	*BM*	0.342	0.118	0.010
Russia	*BM*	5.535	0.108	5.323	5.746	*UK - Italy*	*BM*	0.741	0.130	<0.001
Italy	*BM*	5.136	0.128	4.885	5.387	*Russia - Italy*	*BM*	0.398	0.134	0.008

8.4.3 Data on maternal and environmental exposures influencing Th1/Th2 cytokines detectability in colostrum and breast milk (Final Mixed models)

IL2 detectability

	Estimate	SE	OR	2,50%	97,50%	P.value	AIC	BIC
(Intercept)	-0,68	0,43				0,11	298,99	321,69
Breast Milk vs. Colostrum	0,32	0,30	1,37	0,76	2,49	0,30		
Atopic mothers vs. Non atopic	-0,39	0,40	0,67	0,31	1,48	0,33		
Infections during pregnancy vs. No infections	-0,46	0,35	0,63	0,32	1,25	0,19		
Later Colostrum collection time	-0,02	0,01	0,98	0,97	1,00	0,02		

IL4 detectability

	Estimate	SE	OR	2,50%	97,50%	P.value	AIC	BIC
(Intercept)	-1,83	0,52	0,16	0,06	0,44	<0,001	378,81	401,587
Breast Milk vs. Colostrum	0,72	0,34	2,05	1,05	4,00	0,04		
Atopic mothers vs. Non atopic	-0,20	0,45	0,82	0,34	1,98	0,66		
Infections during pregnancy vs. No infections	0,36	0,36	1,43	0,71	2,88	0,32		
Later Colostrum collection time	-0,01	0,01	0,99	0,97	1,01	0,22		

IL5 detectability

	Estimate	SE	OR	2,50%	97,50%	P.value	AIC	BIC
(Intercept)	0,51	0,43				0,23	315,97	338,68
Breast Milk vs. Colostrum	-0,54	0,32	0,58	0,31	1,09	0,09		
Atopic mothers vs. Non atopic	-0,36	0,38	0,70	0,33	1,48	0,35		
Infections during pregnancy vs. No infections	-0,71	0,35	0,49	0,25	0,98	0,04		
Later Colostrum collection time	-0,03	0,01	0,97	0,96	0,99	<0,001		

IL10 detectability

	Estimate	SE	OR	2,50%	97,50%	P.value	AIC	BIC
(Intercept)	2,08	0,44				<0,001	416,7	435,8
Breast Milk vs. Colostrum	-1,66	0,32	0,19	0,10	0,36	<0,001		
Atopic mothers vs. Non atopic	-0,39	0,31	0,67	0,37	1,24	0,21		
Later Colostrum collection time	-0,02	0,01	0,98	0,97	0,99	0,001		

IL12 detectability

	Estimate	SE	OR	2,50%	97,50%	P.value	AIC	BIC
(Intercept)	-0,90	0,50				0,07	314,90	337,61
Breast Milk vs. Colostrum	-0,11	0,32	0,89	0,47	1,69	0,73		
Atopic mothers vs. Non atopic	-0,36	0,44	0,70	0,30	1,65	0,42		
Infections during pregnancy vs. No infections	-0,34	0,38	0,71	0,34	1,51	0,38		
Later Colostrum collection time	-0,01	0,01	0,99	0,97	1,00	0,12		

IL13 detectability

	Estimate	SE	OR	2,50%	97,50%	P.value	AIC	BIC
(Intercept)	-0,35	0,37				0,34	390,63	413,33
Breast Milk vs. Colostrum	0,09	0,26	1,10	0,66	1,82	0,72		
Atopic mothers vs. Non atopic	-0,46	0,33	0,63	0,33	1,22	0,17		
Infections during pregnancy vs. No infections	-0,12	0,28	0,89	0,52	1,53	0,67		
Later Colostrum collection time	-0,01	0,01	0,99	0,98	1,00	0,09		

IFNγ detectability

	Estimate	SE	OR	2,50%	97,50%	P.value	AIC	BIC
(Intercept)	-0,83	0,39				0,04	378,811	401,59
Breast Milk vs. Colostrum	1,20	0,26	3,32	2,01	5,48	<0,001		
Atopic mothers vs. Non atopic	0,30	0,30	1,35	0,75	2,44	0,32		
Multiparous vs. Primiparous	0,01	0,26	1,01	0,61	1,66	0,98		
Later Colostrum collection time	-0,01	0,01	0,99	0,98	1,00	0,04		

8.5 Appendix E (Sheth, 2014)

The following table is showing the dilutions necessary (in normal saline solution) to obtain detectable protein values in our pilot study, using the Abbott Aeroset Analyser

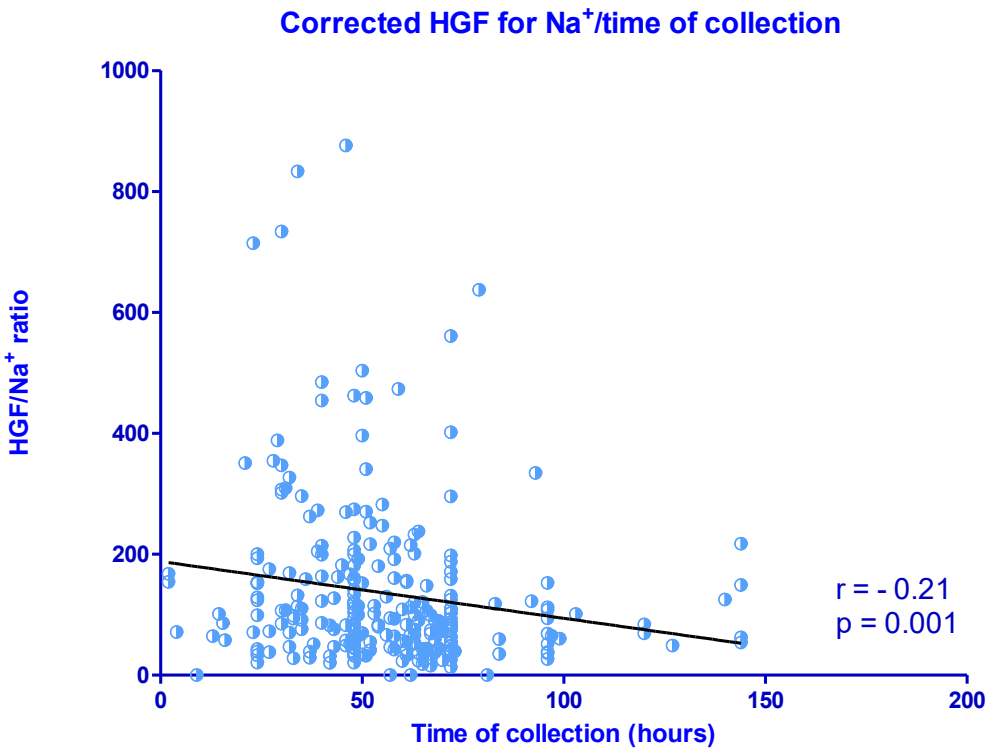
Dilution	**Dilution**	**Dilution**
1:10	1:20	1:40
Protein g/L	Protein g/L	Protein g/L
8.36		
9.07		
10.61		
12.87		
12.98		
15.74		
15.86		
16.53		
16.63		
18.72		
18.85		
>20.00	14.10	
>20.00	14.50	
>20.00	15.40	
>20.00	18.60	
>20.00	19.50	
>20.00	23.60	
>20.00	26.70	
>20.00	29.60	
>20.00	**>39.20**	37.60
>20.00	**>39.20**	39.12
>20.00	**>39.20**	42.96
>20.00	**>39.20**	65.44
>20.00	**>39.20**	**>39.2**

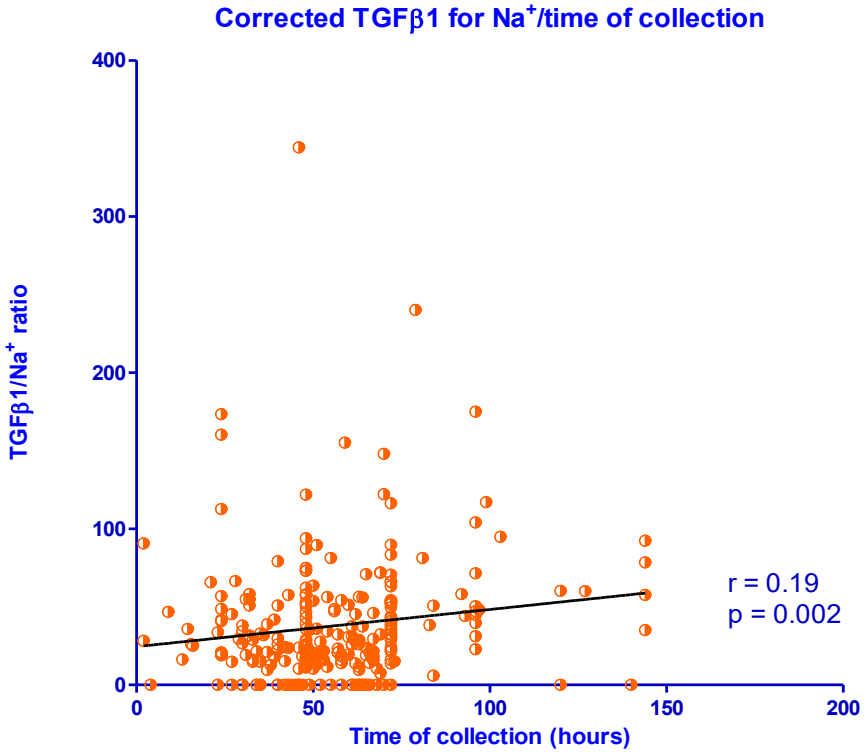
8.6 Appendix F (Sheth, 2014)

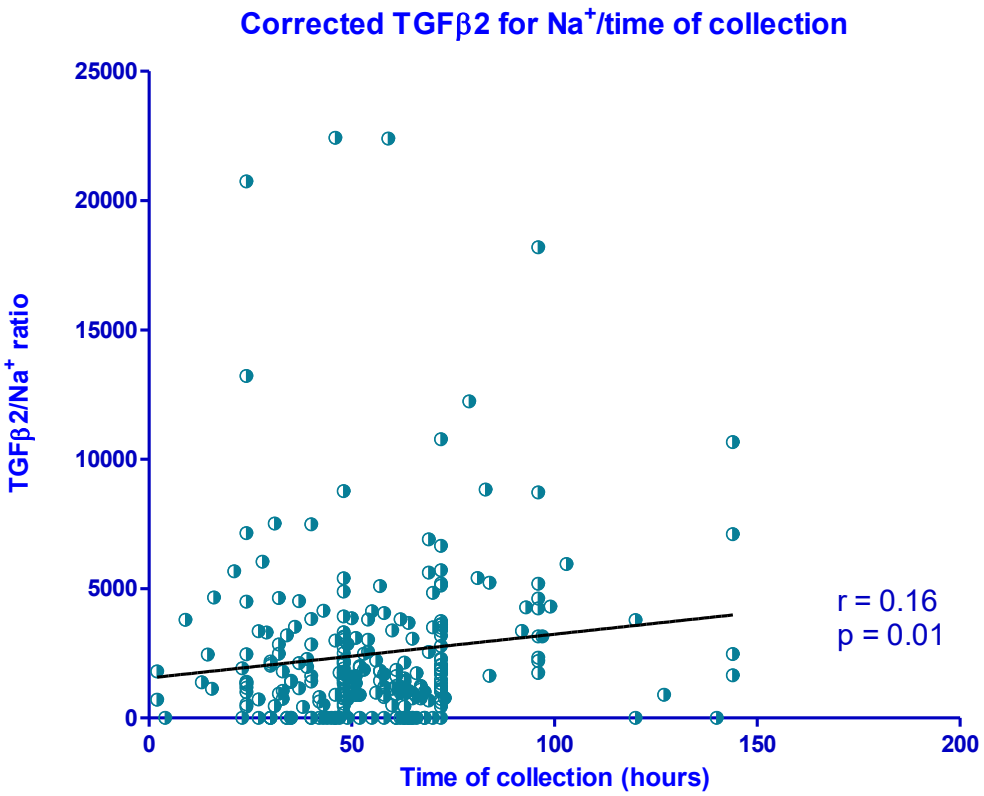
This table shows the results of serial dilutions on two samples, enabling us to establish a suitable dilution factor for an appropriate protein detection range.

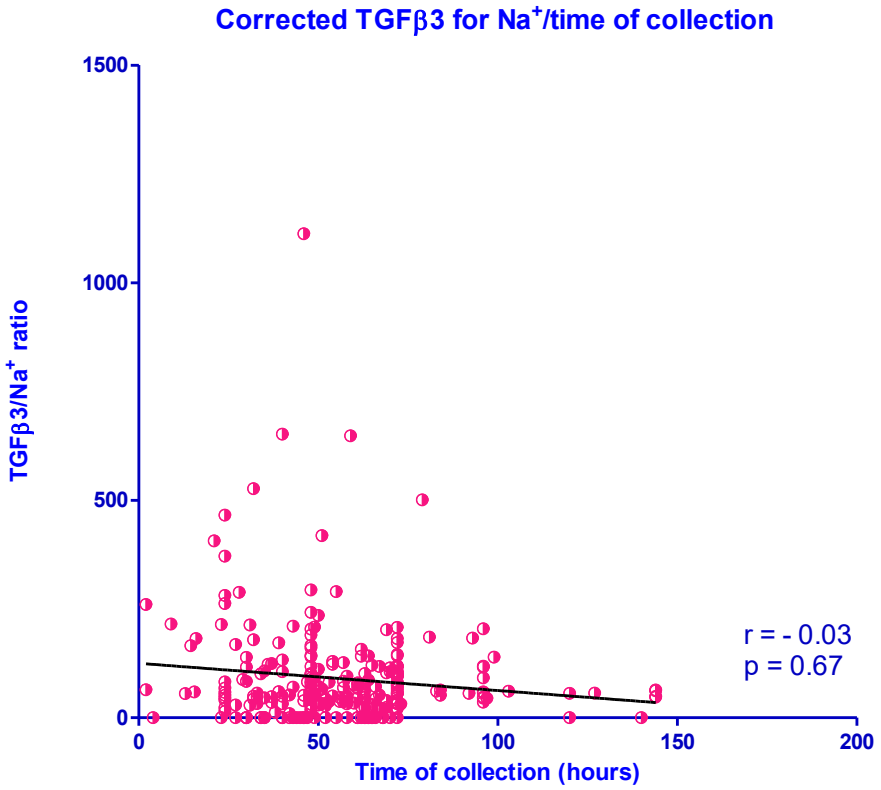
	Dilution factor	**No Dilution**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**
	Protein detection range, g\L	0.1-2	0.2-4	0.4-8	0.8-16	1.6-32	3.2-64
	Protein level g/L		5.2	2.44	1.74	0.7	0.34
Sample 1	Concentration g/L (Protein x Dilution factor)	12.98	10.4	9.76	13.92	11.2	10.88
	Protein level g/L		>10	6.56	2.87	1.83	0.76
Sample 2	Concentration g/L (Protein x Dilution factor)	26.7	>20	26.24	22.96	29.28	24.32

8.7 Appendix G






Corrected TGFβ2 for Na+/time of collection
TGFβ2/Na+ ratio
25000
20000
15000
10000
5000
0
0
50
100
150
200
Time of collection (hours)
r = 0.16
p = 0.01


Corrected TGFβ3 for Na+/time of collection
TGFβ3/Na+ ratio
1500
1000
500
0
0
50
100
150
200
Time of collection (hours)
r = - 0.03
p = 0.67