Thesis for doctoral degree (Ph.D.)
2015

MATERNAL MILK FEEDINGS AND CYTOMEGALOVIRUS INFECTION IN PRETERM INFANTS IN SWEDEN

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Cover: *Eva Charlotte, born at 25 weeks gestation, is being fed mother's milk at a postnatal age of 7 weeks.*
Photo by Andrew Hodges, with permission from Andrew and Marnie Hodges.
Published by Karolinska Institutet.
Printed by E-Print AB 2015

ISBN 978-91-7549-248-3

THE MILK OF HUMAN KINDNESS;
THE ROMAN TALE OF CIMON AND PERO

Roman Charity.
Guido Cagnacci (1601-1681).
Oil on canvas.

Museo de Arte de Ponce,
Puerto Rico.

This painting, known as *Roman Charity*, portrays the exemplary story of Cimon and his daughter Pero, by the ancient Roman historian Valerius Maximus.

Cimon is imprisoned with death penalty by starvation. Pero has recently given birth to a child and secretly breastfeeds her father during her visits in prison. One day, she is discovered by the authorities but her act of selflessness makes such an impression on them that her deed is forgiven and Cimon is released.

The tale of Cimon and Pero has inspired many great artists since the time of Rome resulting in several paintings, carvings and sculptures depicting the story.

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By

Soley Omarsdottir

The defence of the thesis will take place on Friday 12th of June, at 2:00 pm in the Welander lecture hall, Department of Clinical Dermatology, B2:00, Karolinska University Hospital Solna

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To all preterm infants in Sweden and their parents

SUMMARY

In Sweden, preterm infants are preferably fed human milk. Very preterm infants (< 32 weeks), who are unable to breastfeed, are fed with expressed maternal milk via a nasogastric tube. Mothers of these infants often experience difficulties in establishing and maintaining lactation. The majority of women excrete cytomegalovirus (CMV) in their breast milk. CMV transmitted through maternal milk can cause symptomatic infection in preterm infants presenting as a sepsis like syndrome, pneumonitis, hepatopathy or enterocolitis. Routine freezing of maternal milk decreases the CMV load in breast milk and is used in some neonatal centers to reduce CMV transmission to preterm infants.

The aims of the studies in this thesis were to document existing routines pertaining to breast milk use for preterm infants in Sweden, to investigate predictors of maternal milk feedings in extremely preterm infants (EPIs, < 28 weeks), to evaluate the rate and clinical expression of postnatal CMV infection in EPIs, to evaluate the effect of routine freezing of maternal milk on CMV transmission rate, CMV associated disease and neonatal morbidity and mortality in EPIs and to evaluate the prevalence of CMV infection in intestinal specimens from infants with necrotizing enterocolitis (NEC), spontaneous intestinal perforation (SIP) and related surgical conditions.

In a national cross sectional study in 2006 in Sweden, we found that 27 of 36 (75%) neonatal units had their own milk bank. Milk donors were screened for human immunodeficiency virus, human T-lymphotropic virus, and hepatitis B and C viruses by 27 (100%), 14 (52%), and 22 (81%) of the milk banks, respectively. Bacterial culture was performed on donor milk in 24 (89%) milk banks. Donor milk was pasteurized in 22 (81%) milk banks. In 11 of the 36 (31%) neonatal units maternal milk was frozen to reduce the risk of CMV transmission. Nutritional analysis of donor and/or maternal milk was performed in 25 (69%) units.

In a prospective cohort study at the neonatal units in Stockholm, including 97 mothers and their singleton EPIs, predictors of maternal milk feedings in EPIs during the first 6 weeks of life and at discharge were evaluated. Favorable predictors of maternal milk feedings the first 6 weeks of life were high maternal milk feedings (>90%) at second week of life, maternal university education and Nordic origin of the mother. The proportion of maternal milk feedings the first 6 weeks of life and maternal age were positively associated to the provision of maternal milk feedings at discharge while maternal overweight was an unfavorable predictor. High maternal milk feedings (>90%) at second week of life, assisted reproduction technology and maternal employment were predictive factors for exclusive maternal milk feedings at discharge.

Ten EPIs and their 6 mothers were included in a pilot study at the neonatal unit, Astrid Lindgrens Children´s hospital to evaluate the rate and clinical expression of breast milk induced CMV infection. Five (83%) mothers were CMV-seropositive; of these, 4 (80%) excreted CMV-DNA in breast milk and 2 (40%) had a positive CMV culture. CMV was detected in the urine of 2/7 (29%) EPIs fed with CMV-positive milk; both were fed with breast milk positive for CMV culture. One EPI, later diagnosed with cystic fibrosis, developed hepatic affection concurrent with CMV urine excretion.

In a randomized study at the neonatal units in Stockholm, evaluating the effect of routine freezing of maternal milk on postnatal CMV infection and neonatal outcome, 140 EPIs were randomized to be fed only freeze-thawed maternal milk (intervention group, IG) or both fresh maternal milk and freeze-thawed maternal milk (control group, CG). Outcome measures were CMV transmission rate and symptomatic infection in EPIs, neonatal mortality and morbidity during hospital stay. Fifty-six EPIs in the IG and 65 EPIs in the CG were included in the final per protocol analysis. We observed an overall low CMV transmission rate (8%) to EPIs from mothers with detectable CMV in breast milk. Routine freezing of maternal milk did not reduce the rate of CMV transmission (9% in IG vs 6% in CG). Congenital CMV infection was detected in 2% of screened infants. No infected EPI presented with clinical symptoms of CMV infection. Mortality rates were similar; 7% in the IG and 6% in the CG. Neonatal morbidity did not differ except for late onset Candida sepsis; the incidence was 12% in the CG while no case was observed in the IG.

In a retrospective observational study, we investigated the occurrence of the CMV in 70 intestinal specimens from 61 infants with NEC, SIP and related surgical conditions at the Karolinska University Hospital Solna and Uppsala University Hospital. Ten intestinal specimens from autopsied infants without bowel disease were controls. By using immunohistochemistry (IHC), we detected the CMV specific proteins CMV-immediate early antigen (CMV-IEA) in 81% (57/70) and CMV-late antigen (CMV-LA) in 64% (45/70) of the intestinal specimens; 2/10 (20%) of the control specimens were positive for both antigens. Although CMV antigens were prevalent irrespective of pathologic diagnosis, they were most frequent in specimens with the pathologic diagnosis NEC and intestinal perforation; 95% and 89% of these tissue specimens were positive for CMV-IEA and CMV-LA, respectively. CMV infection was confirmed by CMV-DNA analysis in 4/10 (40%) CMV-IHC-positive intestinal samples using Taqman PCR after laser capture microdissection and in 13/13 (100%) CMV-IHC-positive intestinal samples by in situ hybridization.

To conclude, human milk handling routines vary between neonatal units in Sweden and need to be standardized. Mothers of EPIs should aim for a high breast milk production immediately after delivery to optimize lactation success. Mothers who are young, overweight, of non-Nordic origin or without university education may need special lactation support. Postnatal CMV transmission from mothers excreting CMV in breast milk to EPIs was low (8%) and was not reduced by routine freezing of maternal milk. However, congenital CMV infection in EPIs was unexpectedly high (2%). No EPI infected by CMV presented with clinical symptoms. Routine freezing of maternal milk did not affect neonatal death in EPIs although it may have protected against fungal late onset sepsis. CMV infection was prevalent in intestinal specimens from infants with NEC, SIP and related surgical condition implicating a possible role of the virus in disease pathogenesis. More studies are needed to further evaluate the risk/benefit ratio of maternal milk feedings in EPIs with regard to the short-term and long-term effects of postnatal CMV infection.

LIST OF SCIENTIFIC PAPERS

I. **Omarsdottir S**, Casper C, Åkerman A, Polberger S, Vanpée M.
Breast milk handling routines for preterm infants in Sweden: a national cross-sectional study.
Breastfeeding Medicine 2008;3:165-70.

II. **Omarsdottir S**, Adling A, Bonamy AK, Legnevall L, Tessma MK, Vanpée M.
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Acta Paediatrica 2007;96:492-4.

IV. **Omarsdottir S**, Casper C, Navér L, Legnevall L, Gustafsson F, Grillner L, Zweygberg Wirgart B, Söderberg- Nauclér C, Vanpée M.
Cytomegalovirus infection and neonatal outcome in extremely preterm infants after freezing of maternal milk.
The Pediatric Infectious Disease Journal 2015;34:482-9.

V. **Omarsdottir S**, Agnarsdottir M, Casper C, Orrego A, Vanpée M, Rahbar A, Söderberg- Nauclér C.
High prevalence of cytomegalovirus infection in surgical intestinal specimens from infants with necrotizing enterocolitis and spontaneous intestinal perforation; a retrospective observational study.
Manuscript.

RELATED PUBLICATIONS

I. Benard M, Straat K, **Omarsdottir S**, Leghmari K, Bertrand J, Davrinche C, Duga-Neulat I, Söderberg-Nauclér C, Rahbar A, Casper C.
Human cytomegalovirus infection induces leukotriene B4 and 5-lipoxygenase expression in human placentae and umbilical vein endothelial cells.
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CD13 autoantibodies are elevated in sera from mothers of infants with biliary atresia and other neonatal cholestasis.
Manuscript in revision.

CONTENTS

LIST OF ABBREVIATIONS

AD	Anno Domini (in the year of the lord)
BC	Before Christ
BPD	Bronchopulmonary dysplasia
CMV	Cytomegalovirus
CMV-IEA	CMV-immediate early antigen
CMV-LA	CMV-late antigen
EGF	Epidermal growth factor
EPI	Extremely preterm infant
EPO	Erythropoietin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HTLV	Human T-lymphotropic virus
IGF-1	Insulin like growth factor - 1
IgA	Immunoglobulin A
IHC	Immunohistochemistry
ISH	In situ hybridization
IVH	Intraventricular hemorrhage
LOS	Late onset sepsis
NEC	Necrotizing enterocolitis
PDA	Patent ductus arteriosus
PP	Per protocol
PPROM	Preterm premature rupture of the membranes
PVL	Periventricular leukomalacia
RDS	Respiratory distress syndrome
ROP	Retinopathy of prematurity
SIP	Spontaneous intestinal perforation
VEGF	Vascular endothelial growth factor
VLBW	Very low birth weight

1 INTRODUCTION

Human milk is uniquely suited to the newborn infant. The use of human milk to feed all infants, including preterm infants (< 37 weeks), is strongly advocated because of the proven health, cognitive and psychological advantages on the breastfeeding infant extending onto infancy and adulthood. However, mothers of preterm infants often experience difficulties to establish and sustain adequate milk supplies. Very preterm infants (<32 weeks) are unable to feed directly from the breast and mothers of these infants need to express breast milk that is thereafter stored and fed to the infants by a nasogastric tube. Subsequently, when the infant has developed sucking skills, the transition from gavage feeding to breastfeeding may fail.

Cytomegalovirus (CMV) is a virus belonging to the herpes virus family. After primary infection the virus persists in the host and can be reactivated. Almost all mothers that have been infected by CMV excrete the virus in their milk after birth. Infants that are born term are protected by antibodies from their mothers and usually do not get ill when infected by CMV. However, preterm infants, especially extremely preterm infants (EPIs, < 28 weeks), have an immature immune system and lack these protective antibodies and can get seriously ill. One way of eliminating CMV from breast milk is to heat the milk but most heat procedures destroy many important nutritional and immunological constituents in the milk. Freezing maternal milk reduces the amount of the virus and does not have the same harmful effect on all milk components.

In Sweden, breastfeeding and the use of breast milk is highly valued and preterm infants are preferably fed maternal milk. If the mother cannot produce sufficient milk, or the milk cannot be used, donor milk is used. However, the prevailing routines of breast milk use in the care of neonatal infants in Sweden have not been surveyed. Likewise, knowledge is warranted on how to augment breast milk production in mothers of preterm infants during their neonatal stay. In addition, the rate and clinical expression of breast milk acquired CMV infection has not been evaluated in premature infants in Sweden. Furthermore, due to the lack of consistent results in clinical studies, a consensus on how to handle maternal milk to the most preterm infants with CMV transmission in mind is still needed. Moreover, little is known whether CMV can transmit to the intestine in utero, at birth or postnatally via breast milk and contribute to the pathogenesis of bowel diseases in the neonate.

In this thesis I have focused on finding an answer to the following questions:

What are the current routines for breast milk handling in the neonatal units in Sweden?
What factors are predictive for milk production in mothers of EPIs?
What is the rate and clinical expression of breast milk acquired CMV infection in EPIs in Sweden?
Can we prevent CMV transmission to EPIs by routine freezing of maternal milk and does routine freezing of maternal milk affect neonatal outcome?
Can we detect CMV infection in the bowel of infants presenting with necrotizing enterocolitis, spontaneous intestinal perforation or related surgical conditions?

2 BACKGROUND

2.1 HUMAN MILK AND BREASTFEEDING

Human milk is considered the ideal nutrition for both the term and the preterm infant (1). The composition of human milk, comprising both nutritional and nonnutritive bioactive factors, is tailored to promote survival and healthy development in the human infant (2). Today, exclusive breastfeeding for the first six months of life, with continued breastfeeding for at least a year, is recommended for infant feeding (1).

2.1.1 Historical perspectives

2.1.1.1 Mammalia

Human beings belong to the class Mammalia, a group of lactating animals characterized by the presence of breasts (mammae), which after giving birth secrete a fluid that fully comprises the nutritional requirements for to their young during a time period. This very ancient manner of nourishing the offspring dates some 100 million years ago, when the first mammals appeared (3).

2.1.1.2 Early evidence of breastfeeding

Abundant archeological findings of Middle Eastern pottery portraying lactating goddesses implicate that breastfeeding was held in high regard already as early as 3000 years Before Christ (BC) (3). In one of the oldest existing Egyptian medical writings, *The Papyrus Ebers*, recommendations are given on how to increase mothers milk supply, demonstrating that lactation failure was considered a definite problem during ancient Egyptian times (4):

"To get a supply of milk in a woman's breast for suckling a child: Warm the bones of a sword fish in oil and rub her back with it.

Or: Let the woman sit cross-legged and eat fragrant bread of soused durra, while rubbing the parts with the poppy plant."

The Papyrys Ebers (1150 BC)

2.1.1.3 Wet nursing

The use of a wet nurse, or "a woman who breastfeed another's child" was practiced already as early as 2000 BC. Through time, wet nursing evolved from an alternative of need, if the mother experienced lactation failure or died from childbirth, to an alternative of choice for woman of high rank in the society (950 BC to 1800 Anno Domini (AD)) (5).

In Greece, about 950 BC, wet nurses were frequently used in households of high social status, establishing a high position of responsibility taking care of the offsprings until adolescence (4).

In the medical treatise of Soranus of Ephesus (98 AD to 117 AD), addressing the choice of wet nurse in the 2nd century AD, the use of the fingernail test to determine breast milk quality was described (5):

"When a drop of breast milk was placed on a fingernail and the finger moved, the milk was not supposed to be so watery to assess the quality and consistency of breast milk that it ran all over the surface of the nail. When the fingernail was turned downward, the milk was not to be thick enough to cling to the nail. The consistency of the milk should range between the two extremes.".

In the Roman empire, between 300 BC and 400 AD, the wealthy made contracts with wet nurses to feed abandoned infants, often unwanted females, that were subsequently used as slaves in the household (5).

During the Middle Ages (500 to 1500 AD), people believed that breast milk was magic and that physical and psychological characteristics of a wet nurse could be conveyed to the nursed infant. This resulted in aversion against the hiring of wet nurses whereas a mother nursing her own child was highly valued (5).

Throughout the Renaissance period (1400 to 1700 AD), society displayed a preference for mothers breastfeeding their own children and wet nurses were disliked (5). This is noticeably illustrated in the work "*The nursing of children"* by the French obstetrician Jacques Guillemeau published in the beginning of the 17th century. In that work Guillemeau claims that there is *"no difference between a woman who refuses to nurse her owne childe and one that kills her child as soon as she hath conceived"* (6). The main objections that Guillemeau stated against wet nurse use were (5):

"1) the child may be switched with another put in its place, 2) the affection felt between the child and the mother will diminish, 3) a bad condition may be inherited by the child and 4) the nurse may transmit an imperfection of her own body to the child that could then be transmitted to the parents".

As Guillemeau believed that qualities of temperament could be conveyed by the milk he warned against wet nurses with red hair because redheads were known to have a hot temperament. If circumstances necessitated the use of a wet nurse, she should have the following characteristics according to Guillemeau (6):

"She should be physically healthy, with a pleasing countenance, 'ruddie mouth', and rosy complexion, and she should have 'veire white teeth' and broad but not pendulous breasts with good nipples; she should play with her charge and change him often".

Regardless of these recommendations, wet nursing for the societal class remained a popular, well paid profession for many poor women during the Renaissance period. Aristocratic women in high social classes worried that breastfeeding would impair their health and ruin their figures. Essentially, to breastfeed interfered with social activities of the higher class, such as attending theaters and playing cards and prevented them from wearing socially acceptable clothing (6) .

The Dauphin Louis of France (1638-1715) and his Nursemaid, Dame Longuet de la Giraudiere.
Oil on canvas.

Beaubrun, Henri (1603-77) and Charles (1604-92)
Château de Versailles, France.
Giraudon , The Bridgeman Art Library.

With the onset of the Industrial revolution in the end of the 18th century, wet nursing became more common in laboring lower income families. Women were forced to work to contribute financially to their families and were not able to attend and breastfeed their children (5).

In the 19th century, with the emergence of artificial feedings as a safe alternative to feed the infant, the demand of wet nurses diminished (7). Likewise, the establishment of wet-nursing regulations in order to decrease infant mortality led to fewer available wet nurses. In Sweden, the many supportive family policies that were introduced in the first half of the 20th century may have had an additional role for the final disappearance of wet-nurses (8). Today, although the World Health Organization recommends breast milk from a healthy wet-nurse over artificial milk for infants who are unable to breastfeed, wet-nursing is rare in the developed countries (9,10)

2.1.1.4 Artificial feedings as a substitute to breastfeeding

With the decreasing popularity of wet-nursing in the 19th century, dry nursing i.e. feeding infants milk from animals became increasingly practiced. At the same time, physicians were enthusiastic to develop a more adequate substitute for mother´s milk (11). The first commercial infant formula was made by the chemist German Von Liebig in 1867. This formula was composed of wheat flour, malt and potassium bicarbonate that was supposed to be mixed with preheated cow´s milk. As it soon became popular in Europe, some physicians endorsed the use of formula instead of hiring a wet nurse. Soon, in 1874, the first complete formula emerged that contained powdered cow´s milk, wheat flour, malt and sugar and only needed to be blended with water. At that time however, this formula was too expensive for the public majority (12). From 1890, pediatricians mostly recommended the use of artificial milk prepared by the "percentage method" by Rotch; a complex method that aimed at approximating the composition of cow´s milk close to that of human milk. This formula was principally used until 1915 after which commercial formula or home maid formula with evaporated milk took over (11). Concomitant with the developments described above, the

manufacturing of glass feeding bottles and rubber teats helped to encourage the use of breast milk substitutes.

By the establishment of refrigeration, allowing conservation of formulae together with promotional campaigns, the use of artificial feedings increased and breastfeeding rates declined. Moreover, during the feminist movement in the 1960s, feeding bottles were adopted as a symbol of women's liberation, resulting in further decline of breastfeeding rates (12). In the United States in 1970, only 25% of newborn infants were fed any breast milk at 1 week postpartum (13). Thereafter, following the world breastfeeding support movement, breastfeeding popularity increased, and the trend was reversed with inclining breastfeeding rates in the industrialized countries. To date, knowledge about the benefits of breastfeeding is becoming increasingly clear and many countries have regulated the advertisement of artificial feedings and constituted maternal leave with the objective of increasing breastfeeding prevalence rates (12).

2.1.1.5 Breastfeeding rates in Sweden

There is no statistical information on breastfeeding rates in Sweden from earlier than the 20th century (14). In 1945, 95% of infants were fed with breast milk at 2 months; after that the frequency of breastfeeding began to decline. At that time, hospital deliveries were instituted and the maternal wards lacked adequate breastfeeding support for the mothers. In addition, more women began to work outside their homes. Concurrently, manufacturers of artificial feedings began to market their products leading to a further decrease in breastfeeding rates. In the early 1970s, the attitude towards breastfeeding in society altered. The social and medical benefits of breastfeeding where underlined and the trend changed, resulting in a sharp increase in breastfeeding rates (Figure 1). This trend was further reinforced in the mid-1990s by the establishment of the breastfeeding promotion program Baby Friendly Hospitals Initiative. Of infants born in 1998, 93% were being exclusively or partially breastfed at the age of two months and 73 % at the age of six months (15). However, since 2004, the overall breastfeeding rates in Sweden have been successively decreasing resulting in a 10% reduction over a period of eight years in infants being exclusively or partially breastfed at the age of six months (15).

Nonetheless, by international standards the frequency of breastfeeding in Sweden is still high. According to The National Board of Health and Welfare in Sweden, almost 96 per cent of infants born in 2012 were breastfed at the age of one week, about 86 per cent at the age of two months and at six months, 63 per cent were being exclusively or partially breastfed (15). In comparison, in the United States in 2009, the breastfeeding initiation rate was 77% and 47% were at least partly breastfed at 6 months (3). Similarly, according to a recent national cross-sectional study in Ireland, the breast feeding initiation rate was 56% and 8% of infants were at least partly breast fed at 9 months (17).

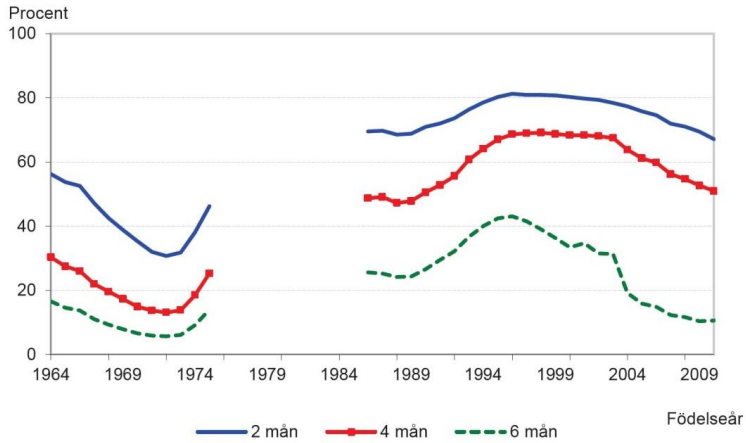

Figure 1 . The proportion of infants born 1964 to 2010 being exclusively breastfed at the age of 2, 4 and 6 months, respectively. (The National Board of Health and Welfare in Sweden, Amning och föräldrars rökvanor. Barn födda 2010 (16))

2.1.2 Breast anatomy and physiology of lactation

2.1.2.1 Breast structure

The female adult breast is composed of two separate functional parts. The first of these contains the glandular tissue that is concerned with milk production. The second part is comprises tissues that make up and support the breast that includes breast fat, connective tissue, and muscles (18).

The glandular tissue is composed of the alveolar gland with tree like ductular branching alveoli. It consists of 15 to 20 lobes that subdivides into 20-40 lobuli, again dividing into10-100 milk secreting units, or alveoli. The alveoli are composed of milk producing secretory cells surrounded by myoepithelial cells responsible for ejecting milk into the duct of the alveoli. Each alveolar gland then opens into a lactiferous duct merging into a larger duct, the mammary duct, that opens on the surface of the nipple (Figure 2) (19).

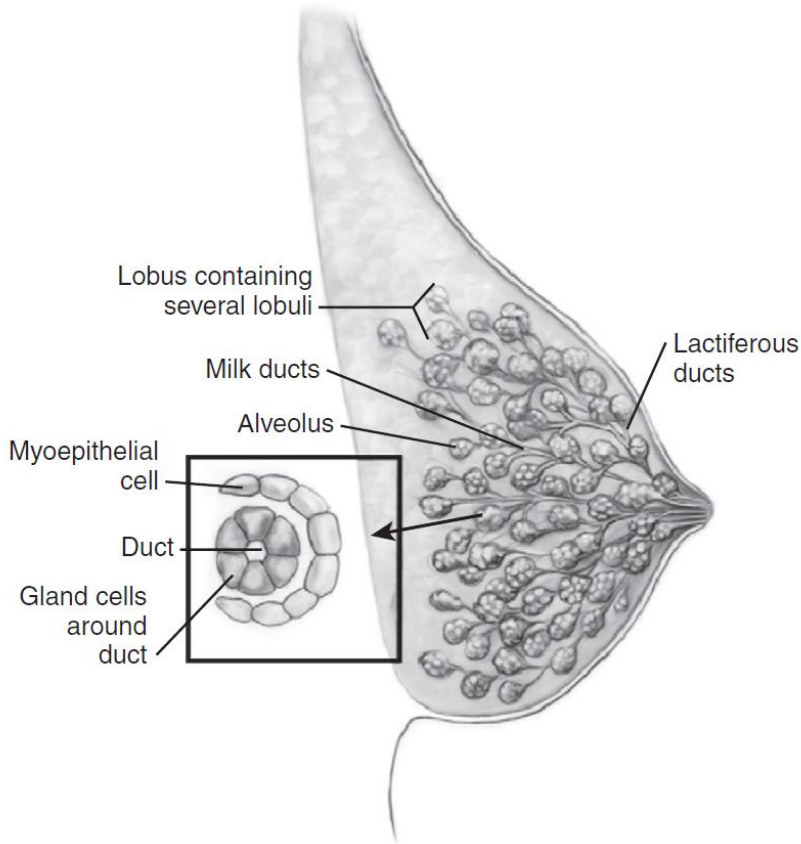

Figure 2. A simplified schematic drawing of breast structure (Breastfeeding: A Guide for the Medical Profession 2011 (19)).

2.1.2.2 Physiology of lactation

In humans, the mammary gland undergoes substantial physiologic adaption postnatally in order to be able to nourish the newborn child. The mammary development after birth are divided into 4 stages; mammogenesis, lactogenesis (lactogenesis 1 and 2), lactation and involution (20).

2.1.2.2.1 Mammogenesis

Mammogenesis, or breast development, takes place under the stimulation of serum hormones before and at puberty, during the menstrual cycles and throughout pregnancy. The hormones estrogen and progesterone stimulate the developmental changes that occur before and at puberty and in association with the menstrual cycles. During pregnancy, accelerated growth and proliferation occurs in response to luteal and placental hormones. The hormones

placental lactogen, chorionic gonadotropin and prolactin stimulate breast growth, estrogen promotes ductular differentiation and progesterone endorses lobular formation (20).

2.1.2.2.2 Lactogenesis

Lactogenesis, or the initiation of milk secretion, is divided into two stages. Lactogenesis 1, or secretory differentiation, is the developing capacity of the mammary gland to secrete milk during pregnancy. Lactogenesis 2, or secretory activation, is the initiation of copious milk production after birth (21)

LACTOGENESIS 1

Lactogenesis 1occurs from midpregnancy to late pregnancy when the epithelial cells of the alveoli differentiate into secretory cells and initiate milk synthesis through prolactin stimulation. Milk is secreted into the alveolar ductules.

LACTOGENESIS 2

Lactogenesis 2 is triggered by the rapid fall of progesterone and estrogen that occurs after delivery of the placenta. Release of prolactin by the anterior pituitary, no longer inhibited by these hormones, is thereby substantially increased. This stage of lactogenesis is controlled by central hormone release, i.e. endocrine controlled. With lactogenesis 2, tight junction complexes between the alveolar cells that previously have been open close tightly, thereby enabling the onset of secretion of copious amounts of breast milk.

2.1.2.2.3 Lactation

Lactation, the maintenance of milk production, begins at about 9 days postpartum. At this stage, continuous milk synthesis is driven by milk removal from the breast, or autocrine control. When milk is removed from the breast the hypothalamus inhibits the release of prolactin inhibiting factor, which in turn stimulates the release of prolactin and milk synthesis (Figure 2) (21).

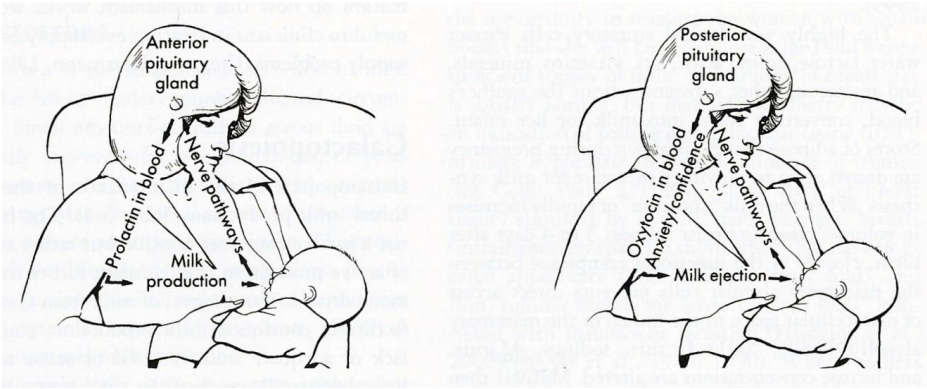

Figure 3. Release and effect of prolactin and oxytocin on milk production and milk ejection. (Breastfeeding and human lactation 2016 (21))

2.1.2.2.4 Oxytocin

The hormone oxytocin is released by the posterior pituitary in response to suckling or nipple stimulation and causes the milk ejection reflex necessary for milk removal from the breast. After release, oxytocin interacts with the myoepithelial cells that contract thereby ejecting milk from the alveoli into the ducts where it becomes available to the breastfeeding infant (21).

2.1.2.2.5 Involution

Involution, the last stage of lactogenesis, occurs on average 40 days after last breastfeeding and is a process where the milk producing epithelial cells die and are replaced by fat cells (21).

2.1.2.2.6 Delayed or failed lactogenesis 2

Delayed lactogenesis 2 is defined as a longer than usual phase between lactogenesis 1 and lactogenesis 2, or an onset greater than 72 hours after delivery (2). Failed lactogenesis 2 is defined as a failure to achieve full lactation; it can be caused by a primary inability of the mother to produce enough breast milk or be secondary to improper breastfeeding management and/or infant related problems (22).

In addition to maternal anatomical or hormonal aberrations that can affect the physiological processes involved in lactogenesis, several maternal and infant factors that delay breastfeeding initiation and/or breast stimulation can cause a failure or delay in lactogenesis 2 (22–24).

Table 1 lists known risk factors for delayed or failed lactogenesis 2.

TABLE 1. Risk factors for primary delayed or failed lactogenesis 2 (Modified from Hurst NM. 2007 (22–24))

Delayed lactogenesis 2
Age ≥ 30 years
Primiparity
Psychosocial stress/pain
Maternal obesity
Diabetes
Hypertension
Preterm birth
Unscheduled cesarean section
Stressful labor and delivery
Long duration of stage 2 labor
Prelacteal feeds, delayed first breastfeed episode
Infant birth weight > 3600 grams
Low perinatal breastfeeding frequency
Flat or inverted nipples
Nipple discomfort at day 0-3 postpartum
Hormonal contraceptive administration during the first week postpartum
Failed lactogenesis 2
Cigarette smoking
Breast surgery/injury
Insufficient mammary glandular tissue
Hypothyroidism, hypopituitarism
Ovarian theca-lutein cyst
Polycystic ovarian syndrome
Retained placental fragments
Postpartum hemorrhage with Sheehan´s syndrome
Maternal medication
Pseudoephedrine, estrogen containing birth control methods
Incomplete breast emptying
Improper latch on, scheduled feedings, supplemental feedings, pacifier overuse
Ineffective suck of infant
Preterm birth, tongue-tie, cleft-palate, congenital heart disease

2.1.3 The composition of human milk

Human milk is the ideal nutrition for the human infant, encompassing unique nutritional and bioactive factors that promote infant growth and development. As a complete overview of human milk composition is beyond the scope of this thesis a selected number of nutritional and bioactive milk components will be characterized.

2.1.3.1 Colostrum, transitional milk and mature milk

The first milk produced by mothers after delivery is colostrum. Compared to transitional and mature milk, proteins are present in high amounts but fat and carbohydrate concentrations are lower, keeping with the needs and reserves of the newborn infant (25). Colostrum is very rich in secretory immunoglobulin A (IgA), lactoferrin, leukocytes and oligosaccharides and contains epidermal growth factor (EGF) highlighting its important immunologic and trophic role in the neonate (2).

With the shift from lactogenesis 1 to lactogenesis 2 and closure of the gaps in the mammary epithelium, a 2-4 week period of ramped up milk production begins. During this period transitional milk is produced with increasing carbohydrate and lipid content to fulfill the infants increasing nutritional demands. By the end of the period, at 4-6 weeks, human milk is considered fully mature and remains relatively similar in composition throughout the rest of the lactation period (2).

2.1.3.2 Nutritional components

2.1.3.2.1 MACRONUTRIENTS

The macronutrient composition (protein, fat, lactose) of human milk varies within mothers and during the course of lactation.

PROTEIN

The mean protein content of mature term human milk is about 0.9 to 1.2 g/dL whereas the concentration in human milk after preterm birth is significantly higher depending on age of gestation at delivery (2,26–28). Regardless of the time of delivery, protein levels in human milk decrease during the first 8 weeks of life (Figure 3) (2,27).

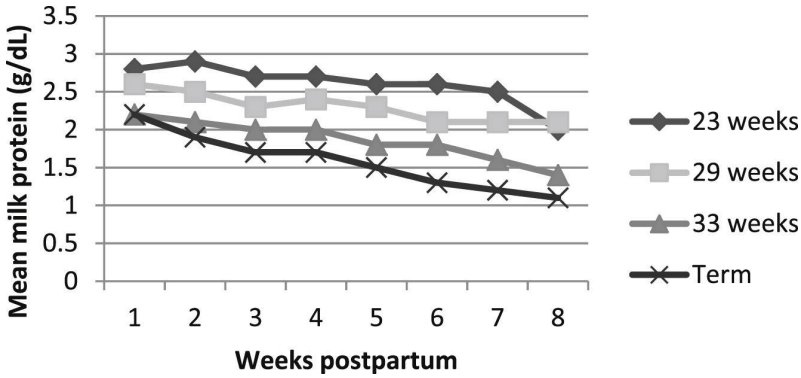

Figure 4. Milk protein concentrations from term and preterm mothers the first 8 weeks of life. (Ballard O, Morrow AL. 2013 (2))

FAT

Fat is the main provider of calories in human milk and is the most variable milk component (2,25). The total fat of human milk ranges from 2.2 to 6.2 g/dL (average 4.1 g/dL) and is higher in milk of preterm mothers, in afternoon and evening feedings and at the end of feedings (27,29,30). Milk fat is mainly composed triacylglycerols (98-99%) with lesser amounts of mono- and diacylglycerols, non-esterified fatty acids, cholesterol and phospholipids. The composition of fatty acids in human milk favors lipid absorption and digestion in the infant thereby supporting an adequate energy supply. Postnatally, human milk is an important source of long-chain polyunsaturated fatty acids such as decosahexanoic acid and arachidonic acid shown to enhance visual acuity and cognitive development by their uptake into the membranes of the retina and brain (31,32). Human milk fatty acids are dependent on maternal diet and body stores; therefore, some expert panels recommend frequent intake of omega-3 rich fish or supplemental decosahexanoic acid while breastfeeding (33,34).

LACTOSE

The disaccharide lactose accounts for most of the carbohydrates in human milk. Lactose in human milk is the most constant of the macronutrients (2). Lactose concentrations are relatively low in colostrum and in early lactation after which it increases to an average concentration of 6g/dL (35). Studies on the effect of preterm birth on lactose milk levels are contradictory; some studies show lower lactose content in preterm milk (36) but others do not (37,38).

2.1.3.2.2 MICRONUTRIENTS

In general, human milk will satisfy the micronutrient requirements of a healthy full term infant. However, the concentration of several micronutrients in human milk rely on maternal diet including vitamins A, B1, B2, B6, B12, D and iodine. If the maternal diet is not optimal, multivitamins during lactation is advocated (39). Vitamin D-supplement is recommended for

all breastfed infants in Sweden from the postnatal age of 1 week (40). When maternal body stores of iron are adequate, the amount of iron in human milk suffices for 4 to 6 months in the full term normal weight infant (41). Breastfed infants with a birth weight below 2500 g are at risk of iron deficiency, due to low iron deposits and rapid postnatal growth, and need supplemental iron from the postnatal age of 6 weeks in order to prevent iron deficiency and iron deficiency anemia(42,43) . Preterm infants fed human milk usually need special oral supplement of micronutrients (44), Injection of vitamin K to avoid hemorrhagic disease of the newborn is recommended in all infants after delivery as vitamin K is present in extremely low amounts in human milk (39).

2.1.3.3 Bioactive components

Bioactive components of human milk are factors with biological qualities that contribute to infant health and survival through enzyme activities, growth stimulation, enhancement of nutrient absorption, modulation of the immune system and defense against pathogens (2,45).

2.1.3.3.1 ENZYMES

LYSOZYME

Lysozyme is an enzyme which is present in high concentration in breast milk (400 µg/ml) and in higher concentrations in the preterm milk (46–48). It is an antibacterial enzyme that exerts its activity against bacteria by degrading their cell wall (45).

AMYLASE

Amylase is an enzyme that is synthesized in the pancreas and salivary glands and is necessary for the digestion of starch. Human milk contains significant amounts of salivary amylase. In newborns, amylase from the pancreas is not released into the gut the first 6 months of life. During this period, human milk amylase is believed to contribute to the infants ability to digest starch (48).

LIPASE

The bile-salt stimulated lipase and lipoprotein lipase in human milk aid in the digestion of lipids and compensate for the immature pancreatic function and the absence of amylase in neonates, especially in the premature infant (48).

2.1.3.3.2 GROWTH FACTORS

EPIDERMAL GROWTH FACTOR

Epidermal growth factor (EGF) stimulates the proliferation and differentiation of the intestinal epithelial cells and exerts protective and healing mechanisms in the infant intestine (2,49). Breast milk from mothers of EPIs contains higher levels of EGF than breast milk from mothers of older preterm infants or mothers of term infants and the EGF levels are stable

during lactation in the first month of life (50,51). In term infants, EGF concentrations are highest in early milk and decrease over lactation (52).

NEUROTROPHIC FACTORS

Brain-derived neurotrophic factor and Glial cell line-derived neurotrophic factor promote development of the enteral nervous system and can be detected in milk in up to 90 days following delivery (2,53).

VASCULAR ENDOTHELIAL GROWTH FACTOR

Vascular Endothelial Growth Factor (VEGF) is important in angiogenesis and its presence in human milk may have a role in retinopathy of prematurity (ROP, see below). VEGF concentration is higher in early milk (day 3) than mature milk (day 28) in both preterm and term human milk but lower in early preterm milk than in early term milk (54).

2.1.3.3.3 HORMONES

ERYTHROPOIETIN

Erythropoietin (EPO) is a hormone critical for the formation of red blood cells and is found in substantial quantities in human milk although variable between mothers and over time (55). EPO receptors have also been found in the intestinal mucosa and experimental studies suggest that enteral EPO may have a stabilizing and protecting role for the intestinal barrier in early life (56,57)

ADIPONECTIN

Adiponectin is a hormone with a metabolic regulatory role. It is found in large quantities in human milk and may contribute to a lower incidence of overweight and obesity later in life in breastfed infants (58,59).

INSULIN LIKE GROWTH FACTOR-1

Insulin like growth factor -1 (IGF-1) is a polypeptide with a growth promoting role found in in human milk (48). Levels are highest immediately after birth, decrease with lactation and do not differ in term and preterm milk (2). IGF-1 is important for early postnatal growth in preterm infants (60), contributes to gastrointestinal development (61) and protects against ROP (62)(see below).

2.1.3.3.4 IMMUNOLOGIC FACTORS

Human milk is enriched with immune factors that protect against infection and promote the development of the infant immune system (63).

HUMAN MILK CELLS

Human milk, especially colostrum, contains a variety of leukocytes derived from the maternal circulation, including granulocytes, macrophages, monocytes and lymphocytes (64). Higher

counts of milk leukocytes are found in colostrum and in milk of preterm mothers (65). Milk leukocytes are thought to confer active immunity and to modulate the development of immunocompetence in the infant (66). Also, apart from immune cells, other cell types have been recognized in human milk including epithelial cells, myoepithelial cells, lactocytes and stem cells (64).

CYTOKINES

Cytokines are proteins released by cells that exert actions on either the cytokine-producing cell or on other target cells (67). As cytokines contribute to the organization, development and function of the immune system they are thus important mediators and regulators of inflammatory responses (68).

The most abundant cytokine in human milk is transforming growth factor- ß known to regulate inflammation and to prevent allergy by inducing tolerance in the gastrointestinal tract (69).

Interleukin-10 inhibits the production of pro-inflammatory cytokines and is thought to be important for the homeostasis of the intestinal barrier and for the regulation of immune responses to foreign antigens (70).
Interleukin-6 is thought to play an important role for the development and differentiation of secretory IgA producing cells in the mammary gland (see below) (71).

The role of pro-inflammatory cytokines in human milk is still under investigation but it is believed that interleukin-1 and interferon- γ may affect the production of secretory IgA and other cytokines by the mammary gland (68).

IMMUNOGLOBULINS

Immunoglobulins, also known as antibodies, are proteins produced by plasma cells. They serve an important role in the immune response by recognizing and binding to particular antigens and aiding in their destruction. Antibodies can occur in two physical forms; a soluble form that is secreted from the cell into a body fluid or a membrane-bound form that act as a receptor on the surface of B-cells.
In human milk the most abundant immunoglobulin is secretory IgA (45). High concentrations of the protein can be found in colostrum (up to 5 mg/mL) decreasing to about 1 mg/mL in mature milk (48). Some studies have found a higher concentration of secretory IgA in preterm milk than term milk (47,72); still lower concentrations were found in infants born < 30 weeks of gestation compared to older preterm and term infants in one study (73). The secretory IgA antibodies prevent microbes from attaching to mucosal surfaces, especially the gut. In that way, maternal secretory IgA antibodies against bacterial, viral and fungal pathogens in human milk is transferred to the infant via the entero-mammary pathway, boosting the immature immune system of the newborn with the acquired immunity of the mother (74) . As the neonate is deficient in secretory IgA in early life, human milk intake of the protein provides an important immunological protection to the newborn infant (48).

2.1.3.3.5 OLIGOSACCHARIDES

Oligosaccharides are carbohydrates composed of a few units of single carbohydrates. Oligosaccharides are an abundant component of human milk found at concentrations ranging from 5-23 g/L (75). Levels of oligosaccharides are higher preterm milk than in term milk and decrease during the first 4 months of lactation (76,77). Oligosaccharides have an important role in establishing the gut flora of infants passing undigested to the colon part of the intestinal tract and promoting the growth of Bifidobacteria and Bacteroides (78). Additionally, human milk oligosaccharides prevent several bacteria and viruses from adhering to the epithelium of the gastrointestinal tract (75).

2.1.3.3.6 LACTOFERRIN

Lactoferrin is a potent multifunctional protein that is abundant in human milk; it is present in mature human milk in concentrations of 1.5-4 mg/ml and in colostrum at 8 mg/ml (79). In preterm milk, the proportion of lactoferrin relative to total protein is higher than in term milk (80). Lactoferrin has antimicrobial properties; it binds to iron and limits the ability of pathogens to grow (45) and it acts together with lysozyme in the stomach to kill gram positive and gram negative bacteria (81). In addition, lactoferricin, a peptide produced by proteolysis of lactoferrin in acidic milieus, disrupts the cell membrane of gram negative bacteria (49). In the small intestine, apart from preventing the adhesion of pathogens to the epithelium, lactoferrin promotes proliferation and differentiation of intestinal epithelial cells, initiates apoptosis of infected intestinal epithelial cells, reduces inflammatory cytokine production and stimulates the growth of commensal bacteria (49,81).

2.1.3.4 The composition of preterm human milk

The composition of preterm human milk differs from that of term milk with higher concentrations of many nutritional and bioactive components. These compositional differences are most pronounced during the first weeks of lactation (82). Energy, carbohydrate, protein and fat amounts are higher in preterm milk than in term milk that may reflect a compensatory lactating mechanism in the mother to fulfill the increased nutrient needs of the premature infant (82). The differences are most pronounced for protein and fat in the most preterm infants (26,83). Preterm milk contains higher concentrations of lysozyme, lactoferrin, secretory IgA and milk cells compared to term milk, providing additional immune protection to the preterm infant (46,47,65,72). Furthermore, preterm milk contains higher levels of EGF and oligosaccharide important for the maturation of the intestinal mucosa and the establishment of the gut flora (50,84).

Although human milk is considered the best nutrition for the preterm infant, due to its many beneficial effects (see below), it does not bring about the specific nutritional needs of the premature infant. The human milk fed preterm infant therefore requires nutrient supplementation to ensure nutritional adequacy to promote growth and development and to prevent nutritional disorders, such as metabolic bone disease and anemia (44,85,86).

2.1.4 Benefits of human milk feedings

Numerous beneficial effects of human milk have been demonstrated for the newborn infant, both the infant born at term and for the infant born preterm.

2.1.4.1 Reduction of morbidity

2.1.4.1.1 Short-term morbidity

Breastfeeding protects against respiratory infections, gastrointestinal infections, asthma, eczema and atopic dermatitis in infanthood (1). In preterm infants, human milk protects against neonatal bacterial infection, necrotizing enterocolitis (NEC) and ROP (87–89). Also, it precipitates full enteral feeding, promotes earlier discharge and reduces the rate of hospital readmissions in the first year after discharge (90–92).

2.1.4.1.2 Long-term morbidity

Infants with a history of breastfeeding have a reduced risk of obesity and diabetes mellitus (93,94). Additionally, infants that are breastfed at the time of gluten exposure have a decreased risk developing celiac disease (95). Furthermore, there is strong evidence that prolonged exclusive breastfeeding has beneficial effect on the cognitive development in children (48,96).

In the preterm infant, human milk positively affects long-term neurodevelopmental outcome (97–99) and skeletal mineralization (100). In addition, human milk decreases risks for high blood pressure in the preterm infant (101).

2.1.4.1 Reduction in mortality

In the developing countries, where infectious diseases significantly contribute to infant death, breastfeeding significantly decreases infant mortality (102,103). However, even in the developed countries, where infectious diseases account for a smaller proportion of infant mortality, breastfeeding is associated with a reduced risk for post-neonatal death (104). In the developed world, a considerate proportion of infant mortality can be attributed to sudden infant death syndrome (SIDS) that can be protected by breastfeeding (105,106).

2.1.5 Risks of human milk feedings

There are a few conditions and circumstances where breastfeeding or feedings of human milk is considered contraindicated.

2.1.5.1 Pathogens in human milk

There are several possible transmission routes for pathogens from the infected mother to the breastfed infant; the infant can be infected by respiratory secretions and droplets, via direct

contact with lesions in the nipple or the skin of the breast or through breast milk (107). Several known pathogens can be transmitted through maternal breast milk.

2.1.5.1.1 Viral pathogens

Human T-lymphotropic virus (HTLV) type I , associated with an increased risk for T-cell leukemia/lymphoma after early life infection, can be transmitted through breast milk (108). Infection with HTLV type I and HTLV type II both have been associated to the development of neurological diseases (109). Mothers positive for HTLV type I or II should be advised not to breastfeed or to give expressed milk to their infants (107).

Human immunodeficiency virus (HIV) can be transmitted through breast milk and in the industrialized world, HIV infection in the mother is a recognized contraindication for breastfeeding. In developing countries however, the risk of malnutrition and infectious diseases in non-breastfed infant are thought to outweigh the risk of HIV infection from human milk (1).

Transmission of Hepatitis B virus (HBV) occurs through breast milk. However, if infants of HBV surface antigen positive mothers are given hepatitis B immunoglobulin and HBV vaccine at birth, HBV transmission is prevented (107).

Although Hepatitis C virus (HCV) has been detected in low levels in breast milk, HCV infection through breast milk has not been proven, and transmission of infection seems similar in formula-fed and breast-fed infants of Hepatitis C positive mothers (107,110,111).

Human cytomegalovirus (CMV) is excreted in breast milk by the majority of breastfeeding carrier mothers (112). Whereas breast milk acquired CMV infection is usually asymptomatic in term infants, very preterm infants may develop severe clinical manifestations at the time of CMV transmission (113–115). For this reason a current debate is ongoing whether to treat maternal milk before feeding it to very preterm infants to reduce the risk of CMV transmission (see below).

2.1.5.1.2 Bacterial and fungal pathogens

The transmission of bacterial infections from mother to infant through breast milk is rare. However, if the mother has a severe contagious infection such as Neisseria gonorrhea, Haemophilus influensae, Group B streptococcus or Staphylococcus aureus, breastfeeding should be stopped temporarily whereas a longer period of abstinence for breastfeeding is required for infection such as Borrelia burgdoferi, Treponema pallidum and Mycobacterium tuberculosis (117).

Candida fungi can be transmitted from the maternal skin to the oral mucosa of the infant, particularly in mothers with Candida mastitis (118) . In the healthy term infant breastfeeding can be continued during fungal treatment of the mother-infant dyad (119).

2.1.5.2 Maternal medications, smoking and alcohol use

Some maternal medications may induce a risk of drug exposure to the infant through breast milk. Mothers medicating with amphetamines, ergotamines, statins and chemotherapy agents are generally advised against breastfeeding. Likewise, milk from mothers abusing phencyclidine, cocaine and cannabis may negatively affect long-term neurobehavioral development in the infant and is contraindicated (1).

Alcohol intake affects oxytocin and prolactin release and decreases milk output in lactating mothers; therefore limited ingestion of alcoholic beverages is recommended for breastfeeding mothers (1,120).

Maternal smoking while breastfeeding is discouraged as smoking decreases milk volumes (121,122) and postnatal maternal smoking increases the risk for sudden infant death syndrome and respiratory allergy (1,123,124).

2.1.5.3 Infant metabolic diseases

Infants with diseases that alter metabolism of certain nutrients may need to be nourished partly or exclusively with special modified formulas (41).

For instance, in classic galactosemia, the infant is unable to metabolize lactose and intake of galactose results in multi-organ dysfunction. As human milk contains high amounts of lactose, breastfeeding is strictly contraindicated.
In phenylketonuria, the enzyme that converses phenylalanine to tyrosine is defect, and dietary phenylalanine must be limited to avoid accumulation of abnormal metabolites in the tissues; breastfeeding is then alternated with phenylalanine free formula while closely monitoring phenylalanine blood levels (125).

2.1.6 Human milk banking

The World Health Organization and the United Nations Children's Fund created a ranking of feeding choices for infants in 2002. After breastfeeding the infant or feeding with mother's expressed milk, the choice is use of pasteurized milk from another mother via a milk bank (10).

2.1.6.1 History of human milk banking

By the early 20th century, when services of wet nurses abated and artificial feedings were still lacking, lactating women were encouraged to express extra milk for use in premature and ill children. With better technology and improvement in hygienic standards human milk donation evolved to a sophisticated system of operational milk banks (126).

The first operational donor milk bank was established in Vienna in Austria in 1909; shortly after that two more were opened in Boston, Massachusetts, USA and in Magdeburg in

Germany. At that time, more infants of earlier gestational ages with complicated medical conditions were surviving, increasing the interest in human milk banking. German and English guidelines for the operation of donor milk banks were designed in 1930, and these were expanded and adapted by the American Academy of Pediatrics in 1943. However, in the 1950s and 1960s, improvement in artificial feedings and the common belief that human milk could be replaced by formula led to a decline in milk banking both in Europe and in North America (126–128). As the interest in human milk banking again was renewed by the mid-1970s, raw or pasteurized donor milk was primarily used for medical purposes in preterm sick infants (129).
By the mid-1980s however, concerns regarding the risk of CMV and HIV transmission via human milk together led to increased used of specialty preterm formulas decreasing the demand of donor milk and closure of many milk banks (126,128–130). Accordingly, the Human Milk Banking Association of North America was founded in 1985 so as to standardize donor milk bank operations and establish guidelines to make the use of donor's breast milk safe. Recommendations on donor milk practices were first published in 1990 and are reviewed and updated annually and have been implemented by many other milk banks around the world (130). With increased awareness of the benefits of human milk and the safety of using processed bank milk around the world, corresponding regional and individual country organizations for milk banking have been established and in many countries national guidelines regarding the use of donor milk and the operation of milk banks have been issued (131–134).

In Sweden the network Milknet was established in 2001 by representatives of neonatal care with the purpose to maintain and improve access to donated milk, and to exchange experiences in breast milk handling and breast-milk feeding of newborn infants in neonatal units. The group formed Swedish national guidelines for use of human milk and milk handling that were published in 2008 and in a revised version in 2011 (135).

2.1.6.2 Donor milk use

Donor milk is the second choice of feedings for very preterm infants and sick infants with feeding intolerance treated in the neonatal intensive care unit. The use of donor milk is indicated if the mother is not able to express milk, if the use of maternal milk is contraindicated or to supplement the mother's own milk supply (135–137).

2.1.6.3 Milk donors

Milk donors come from different backgrounds. Mothers with large milk supplies may wish to donate so that their milk is not wasted. Other mothers might choose to express extra milk to help infants in need. For instance, mothers of preterm infants might want to contribute to saving the life of another preterm infant. Moreover, when mothers have lost an infant, banking milk could help contribute to healing process (138).

2.1.6.4 Donor milk screening

In Sweden, mothers that wishes to donate milk are screened according to the Swedish national guidelines for human milk and milk handling (135). Before acceptance, mothers submit a health declaration including health and risk history.

Mothers who smoke, use snus, use excess alcohol, or illegal drugs are not allowed as donors. Donors should not take any medications; some hormonal substitutions, topical inhalations steroids, topical treatment of skin, eyes and nose, gestagen contraceptives and occasional use of analgesics are though permitted. Women with a history of intravenous drug abuse, who have received an organ or tissue transplantation or a transfusion of blood products or have had body-piercing or tattooing for the last 12 months are not considered suitable as donors. Neither are women with a hemophiliac sex partner or a partner with suspected HIV, HTLV, hepatitis or intravenous drug abuse the last 12 months. Woman with cancer are not recruited as donors. Every mother should have a negative blood test for HIV, HTLV, HBV, and HCV before donation.

Donated milk should be cultured and proved to be free of pathogenic bacteria and have a content of less than 10,000 colony forming units/mL of Staphylococcus aureus and less than 100 colony forming units /mL of Enterobacteriaceae. During continued milk donation, bacterial testing shall be performed once a month.

2.1.6.5 Storage and treatment of human milk

Human milk banks collect, pasteurize, store, and distribute the human milk that has been donated. In Sweden donor milk is used primarily for preterm infants and almost all handling of donor milk is performed in milk banks stationed in neonatal care units.

In most milk banks, donated milk is pasteurized prior to its use. The most common heat treatment is rapid heating to 62.5°C for 30 minutes i.e. Holder pasteurization (135).

Holder pasteurization effectively eliminates viruses such as HIV, HTLV and CMV as well as most of the common bacterial contaminants (139–141). However, treatment by Holder pasteurization has unfavorable effect on many nutritional, bioactive and immunological components in the milk. It completely inactivates all human milk cells and reduces the levels of sIgA, lactoferrin, lysozyme, IL-10 and EPO (142–144). Likewise, it significantly reduces the fat and energy content of the milk and completely inactivates the milk lipases affecting the infants' ability to lipid absorption (139,145–147). In addition, the pasteurization process usually includes additional steps of changing containers and freeze-thawing that further reduces milk fat content (145,148).

In Sweden, mother's own milk is generally given succeedingly, as it is expressed, to avoid great variation in nutrient intake. Maternal milk is given either fresh or after freezing and defrosting (135). Compared to pasteurization, short-term freezing does not have the same

detrimental effects on the immunological and bioactive constituents of human milk (142,149–151).

2.2 PRETERM BIRTH

Worldwide, neonatal mortality is a major cause of death in infancy and childhood (152). In the developed countries, preterm birth and low birth weight (LBW) are the leading cause of neonatal death and infant death. In addition, prematurity and LBW conduce to more than half of all neurodevelopmental and other disabilities in infancy, childhood and adolescence (153).

2.2.1 Definitions of preterm birth, low birth weight and small for gestational age

In humans, an uncomplicated gestation is 40 weeks long, or 280 days, from the first day of the last menstrual period.

Preterm birth is defined as delivery occurring at less than 37 weeks' gestational age. Preterm birth can further be classified into very preterm birth and extremely preterm birth with delivery occurring at gestational age less than 32 weeks and 28 weeks, respectively.

Low birth weight is defined as a birth weight less than 2500 grams, birth weight less than 1500 grams as very low birth weight (VLBW) and less than 1000 grams as extremely low birth weight (153).

Infants that have lower birth weights than the 10th centile of the index population's distribution of birth weights by gestation are defined as small for gestational age. These infants birth weight lies below the 10th centile of the index population's distribution of birth weights by gestation (154).

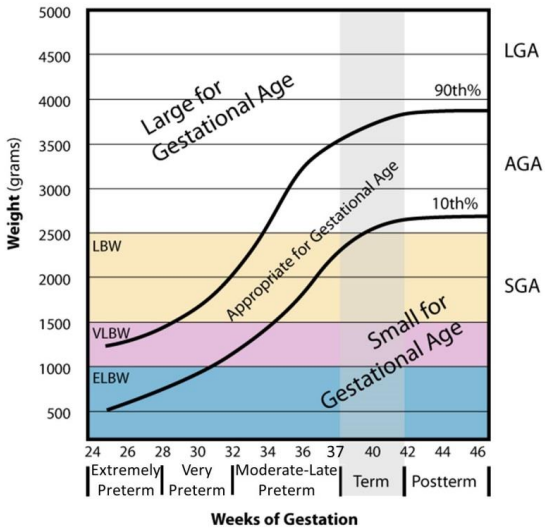

Figure 5. Categorization of preterm birth, low birth weight and small for gestational age. (Adapted from http://en.wikipedia.org/wiki/Small_for_gestational_age, accessed 2015-05-02)

2.2.2 Epidemiology of preterm birth

According to the World Health Organization, the global rate of preterm births in 2010 was 11%. The rate of preterm births varies, low-income countries have higher rates on average than high-income countries; estimated rates vary from 5% in many Northern European countries to 18% in Malawi (155). Consistent with data from the The National Board of Health and Welfare in Sweden, 6% of all deliveries in 2013 in Sweden were preterm births; of these 1% were very preterm deliveries before 32 weeks of gestation (156).

The rate of preterm birth is increasing in most industrialized countries around the world. In some countries an increase in preterm births occurring at 32 to 37 weeks is recognized (155). In addition, with advances in perinatal medicine even the most immature preterm infants can be saved. In Sweden from 1990 to 1992, the infant mortality (0-1 year) of EPIs born at 23 and 24 weeks were 92% and 72%, respectively (157). From 2004 to 2007, the corresponding numbers were 48% and 33% (158).

Likewise, the increased number of indicated preterm deliveries of artificially conceived pregnancies are important contributors to increased premature birth rates (159).

2.2.3 Causes of preterm birth

Causes of preterm birth can be divided into: 1) *indicated preterm labor* when delivery is brought about for maternal or fetal indications and 2) *spontaneous preterm labor* that follow spontaneous preterm labor with intact membranes or preterm premature rupture of the membranes (PPROM) (159).

Spontaneous preterm labor is defined as regular contractions accompanied by cervical change at less than 37 weeks of gestation.

PPROM is defined as spontaneous rupture of the membrane at less than 37 weeks' gestation at least 1 hour before the onset of contractions.

Approximately 30-35% of preterm deliveries are indicated whereas 40-45% follow spontaneous preterm labor and 25-30% follow PPROM (159).

Common reasons for indicated preterm delivery are pre-eclampsia or eclampsia in the mother or fetal intrauterine growth restriction (159). Spontaneous preterm labor can be initiated by various conditions including inflammation/infection, uteroplacental ischemia or hemorrhage, uterine overdistension, cervical disease, stress, endocrine disorders and other immunologically mediated processes (160). It is thought to be a multifactorial process where a number of risk factors, often associated to systemic inflammation, interact leading to preterm delivery or PPROM (159).

Some of the recognized risk factors for preterm labor are listed in table 2.

TABLE 2. Risk factors for preterm delivery (Adapted from De Bonis et al. Neonatology 2012 (160))

Risk factors for preterm delivery
Preconceptional
- Socio-economic characteristics
- Previous preterm delivery
- Interval between pregnancies
- Nutritional status
Maternal disorders
- Systematic diseases
- Local or systemic infections
- Previous uterine surgery
Pregnacy-associated risk factors
- Multiple pregnancy
- Intrauterine infection
- Vaginal bleeding
- Bacterial vaginosis
- Cervical shortening and insufficiency

2.2.4 Prognosis after preterm birth

2.2.4.1 Preterm mortality

In Sweden, the overall neonatal mortality (day 1-28 of life) in preterm infants has sequentially decreased from 1973 to 2013 with the most pronounced reduction in infants born before 33 weeks of gestation (156).

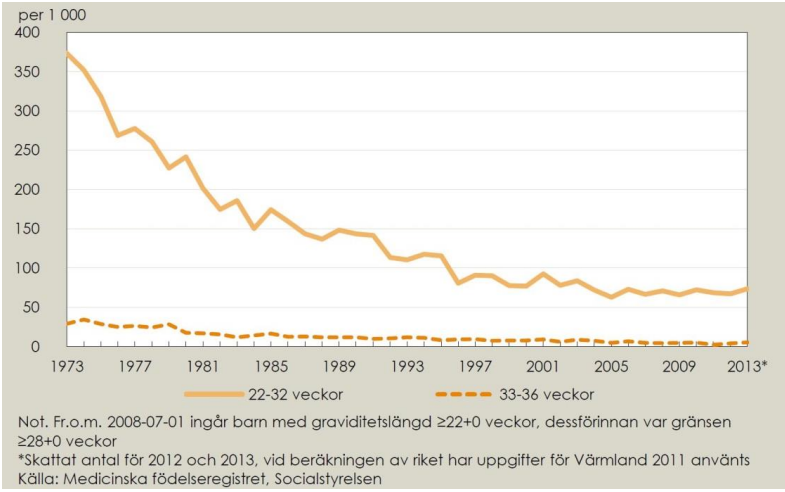

Figure 6. Decrease in neonatal mortality in preterm infants in Sweden from 1973 to 2013. (The National Board of Health and Welfare in Sweden, 2014 (156))

Both early neonatal death rate (day 0-6 of life) and late neonatal death rate (day 7-28 of life) increase inversely to gestational age in preterm infants.

In Sweden, early neonatal death for infants born at 22- 24 weeks of gestation 2004 to 2013 was 31%, decreasing to 9% in infants born at 25 to 26 weeks and to 5% in infants 27 to 28 weeks of gestation, respectively. Corresponding numbers for late neonatal deaths were 9%, 4% and 2%. Early neonatal death rate of all neonates was 0.12% during the time period late neonatal death rate was 0.04% (156).

2.2.4.2 Preterm morbidity

Due to the increased rate of preterm births and with the medical and technological advances increasing survival of the most preterm infants, preterm births are becoming a significant health problem in the developed countries (161,162).

Preterm birth causes injuries to many organ systems not yet prepared for the extra-uterine environment which result in the many complications of prematurity. Consequently, neonatal morbidities occur most frequently in the most preterm survivors due to the immaturity of their organ systems combined with prolonged hospital stays (163).

Although many preterm infants with major neonatal morbidities develop normally, neonatal morbidities are associated with high mortality rates and later adverse health, growth, and neurodevelopmental outcomes (161,164)

2.2.4.2.1 Major morbidities

RESPIRATORY DISTRESS SYNDROME

Respiratory distress syndrome (RDS) is an acute respiratory illness due to lack of surfactant, a substance produced after approximately 30-32 weeks of gestation that helps keep the air sacs (alveoli) open (165). The incidence of RDS increases with decreasing gestational age; about 5% of near-term infants are affected, 30% of infants of gestational age less than 30 gestational weeks and 60% of infants born before 28 weeks of gestation. The incidence and severity of RDS can be reduced by maternal administration of glucocorticoids prenatally to increase fetal lung maturity (166). After delivery, exogenous surfactant provided into the lungs improves lung function and decreases the risk of later chronic lung disease (see below) (167).

BRONCHOPULMONARY DYSPLASIA/CHRONIC LUNG DISEASE

Chronic lung disease, or bronchopulmonary dysplasia (BPD), is defined as a requirement for oxygen at 36 weeks of postmenstrual age (168).

Classically, before the introduction of antenatal corticosteroid and postnatal surfactant therapy, BPD occurred in preterm infants who had been treated with high ventilation pressures and oxygen concentrations for severe RDS. The condition was characterized by airway inflammation, fibrosis and smooth muscle hypertrophy (169). With advances in neonatal care a new disease entity developed where lung development is arrested before alveolarization resulting in lungs with larger but fewer alveoli. Likewise, impaired vasculogenesis results in a smaller vascular bed with increased vascular tone and reactivity (169). A number of factors have been implicated to contribute to the abnormal lung development; neonatal sepsis, patent ductus arteriosus (PDA), mechanical ventilation, oxygen therapy and possibly fetal response to chorioamnionitis and colonization by the bacteria Ureoplasma (169).

BPD results in chronic respiratory insufficiency with prolonged oxygen dependence. Infants with BPD have reactive airways, an increased vulnerability to respiratory infections and nutritional and fluid problems due to increased metabolic needs and fluid sensibility (165,170). BPD increases the risk of other neonatal complications such as patent ductus arteriosus (PDA), sepsis, intraventricular hemorrhage (IVH), ROP and death (170).

The risk to develop BPD is inversely related to both birthweight and gestational age at birth. In Sweden during 2004–2007 the incidence of BPD in infants born before 27 gestational weeks was 73%; 61%, 75%, 81%, 88% and 100% for gestational ages 26, 25, 24, 23 and 22, respectively (164).

Acknowledged measures to reduce the incidence of BPD include non-invasive ventilation and careful oxygen delivery whereas pharmacologic treatments comprise prophylactic surfactant therapy, methylxantines and vitamin A supplementation (171).

Today, BPD is the most common chronic respiratory disease in infancy, causing reduced lung function through childhood and into adult life (172). Furthermore, children with BPD born very preterm have an increased risk for cognitive, educational and behavioral impairments compared to very preterm infants without BPD (173).

PATENT DUCTUS ARTERIOSUS

Ductus arteriosus is a temporary fetal blood vessel between the pulmonary artery and aorta allowing fetal blood to bypass circulation to the lungs in utero. The ductus arteriosus normally closes after birth, when air enters the lungs and the lungs expand, redirecting the blood to the lungs.

In preterm infants, the duct may not close properly, shunting too much blood to the lungs which can lead to heart failure and reduced blood flow to vital body organs (165).

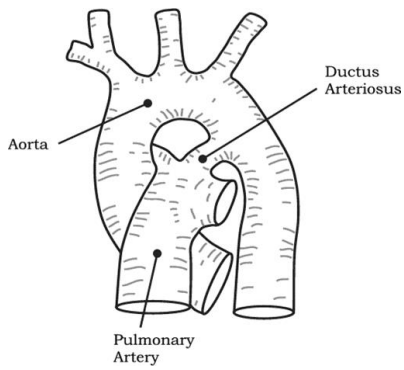

Figure 7. The great arteries and the ductus arteriosus (Schneider DJ 2012 (174))

The incidence of PDA in preterm infants increases almost linearly with decreasing gestational age, or circa 9% for each week of gestation (175).

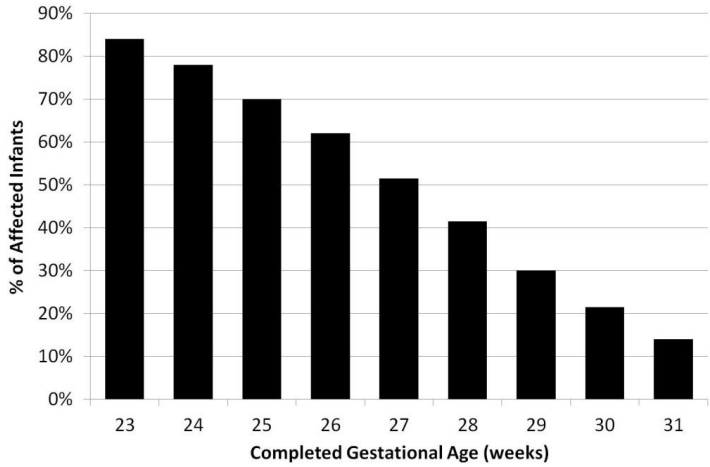

Figure 8. The incidence of PDA increases with decreasing gestational age. (Adapted from Hajj H 2012 (175))

A PDA may close spontaneously, or, it can complicate a preterm infant's clinical course with an increased the risks for neonatal complications such as IVH, NEC, BPD or death (165). PDA closes spontaneously within a few days in the majority of the more mature preterm infants. In extremely low birthweight infants however, the rate of spontaneous closer is only 33% within the first week of life (176).

Current treatment approaches to a PDA today include pharmacologic treatment with nonselective inhibitors of cyclooxygenase (Indometacin or Ibuprofen) or surgical ligation. However, as spontaneous closure occurs in a substantial proportion of preterm infants randomized controlled trials to evaluate the risk/benefit of these treatment are lacking and these treatment options are currently debated (177,178).

INTRAVENTRICULAR HEMORRHAGE AND PERIVENTRICULAR LEUKOMALACIA

In preterm infants, the incomplete formation of the central nervous system renders it vulnerable to injury, especially the highly vascular germinal matrix and the white matter around the ventricles that have difficulties with auto-regulation of cerebral blood flow (165).

Brain injury constitutes a main complication in the perinatal period with long-term consequences as early injury can interrupt normal brain maturation with the risk of subsequent neurodevelopmental disabilities (179).

2.2.4.2.1.1.1 INTRAVENTRICULAR HEMORRHAGE

IVH is a major complication of preterm birth. Massive IVH may result in death from hypovolemic shock, while large hemorrhages may result in severe disability in the affected infant (180).

IVH is a complex, developmental disorder where multiple environmental and genetic factors interact (181). IVH usually occurs in infants born before 32 weeks of gestation and the incidence is inversely related to gestational age (182). In the national Swedish EXPRESS study, 10% of infants born before 27 weeks of gestation had severe IVH (grade 3 or more, see classification below) (164). Even if IVH can occur in utero, IVH is usually an early postnatal event ensuing within the first 72 hours after birth (165,180).

IVH originates from the capillaries of the germinal matrix just below the ventricles. This sub-ependymal germinal matrix, rich in immature vessels poorly supported by connective tissue, is vulnerable to fluctuations in cerebral blood flow and swings in intra-thoracic and venous pressure that occurs with severe respiratory problems in the preterm infant. Subsequent to bleeding in the subependymal germinal matrix, blood filling the lateral ventricles may lead to ventricle dilatation (165,180).

IVH is graded according to Papile's classification; Grade I is confined to the sub-ependymal germinal matrix with no blood clot in the lumen, Grade II is defined by the presence of blood within the ventricular lumen but without ventricular dilatation, Grade III consists of IVH with ventricular dilatation, and Grade IV is IVH accompanied by parenchymal hemorrhagic infarction (183).

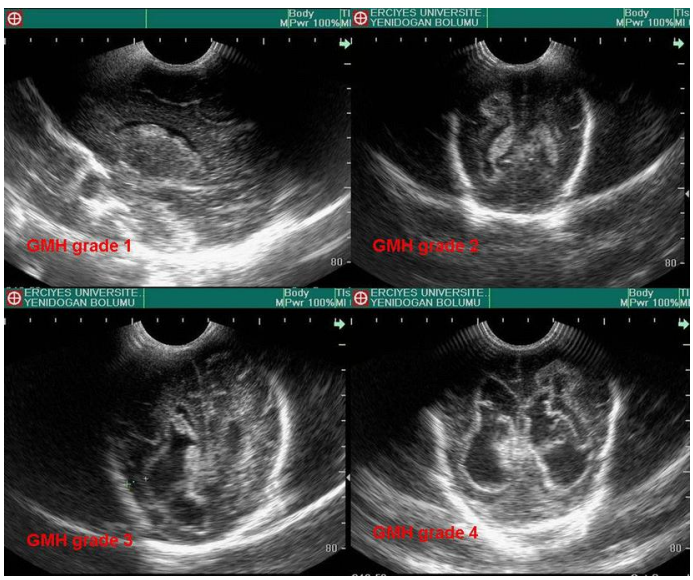

Figure 9. IVH grading according to Papile (Köksal V 2010 (184))

Management of IVH encompasses: prophylactic care by minimal handling of the EPT infants; screening for symptoms of IVH; supportive care with correction of underlying medical disturbances, such as blood pressure, respiratory status and coagulopathies, which might influence progression of IVH; and treatment of the ensuing complications such as seizures and post-hemorrhagic hydrocephalus (182).

The outcome of IVH depends on gestational age and the severity of the bleeding. Short-term, 5 to 10% of preterm infants with Grade III or IV IVH suffer seizures in the neonatal period and up to 50% develop post-hemorrhagic hydrocephalus (180) and the overall mortality is higher than in gestational age-matched infants without IVH (182).

In the long-term, IVH conveys an increased risk of cerebral palsy, visual impairment and delayed psychomotor and mental development with the risk for sequelae inversely related to the gestational age of the infant and correlating to the grade of bleeding (185).

2.2.4.2.1.1.2 PERIVENTRICULAR LEUKOMALACIA

Periventricular leukomalacia (PVL) is the predominant brain injury underlying neurologic morbidity in preterm infant; it is the main cause of cerebral palsy and cognitive impairment in these infants (186). Using magnetic resonance imaging, some degree of cerebral white matter injury can be detected in at least 50% of VLBW infants (187) .

PVL is caused by white matter necrosis due to ischemia-reperfusion injury in the cerebral artery watershed area and is expressed as either focal periventricular necrosis with subsequent development of cysts or diffuse cerebral white matter injury (163,188). PVL is highly correlated with prematurity. Other contributing factors include chorioamnionitis, neonatal hypocarbia and hypotension (188).

There are no effective treatments for periventricular white matter damage, though promising neuroprotective strategies are emerging (189). Adequate supportive neonatal care contributes to improve the final neurological outcome (190).

NECROTIZING ENTEROCOLITIS

NEC is an acute inflammatory necrosis of the intestinal tract primarily affecting preterm VLBW infants (191). As a major cause of morbidity and mortality in neonates it has become one of the most dreaded diseases in neonatal intensive care units (192). The incidence of NEC is increasing as a result of advances in neonatal care and shows a clear, inverse relationship with birth weight and gestational age (193,194). NEC manifests in 7 to 11 % of infants born weighing less than 1500 g (195); about half of these infants will require surgery of which 30% will not survive (196).

NEC is a multifactorial illness with an incomplete understood pathogenesis. The combination of bowel immaturity, abnormal pathogenic colonization of the bowel , enteral feedings and intestinal ischemia are thought to provoke an aggravated intestinal inflammatory response that lead to damage of gut epithelium, translocation of intraluminal contents and induction of a systemic inflammation response (191,197).

Currently acknowledged risk factors include formula feedings, prolonged empirical antibiotic treatment and the use of acid blockade (198).

The use of human milk is of major importance in the prevention of NEC (198). Human milk reduces the incidence and severity of NEC with some studies demonstrating a dose-

dependent effect (199–201). The protective effect of human milk can be attributed to its content of bioactive factors; these are of importance for the development of the infant´s gut mucosa, the establishment of a favorable intestinal microbial ecology and due to their anti-pathogenic and immunomodulary properties (2,202). Standardized feeding regimens, minimized antibiotic and acid blockade treatment are additional protective measures against NEC (198). Among other proposed preventive strategies against NEC are the use of probiotics and oral lactoferrin; however, these alternatives need more study (203,204).

The classical manifestations of NEC in a preterm infant are abdominal distension, feeding intolerance and bloody stools presenting at about 29-32 weeks of postmenstrual age. The progress of the disease may be rapid, succeeding from subtle signs to abdominal discoloration, intestinal perforation and peritonitis, advancing to systemic hypotension requiring intensive medical and frequently surgical support (205).

In the field of neonatology and pediatric surgery, Bells staging is used to gauge the severity of NEC (206):

TABLE 3. Modified Bell´s staging for NEC (Adapted from Gordon 2007 Journal of Perinatology (206))

Review of Bell's stages	Clinical findings	Radiographic findings	Gastrointestinal findings
Stage I	Apnea and bradycardia, temperature instability	Normal gas pattern or mild ileus	Gastric residuals, occult blood in stool, mild abdominal distention
Stage II A	Apnea and bradycardia, temperature instability	Ileus gas pattern with one or more dilated loops and focal pneumatosis	Grossly bloody stools, prominent abdominal distention, absent bowel sounds
Stage II B	Thrombocytopenia and mild metabolic acidosis	Widespread pneumatosis, ascites, portal-venous gas	Abdominal wall edema with palpable loops and tenderness
Stage III A	Mixed acidosis, oliguria, hypotension, coagulopathy	Prominent bowel loops, worsening ascites, no free air	Worsening wall edema, erythema and induration
Stage III B	Shock, deterioration in laboratory values and vital signs	Pneumoperitoneum	Perforated bowel

Today, NEC is treated either by medical interventions or, in case of deteriorating status with intestinal perforation, surgery as well. Apart from intravenous antibiotics, medical interventions include abdominal decompression, bowel rest and intravenous fluid resuscitation whereas surgical procedures encompass drain placement or exploratory laparotomy with resection of diseased bowel (207).

Short-term complications of NEC survivors comprise recurrent disease, strictures and stoma complications. The most common long-term gastrointestinal complication is short bowel syndrome, affecting about one-fourth of NEC survivors. Abnormal growth may occur, especially in those children with short bowel syndrome. Likewise, infants surviving NEC have an increased risk for neurodevelopmental dysfunction (207).

RETINOPATHY OF PREMATURITY

ROP is a leading cause of visual impairment and blindness among preterm infants (208) It is a neovascular retinal disorder with multifactorial etiology. Preterm infants have incompletely vascularized retinas with a peripheral avascular zone. As the infant matures, the nonvascularized retina becomes increasingly metabolically active, leading to tissue hypoxia. Hypoxia stimulates up-regulation of proangiogenic growth factors such as VEGF and EPO which can lead to uncontrolled vascular growth into the vitreous (209).

Multiple risk factors have been suggested in the genesis of ROP. Prematurity, low birth weight and postnatal oxygenation are recognized risk factors (62,210). Likewise, low early-postnatal serum IGF-1 concentrations increase later risk of ROP. In utero, IGF-1 concentrations increase with length of gestation; after birth, IGF-1 levels are nutrition dependent and decrease with starvation, infection and stress. Preventive postnatal measures against ROP thus comprise careful oxygenation, and provision of adequate nutrition and treatment of infections (62,211).

ROP is classified according to the international classification of ROP (212). Staging indicates the severity of the disease; stage 1-2 comprise mild ROP and stages 3-5 severe ROP. Stage 1 is defined as a distinct line between the vascularized and avascularized regions of the retina. Stage 2 occurs when the line noted in Stage 1 gains both depth and height. When the blood vessels extend into the vitreous, it is classified as Stage 3. Stage 4 is defined as partial retinal detachment; Stage 5 is total retinal detachment (213).

In a national Swedish study conducted 2008 to 2009 and including 1784 infants less than 32 weeks of gestation, ROP was found in 25.1% of infants; among these 8.5% had severe ROP (214). Comparable, the reported incidence of severe ROP in infants less than 27 weeks of gestation in the Swedish express study was 34% (164).

Early detection of ROP in preterm infants is ensued by appropriate screening protocols during the first weeks of life. Severe ROP is managed with ablation of the non-vascularized retina with trans-pupillary laser treatment thereby avoiding aberrant preretinal neovascularization (62). A new promising impending approach against ROP in clinical practice is intravitreal anti-VEGT therapy; however the safety profile of this treatment must be better elucidated as these drugs may interfere with vasculature development elsewhere in the body (215,216). Apart from visual loss and blindness, ROP is associated with other eye problems, such as strabismus, myopia and astigmatic refractive errors, prompting the need of continuous follow up in these infants (209).

INFECTIONS

In low birth weight and preterm infants neonatal infection is a significant cause of mortality and short and long-term morbidity. The pro-inflammatory state associated with infection may interrupt developmentally regulated processes involving the central nervous and respiratory

systems resulting in an increased risk for death, chronic lung disease, and adverse neurodevelopmental outcome (217).

Neonatal infections can have an early onset within 72 hours of birth or a late onset when occurring after the first 72 hours. Septicemia accounts for the majority of all infections (45-55%) preceding low respiratory tract infections (16-30%) and urinary infections (8-18%). Early onset sepsis is often related to maternal pregnancy complications and usually involves pathogenic organism of the maternal genitourinary tract whereas late onset sepsis (LOS) is usually caused by nosocomial or environmental pathogens (218).

Nosocomial infection rate is inversely related to birth weight and gestational age (219). In a report from The National Institute of Child Health and Human Development Neonatal Research Network by Stoll et al., the incidence of culture proven LOS in VLBW infants over a 2 year period (1998-2000) was 21% ; 46% for infants less than 25 weeks' gestation, 29% for infants 25 to 28 weeks' gestation, and 10% for infants 29 to 32 weeks' gestation (220).

Very preterm infants are susceptible to infections due to naive immune systems, defective regulation in immune homeostasis and lack of acquired maternal immune factors (221).

While infants born at term are protected by maternal antibodies, transferred to them through the placenta, preterm infants may lack maternal antibodies, which largely occur during the third trimester of gestation (221). Furthermore, ill preterm infants undergo medical interventions such as mechanical ventilation, intravenous lines and drains that interfere with the body's protective mucosal and epithelial barriers (222).

Postnatal infections in preterm infants can be acquired through their immature skin, lungs, or gastrointestinal tract, which lack fully developed immunoprotective functions. Due to their immature immune system, they have difficulty confining these infections to where they arise so sepsis frequently develops (165).
Apart from low birth weight and prematurity, risk factors for early onset sepsis include maternal group B streptococcal colonization, maternal intraamnionic infection and prolonged rupture of membranes (> 18 hours) (223). Risk factors for LOS comprise postnatal use of steroids and H2 antagonists, poor hand hygiene, previous antimicrobial exposure and central venous catheters (218,224).To the contrary, the use of human milk feedings and oral administration of oral lactoferrin, a component of breast milk (see above), have protective effect against neonatal sepsis in preterm infants (87,203)

Clinical manifestations of neonatal infection can vary. Symptoms can present very subtly and progress gradually or a rapid deterioration with sudden collapse can occur. In general, infected neonates present with systemic signs such as recurrent apneas that may necessitate mechanical ventilation. Hypotension, shock, renal and hepatic dysfunction, coagulopathy, thrombocytopenia, glucose dysregulation and seizures can also be accompanying features (221).

The most prevalent pathogens of neonatal infection are listed in table 4.

Table 4. Important organisms presenting as neonatal sepsis (Ghazal P 2013 (221)).

Early-onset bacterial infection: infection presenting within 72 h of birth	*Streptococcus agalactiae* (Group B streptococcus) *Escherichia coli* *Haemophilus influenzae* *Listeria monocytogenes*
Late-onset bacterial infection: infection presenting later than 72 h after birth	Coagulase-negative staphylococci *Staphylococcus aureus* *Escherichia coli* and other Gram-negative organisms *Enterococcus* species *Klebsiella* species *Streptococcus agalactiae* (Group B streptococcus)
Viral infection	Cytomegalovirus Enterovirus Herpes simplex virus
Fungal infection	*Candida* species

Most prevalent organisms will vary between regions.

Neonatal infections require prompt treatment with antibacterial, antiviral or antifungals drugs depending on the suspected pathological agent (223). Currently available strategies to prevent neonatal infections include policies related to neonatal management, and the use of drugs/bioactive substances that may be helpful in preventing diseases caused by specific pathogens. These include hand-hygiene, prevention of central-line associated blood stream infections, skin care, early feedings with human milk, appropriate use of drugs for therapy and prophylaxis and restricted use of steroids and antacids (225,226).

2.2.4.2.2 Long-term complications

Infants born preterm are at increased risk of long-term developmental, behavioral and health problems. Although these sequelae are most common and most serious for infants born extremely preterm, they also apply to infants with minor degrees of prematurity (227). Encountered problems include cognitive and motor impairments, asthma, visual and hearing impairments, behavior disturbances, impaired growth, osteopenia and reduced bone mass (227–229). Furthermore, preterm birth is an important independent risk factor for the development of cardiometabolic disease and renal disease in later life (230,231).

On the other hand, according to available studies, the majority of infants born preterm live an independent productive life, and, by their own rating, their life has a more or less the same quality as their term born peers (232,233).

2.2.5 Human milk in preterm infant feeding

As described earlier, the benefits of human milk in the management of preterm infants are well recognized, and as such, all preterm infants are recommended to receive human milk (1). Furthermore, the provision of mother's own milk to her infant has emotional bearing for the mother, allowing her a distinct role in the intensive care of her infant (234). However, the route to successful lactation often presents unique challenges to mothers of premature infants, including establishing and maintaining a milk supply and transitioning from gavage feeding to breastfeeding (235).

2.2.5.1 Infant factors affecting lactation

Preterm infants, especially very preterm infants, may not have fully developed skills at birth to feed independently at the breast (236). In preterm infants, the emergence of sucking competence has been observed as early as 27 weeks postconceptional age (237). However, in the most preterm infants and the transition time from starting suckling feedings to exclusive suckle feeding is longer than in more mature preterm infants. Likewise, neonatal conditions affecting the respiratory and circulatory systems that are more common in the most preterm infants, can further delay the accomplishment of full oral feedings (236). Thus, until the infant can take oral feedings, mothers need to pump or express breast milk that is thereafter stored and fed to the infants by nasogastric tube (238,239).

2.2.5.2 Maternal factors affecting lactation

Mothers of preterm infants are at greater risk for lactation failure after birth than mothers of term infants (240). This is especially true of mothers giving birth at extremely preterm gestational ages, due to the earlier stage of breast development at parturition since the mammary epithelium may not be sufficiently prepared by the pregnancy hormones to synthesize milk efficiently (85). Furthermore, research has shown a significant delay in the onset of lactogenesis II among mothers of EPIs (241).

2.2.5.3 Optimizing lactation success

Early, frequent and effective milk expression is crucial to the initiation of preterm lactation. A positive relationship has been shown between milk volume and the time point of initiation of pumping after delivery and the frequency and duration of pumping (242–244). In order to keep up with the increasing nutritional needs of the infants, mothers should strive at a milk volume of 750 ml/day at day 10 postpartum (245). Early initiation of kangaroo care can increase milk volumes and thereby counteract insufficient lactation (246). Oxytocin and the prolactin enhancer domperidone may have a role in mothers not responding to these interventions, but at present, safety data regarding the use of these galactogouges is missing (247).

2.2.5.4 Donor milk feedings in preterm infants

Donor milk is primarily used for preterm infants as a supplement to maternal milk feedings the first days after delivery until mother's own milk production is sufficient to fulfill the infants' needs. It is also used if the mother is unable to express milk or if the milk cannot be used for medical reasons (135). Compared to formula, donor milk reduces the risk of NEC, may protect against BPD and possibly improve feeding tolerance and reduce adolescent risk factors for cardiovascular disease (248,249).

In most neonatal units in Sweden, in order to guarantee optimal nutritional composition of the donor milk before feeding it to the preterm infant, nutritional analysis for protein, fat, and

carbohydrate contents is performed, with subsequent fortification of the milk according to the nutritional content determined by the nutritional analysis (135).

2.2.5.5 Breastfeeding the preterm infant

Mothers and their preterm infants often experience difficulties in making the transition from expressing milk and gavage feeding to feeding at the breast. Indeed, the initiation rate and duration of breastfeeding for preterm infants are lower than for full term infants (235,250). However, interventions in the neonatal units such as maternal support and education, kangaroo care or skin to skin care, pre-feeding oral stimulation, infant's nonnutritive sucking on the breast, and the avoidance of bottles during the transition process can facilitate the transition to breastfeeding (251,252). Regarding the initiation and establishment of breastfeeding in the preterm infant, reference is made to the expert group recommendations of the Baby-Friendly Hospital Initiative Ten Steps to Successful Breastfeeding into Neonatal Intensive Care (253).

Figure 10. A preterm infant being fed human milk by nasogastric tube while skin to skin holding. (With permission from the caregivers. Photo by Ann Sofie Ingman.)

2.3 HUMAN CYTOMEGALOVIRUS (CMV)

CMV infection is a leading source of congenital infection worldwide and a cause of significant morbidity and mortality in immunocompromised patients (254,255). In the developed world, it is the most common cause of nonhereditary sensorineural hearing loss and an important contributor to developmental delay (255).

2.3.1 The discovery of CMV

In the literature, the histopathological changes in tissue typical for CMV were first described in 1881 by the German scientist Ribbert who described large unidentifiable cells in kidney sections of a stillborn syphilitic infant and also in the parotid glands of other children.

Shortly thereafter, analogous findings were reported by other scientists, describing protozoan like cells in lung, kidney and liver tissue of a luetic fetus that had eccentrically placed nuclei containing a “central nuclear body” surrounded by clear halo.

Figure 11. CMV infected cytomegalic cell with intranuclear inclusion with halo. (Ho 2008 (256))

Subsequently, in 1921, when similar intranuclear inclusion containing cells were also described in lesions of patients infected by herpes zoster, these histopathologic findings were thought to be caused by viruses that were related (256).

In 1950, Wyatt et al. described the disease entity “generalized cytomegalic inclusion disease” after the detection of cells with the intranuclear inclusions in tissues of infants that had died of a congenial infection characterized by hepatosplenomegaly, petechiae, and intracerebral calcification (257). Following that, cytomegalic inclusion disease was diagnosed based on the presence of inclusion-bearing cells in the urine of infected infants.

In 1953, a viruslike particle could be detected with low-resolution electron microscopy in the halo around the intranuclear inclusion of a pancreatic cell of a case with cytomegalic inclusion disease.

However, only after the achievement of growing human cells in culture, could the virus finally be isolated in by 3 independent research groups; Smith and Rowe et al. in 1956 and by Weller et al. in 1957 (258).

A few years later, the name cytomegalovirus (from the Greek cyto-, "cell", and -megalo-, "large") replaced the previous used terms of “cytomegalic inclusion disease virus” or “salivary gland virus” (259) .

2.3.2 The herpes virus family

CMV belongs to the herpes virus family, or *herpesviridae*, distinguished by a common virion morphology. The herpesviruses are large viruses (150-200 nm) with double stranded DNA enclosed in a core within an icosahedral capsid, protein tegument, and lipid envelope (260).

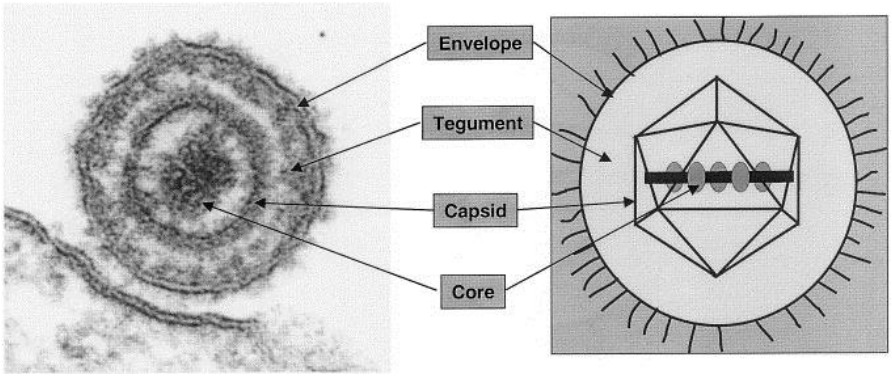

Figure 12. Morphology of herpesviruses. (Mettenleiter TC 2003(260))

Based on their growth characteristics and tissue tropism, herpes viruses are further subdivided into three subfamilies. Among the 8 herpesviruses infecting humans, herpes simplex virus 1 and 2 and varicella virus are neurotropic and belong to the α-subfamily. Ebstein Barr virus and and Kaposi's sarcoma-associated herpesvirus are lympothotropic and have a place in the γ-subfamily. CMV and human herpesvirus 6 and 7 belong to the β-subfamily and are able to establish infections in many cell types (261). After primary infection, viruses of the herpes family persist in a latent state in the host thereby avoiding virus elimination by the immune system or host destruction by the infection. As the viruses have coevolved with the human host, they have managed to develop an array of strategies to escape the multifaceted antiviral immune response in humans (262).

2.3.3 Epidemiology and transmission

CMV infection is prevalent in human populations worldwide with a seroprevalence in the adult population ranging from 45%-100% (263). CMV seroprevalence is higher in the developing countries and increases with age. In the developed countries a higher prevalence has been observed among non-whites, immigrants from the developing countries and in the lower socioeconomic strata (264). In a population based serological study conducted in Sweden 1973-1982, CMV seroprevalence was 40% in the age group 1-4 years increasing to 80-100% in the older age groups (265). In a more recent study by Engman et al. performed in 2003 and 2004, the CMV seroprevalence among 1000 pregnant Swedish women was 72% (266).

CMV can be transmitted via placenta, at delivery via the birth canal, or postnatally through intimate contact via infected body fluids, transfusion of blood products or transplantation of solid organs from CMV seropositive donors (267).

After initial acquisition of CMV, the immune system of the host is unable to eliminate the virus, leading to a latent infection that can be reactivated at any time when the delicate

balance with the host immune system is disrupted. Reactivation from the latent state can be silent or cause severe disease in the host for example in the immunosuppressed or critically ill patient (268).

Following primary infection or reactivation from its latent state, CMV can be shed in body fluid by the host for months to years thereby promoting further spread of the virus (264).

2.3.4 Clinical manifestations

2.3.4.1 Congenital CMV infection

CMV is the most common cause of human congenital infection with reported prevalence ranges globally between 0.3 to 6%, with higher prevalence in the developing countries (269). Approximately 10-15% of infants with congenital CMV infection present at birth with clinical manifestations, i.e. a *systemic congenital CMV disease* (270). The clinical symptoms derive primarily from organs of the reticulotendothelial system and central nervous system and comprise jaundice, hepatosplenomegaly, petechiae, microcephaly, seizures, hypotonia and lethargy. The infected newborn is often a growth retarded and prematurely born baby. About one third of the most severely affected infants die of hepatic dysfunction, bleeding, coagulopathy or secondary bacterial infections (271). Of those infants surviving, the majority (50% to 90%) will suffer from later neurological sequelae including mental retardation, cerebral palsy, impaired vision and sensorineural hearing loss (264).

About 85-90% of infants infected by CMV in utero have an *asymptomatic congenital CMV infection* with no overt clinical symptoms at birth. However, approximately 7% to 25% of these asymptomatic infants will also develop CNS sequelae, mainly sensorineural hearing loss (264).

Today, congenital CMV infection is the most important cause of sensorineural hearing loss during childhood affecting about 10-15% of all infants infected by CMV in utero (271).

Figure 13. A newborn infant with systemic congenital CMV disease. This infant presented at birth with disseminated violaceous cutaneous nodules , hepatopathy, thrombocytopenia and coagulopathy (Martins 2011 (272))

2.3.4.2 CMV infection in the immunocompetent host

Primary CMV infection is usually clinically silent in the immunocompetent individual but can at times cause an acute febrile illness resembling mononucleosis with fever, malaise, myalgias, headache, and fatigue. Splenomegaly, hepatomegaly, adenopathy, and rash can occur (264,273). Occasionally, CMV infection present in the apparently immunocompetent host with similar manifestations as encountered in the immunocompromised host or even with life-threatening illness (264,274,275).

Several medical conditions have been linked to CMV infection in the immunocompetent host. CMV has been implicated as a cofactor in the pathogenesis of atherosclerosis and coronary heart disease (276,277). The virus has been detected in the bowel of patients with inflammatory bowel disease and in patients with plaque psoriasis, rheumatoid arthritis, systemic lupus erythematosus and Sjögrens syndrome (278,279). In addition, CMV has been identified in tumors of the colon, breast, prostata and brain; likewise, the virus is frequently found in brain metastases of primary breast and colon tumors (280,281). Today, extensive research is further evaluating the role of CMV in the genesis of these conditions and if anti CMV treatment may improve patient outcome (282).

2.3.4.3 CMV infection in the immunocompromised host

CMV infection is a common complication in the care of immunocompromised patients resulting from either reactivation of latent virus, reinfection or primary infection. Although the infection can be clinically asymptomatic, it can cause life-threatening and debilitating disease, especially in the most immunosuppressed patients; recipients of allogeneic stem cell transplants, solid organ transplant recipients, patients with AIDS and very low CD4 counts,

patients receiving immunosuppressive chemotherapy, infants with congenital immunodeficiency and infants born very prematurely (264).

In transplant patients, CMV infection is defined as isolation of the virus or detection of CMV proteins or nucleic acid in a body fluid or tissue specimen; CMV disease is defined as CMV infection accompanied by symptoms and signs of an affected end-organ (283,284).

CMV can cause a variety of end-organ diseases in immunocompromised patients. Amongst allogenic stem cells recipients, the most common clinical presentations of CMV disease are gastrointestinal disease and pneumonitis, the latter being a cause of mortality in more than 50% of patients afflicted (285).

In solid organ transplant recipients, CMV infection can present as CMV syndrome; an acute, systemic, febrile illness with laboratory signs of neutropenia, thrombocytopenia, and elevated hepatic transaminases. The CMV infection can also target specific end-organs causing pneumonitis, gastrointestinal lesions, hepatitis, retinitis, pancreatitis, myocarditis, and rarely, encephalitis or peripheral neuropathy. Furthermore, CMV infection in solid organ transplant recipients has been linked to graft rejection, accelerated coronary artery atherosclerosis and new-onset diabetes mellitus (264,286).

An indirect effect attributable to CMV infection in both allogenic stem cells recipients and solid organ transplant recipients is an increased risk for opportunistic fungal and bacterial infections (264,287).

Before the use of combined treatment in HIV patients in 1995 with at least three antiretroviral drugs of different classes, CMV infection was one of the most important opportunistic infections in patients infected by HIV, prevalent in 40% of patients with advanced disease. Today the risk of CMV disease chiefly remains in those patients with low CD4 cell counts (< 100/ μL). Retinitis is the most common clinical manifestation accounting for about 85% of CMV disease followed by gastrointestinal disease accounting for about 15% of cases. CMV encephalitis is a rare (<1%) but important manifestation of CMV disease in HIV-infected patients with a detrimental outcome if left untreated with a nearly 100% mortality rate (288,289).

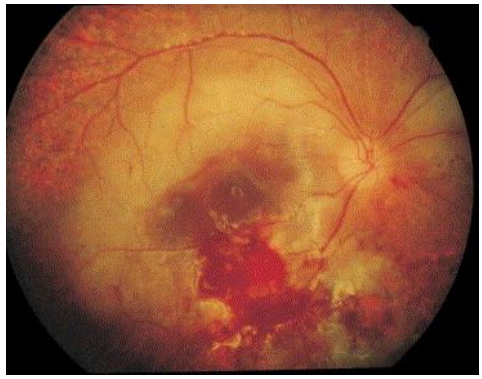

Figure 14. CMV retinitis in a patient with AIDS (Steininger 2006 (288))

2.3.4.4 Postnatal CMV infection in the preterm infant

The most common source of postnatal CMV infection in the preterm infant is maternal breast milk (290). CMV is excreted in breast milk of the majority of CMV seropositive mothers (116). Nearly half of all infants nursed for a longer period by mothers that have been infected by CMV acquire the virus postnatally (290).

Postnatal CMV infection in infants born at term, that are protected by maternal derived antibodies, usually are asymptomatic and without sequelae. Preterm infants on the other hand, having an immature immune system and lacking these protective antibodies are more prone to develop a symptomatic infection (291).

In 2001, Hamprecht et al. reported a transmission rate of 37% of CMV through fresh breast milk in very preterm low birth weight infants at their neonatal unit in Tübingen, Germany (116). Half of the infected infants developed clinical symptoms or laboratory abnormalities. The clinical symptoms included sepsis like symptoms with apnea and bradycardia, distended bowel, hepatosplenomegaly and myoclonia while laboratory abnormalities comprised neutropenia, thrombocytopenia, raised liver enzymes and elevated C-reactive protein (292). Since then many neonatal studies from around the world have been published addressing this subject. According to a recent review by Kurath et al., reported transmission rates in preterm infants have varied between 6-59% with symptomatic disease in 0-35% of infected infants (293). Apart from the clinical picture described above, pneumonitis, cholestasis, enterocolitis, bowel perforation and bowel stricture have been reported while more uncommon manifestations are hemophagocytic lymphohistiocytosis and lung cysts (294–300) . Risk predictors of severe symptomatic postnatal infection are extreme prematurity (<26 weeks), early postnatal infection and high pre-infection comorbidity (301). Illustrative of these risk factors,in a retrospective study including only preterm infants with a gestational ages 22-24 weeks, 65% of infants born to CMV-seropositive mothers presented with a symptomatic infection of which 55% developed sepsis like disease (114).

Data on the long-term outcome in preterm infants after postnatal CMV infection are limited, especially in adolescents; some studies have implied an increased risk for BPD (302,303) and a poorer neurodevelopment outcome (304–307), whereas other studies do not confirm these findings (296,308–312).

To date, we don't fully know the clinical burden of breast milk acquired CMV infection. Consequently, we don't know if treatment of maternal milk is needed to protect the preterm infant from postnatal CMV infection. Furthermore, if preventive measures are needed, the risk- benefit ratio of these interventions needs to be better evaluated (313).

2.3.5 Diagnosis

Commonly used diagnostic tests for CMV include serology, antigenemia assay, quantitative nucleic acid testing, culture and histopathology.

2.3.5.1 CMV serology

CMV serology detects CMV specific antibodies and is useful to test whether a patient has been previously exposed to CMV, or, if a seroconversion from negative to positive occurs for a recent CMV infection. The specificity of IgM antibodies against CMV to prove a primary infection is inferior as a false–positive test can occur; likewise patients with reinfection or reactivation of a past infection can have IgM antibody to CMV. A primary CMV infection can also be confirmed by demonstrating a low CMV-IgG avidity that improves over time (264).

2.3.5.2 Antigenemia assay

Antigenemia assay is a quantitative method for detection of CMV that uses monoclonal antibody to detect the tegument protein pp65 in blood leukocytes by immunostaining. According to the clinical setting, the number of pp65-positive leukocytes correlates with risk of disease. The assay is easy to perform and does not require expensive equipment but is operator dependent and needs to be performed within hours of sample collection (264) .

2.3.5.3 Quantitative nucleic acid testing

Quantitative nucleic acid detection is progressively replacing antigenemia assay as a method of monitoring CMV load, especially in Europe and North America (314). These techniques are highly sensitive and provide viral load measurements that give important prognostic information (315). The real time PCR assay, in particular, is a simple reproducible technique able to measure DNA-load in several kinds of specimens (314). However, results can vary widely across different testing centers due to the variation in nucleic acid extraction and assay design (316).

2.3.5.4 CMV culture

CMV culture is performed by co-culturing clinical specimens with fibroblasts subsequently identifying the characteristic cytopathic effect of the virus. Although slow, expensive and labor intensive, CMV culture remains a diagnostic option in tissue invasive CMV disease where antigenemia or polymerase chain reaction (PCR) testing on blood may not always be positive (316).

2.3.5.5 Immunohistochemistry (IHC)

In immunohistochemistry, antibodies are used to detect CMV specific antigens in cells of tissue specimens (317) or blood samples. Immunohistochemistry for CMV can be used on biopsy specimens to maximize diagnostic sensitivity where CMV disease is suspected. Identification of inclusion bodies or viral antigens in the cells of the biopsy material or specimens is characteristic for CMV disease, especially combined with a positive culture (316).

2.3.5.6 In situ hybridization (ISH)

In situ hybridization is a technique used to localize specific nucleic acid sequences within cells in tissue sections or whole cell preparations (318). DNA and RNA sequences are visualized by hybridization with labelled complementary strands of nucleic acid. An advantage of the methodology is that it allows localisation and visualisation of target nucleic acid sequences within morphologically identifiable cells or cellular structures (319).

2.3.6 Treatment

To date, the main antiviral agents used to treat CMV infections comprise ganciclovir, valganciclovir, cidofovir and forscarnet. Of these, ganciclovir and valganciclovir are the gold standard drugs for prophylaxis and treatment of CMV. Second line theraphy options against CMV include foscarnet or cidofovir, or the off-label compound leflunomide. High-dose valacyclovir, penciclovir, famciclovir, and acyclovir have been used for CMV prophylaxis in organ transplant recipients. Efficacy is different depending on the transplant population, but currently valganciclovir is the most effective drug in use for both prophylaxis and treatment of CMV infections. The patent for this compound is expiring in August 2015 and several new anti-CMV drugs are in phase II/III trials to be evaluated for CMV prophylaxis and treatment; brincidofovir, maribavir and letermovir. Of these brincidofovir has a broad antiviral activity and great hope is set to its ability to control multiple infections in immunocompromised patients within the next few years. Below is a brief description of the currently used drugs for CMV infections.

2.3.6.1 Ganciclovir

Ganciclovir was the first antiviral agent licensed for treating CMV infection. It blocks viral DNA synthesis by inhibiting the CMV- DNA- polymerase in CMV infected cells. Ganciclovir requires phosphorylation by the viral protein UL 97 to convert to its active form. Ganciclovir can only be administrated intravenously. It´s major adverse effects include neutropenia, thrombocytopenia and kidney and liver toxicity (320,321).

Ganciclovir prophylaxis is recommended for CMV- seropositive HIV infected patients with low CD4+ T lymphocyte counts (< 50 cells) and in HIV and AIDS patients with previous CMV end- organ disease. It is used as antiviral therapy against CMV end-organ disease in adults and adolescents with AIDS. Ganciclovir is also used for prevention and treatment of CMV disease in transplant patients (322).

Ganciclovir treatment is recommended in neonates with severe symptomatic congenital CMV infection involving the central nervous system as it can improve hearing outcome and possibly neurodevelopmental outcome (323). No controlled study has evaluated the impact of ganciclovir on the outcome of postnatal CMV infection; however, individual reports have described a positive effect of ganciclovir treatment for severe postnatal CMV disease in preterm infants (294,295,324).

2.3.6.2 Valganciclovir

Valganciclovir is the ester prodrug of ganciclovir and has the same mechanism of action. It is only available in enteral form and is very well absorbed. In adults common side effects include diarrhea, nausea, neutropenia, and anemia (325). In neonates neutropenia is a reported adverse effect (326).

Valganciclovir is used as treatment for end-organ disease in AIDS patient, as an alternative to ganciclovir in prophylaxis in HIV or AIDS patients with previous CMV end-organ disease, for prevention of CMV disease in solid organ transplant patients and for treatment of CMV disease in adult and adolescent transplant populations (322). It is also recently accepted as an treatment option against symptomatic CMV infection in neonates (326).

2.3.6.3 Cidofovir

Cidofovir is a nucleotide analog that requires phosphorylation by cellular kinases (327). It is incorporated into the viral DNA in its active form where it inhibits DNA synthesis (320). Cidofovir is administrated intravenously. Nephrotoxicity is the main adverse effect. Cidofovir is used as a second-line therapy for treatment of CMV end-organ disease and prevention of recurrent CMV end-organ disease in adults and adolescents with HIV or AIDS (322).

2.3.6.4 Forscarnet

Foscarnet is an inorganic pyrophosphate analogue administrated intravenously that inhibits DNA polymerase by blocking the pyrophosphate binding site. The most common side effects are nephrotoxicity and metabolic derangements (328).

Foscarnet is an alternative therapy in adults and adolescents with AIDS and end- organ disease and to prevent recurrence of CMV end-organ disease. It is also used as prophylaxis in recipients of allogeneic hematopoietic cells and in CMV disease in transplant patients with myelosuppression, or whose virus is resistant to ganciclovir or those who cannot tolerate ganciclovir (322) .

3 AIMS

When this thesis project was initiated, the benefits of feeding preterm infants with maternal milk were fully acknowledged. However, there was a lack in knowledge and consensus regarding the routines used for handling breast milk to limit transmission of CMV to preterm infants, as well as the risk of acquiring CMV infection via breast milk.

The specific aims of this thesis were the following:

I. To document existing routines pertaining to breast milk use for preterm infants in Sweden.

II. To investigate predictors of maternal milk feedings in EPIs during the first 6 weeks of life and at discharge from hospital or home care.

III. To evaluate the rate and clinical expression of postnatal CMV infection in EPIs in a pilot study.

IV. To evaluate the effect of routine freezing of maternal milk on CMV transmission rate and CMV associated disease and neonatal morbidity and mortality in EPIs.

V. To assess whether NEC, SIP and related surgical conditions are associated with CMV infection as a potential consequence of exposure of CMV to the bowel in utero, at birth or postnatally through breast milk.

4 RESULTS AND BRIEF DISCUSSION

4.1 BREAST MILK HANDLING ROUTINES FOR PRETERM INFANTS IN SWEDEN: A NATIONAL CROSS-SECTIONAL STUDY. (PAPER I)

This national cross-sectional study was performed as a questionnaire survey in 2006 to document existing breast milk handling routines in neonatal units in Sweden awaiting the implementation of updated national recommendations on human milk use.

The strength of the study is that it included all neonatal units in Sweden enabling a national overview of the use and handling of human milk for preterm infants.

We confirmed that in all neonatal units, preterm infants <32 weeks were preferably fed donor milk as a complement to maternal milk. Human milk banking was established in the care of preterm infants with 27 of the 36 neonatal units having access to a milk bank. Since this paper was published, still one more Swedish milk bank has been established and to date, after France, Sweden has the second most human milk banks among the European countries (329).

Although well constituted, none of the 27 milk banks completely followed the prevailing Swedish national guidelines from 1998 regarding the recruitment of breast milk donors and the handling of donor milk (330). In 7% of the milk banks, donors were not given a health declaration in the recruitment process. A complete virology screening of breast milk donors against HIV I and II, HTLV type I and type II, HBV and HCV was not performed in 48% of the milk banks and donors were not screened for active lung tuberculosis in 85% of the milk banks. Donor milk was not routinely pasteurized in 19% of the milk banks and bacterial culture was not consistently performed in 11% of the participating milk banks.

While not endemic in Sweden, the risk of transmission of HTLV type I through the breast milk of an infected mother is considerable, with increased the risk of adult T-cell leukemia/lymphoma in the offspring (108). Heat treatment of milk by Holder pasteurization effectively eliminates HTLV (139). Still, in our study, 2 of the milk banks not screening milk donors for HTLV type I did not routinely pasteurize donor milk.

Five of the milk banks did not screen their donors for HBV and HCV. As the effectiveness of Holder pasteurization against HBV and HCV in breast milk has hitherto not been proven or evaluated, consistent screening of breast milk donors against these viruses is essential to avoid their transmission to donor milk recipients (331). Transmission of tuberculosis through breast milk has never been documented in the absence of tuberculosis mastitis, which is rare (117). In the newly revised national guidelines by Milknet, screening of milk donors with a lung X-ray to exclude active tuberculosis is mainly underscored in those milk banks where unpasteurized breast milk is used (135) .

The Holder pasteurization was the most common mode of heat treatment used on donor milk. Although this technique is known to eradicate the most common bacterial contaminants in human milk, endospores of the Bacillus species and enterotoxin of Staphylococcus aureus can resist the pasteurization process and cause disease in susceptible preterm infants

(141,332) . Likewise, if donor milk becomes contaminated by microorganisms after pasteurization, their growth will be enhanced as the pasteurization process damages antimicrobial components of the milk (139). Therefore, in some countries, postpasteurization bacterial culture is considered an important part of the donor milk screening process (135). However, at the human milk bank of Rikshospitalet University Hospital in Oslo, Norway, donor milk with low bacterial count is used for the most preterm infants, and, remarkably, although the donor milk is dispensed unpasteurized the reported incidences of both NEC and LOS among preterm infants at that centre are low (333,334).

In this study, preventive treatment of maternal milk with regard to CMV transmission was inconsistent among the neonatal units. In accordance with the recommendations of the Swedish National Board of Health and Welfare, 31% of the neonatal units routinely fed their preterm infants with freeze-thawed maternal milk in order to reduce the risk for CMV transmission. At that time, in vitro studies had shown that the CMV load in breast milk could be decreased considerably by freezing the milk 3-4 days (149,335).

However, the time the mother's own breast milk was kept frozen before use varied between the units from a few hours to 7 days.

In the remaining 69% of neonatal units in Sweden, and contradictory to prevailing recommendations, early feedings with fresh maternal milk were permitted. This may reflect a common reluctance to routine freezing of maternal milk as no randomized clinical study had yet shown true effect of freezing on postnatal CMV transmission. Likewise, the reputed beneficiary anti-inflammatory and trophic effects of early human milk feedings may have affected the breast milk handling routines (336).

In our study, nutritional analysis of donor milk was performed in 63% of the milk banks and of maternal milk in 61% of the neonatal units. The composition of human milk is influenced by gestational age and is variable within feedings, diurnally, over lactation, and between mothers (26,27,29,30). Therefore, and because of the special nutrient requirements in the preterm infant, protein and energy fortification of breast milk after nutritional analysis is most desirable to improve outcome and to prevent nutritional disorders (337).

4.2 PREDICTORS OF SUSTAINED MATERNAL MILK FEEDS IN EXTREMELY PRETERM INFANTS. (PAPER II)

In this prospective cohort study, including 97 singleton EPIs and their mothers at the Karolinska University Hospital and the Stockholm South General Hospital, predictive factors of maternal milk feedings in EPIs during the first 6 weeks of life and at discharge were investigated.

This is the first study in a country with high breastfeeding rates to examine predictors for sustained maternal milk provision in EPIs. A weakness in the study was that the cohort derived from another randomized study (study IV) where study subjects were initially

allocated to separate feeding groups. However, no associations were found between the randomization groups and the outcome measures in our study.

At the end of the sixth week, 80% of mothers were still producing breast milk. In comparison, in the study by Stoltz Sjöström et al., using data of EPIs born < 27 weeks between 2004-2007 from the Swedish Express study, 71% of infants were at least partly fed with maternal milk during postnatal week 4 to 8, indicating that the same proportion of mothers of these infants were producing breast milk (28).

We found that the mean proportion of enteral feedings that were maternal milk feedings during the first 6 weeks of life was 69%. The proportion of maternal milk feedings reached a maximum during the third week of life (76%) after which expressed volumes of maternal milk could not keep up with the infants' increasing enteral feedings. We believe that the observed diminishing milk production may reflect the occurrence of the physiological switch from central to autocrine control of milk production, during which lactation becomes more dependent on the frequent and total emptying of the breast than on hormonal control (338).

At discharge, 66% of EPIs were provided with maternal milk; 13% were exclusively breastfed, 13% were breastfed and bottle fed with maternal milk only and 39% were fed a combined diet of maternal milk and formula. Correspondingly, in the study by Stoltz Sjöström et al., 50% of infants were fed maternal milk at discharge; of these 89% were exclusively or partly breastfed whereas 11% were bottle fed with maternal milk (28). In a previous study on breastfeeding rates at discharge in preterm infants at Danderyds Hospital, Stockholm, 76% of EPIs were exclusively or partly breastfed at discharge; in that study, a much higher proportion (38%) of the EPIs were exclusively breastfed than in our study (250). Since that study was performed, however, the overall rates of exclusive breastfeeding have been decreasing in Sweden (15). Other possible reasons that could have contributed to lower breastfeeding rates observed in our study may be that the EPIs in our study were more ill and/or that mothers of EPIs received less support during the process of transition to breastfeeding.

We found that high maternal milk feedings in EPIs during week 2 were positively associated with both the proportion of maternal milk feedings the first 6 weeks of life and with exclusive maternal milk feedings at discharge. Additionally, the proportion of maternal milk feedings during the first 6 weeks of life was related to the provision of maternal milk feedings at discharge. Consistently, Hill et al. demonstrated that milk volumes at week 2 were predictive of milk volumes at week 5 in mothers of very preterm VLBW infants and that high milk volumes during the first 6 weeks were predictive of sufficient maternal milk at 12 weeks postpartum (339,340). Another recently published study from Sweden found a relationship between volumes of maternal milk provided at day 3 and 7 and exclusive maternal milk feedings at 36 weeks post menstrual age in preterm infants born between 28 and 32 weeks but this association was not found in infants < 28 weeks of gestation (341) .

Interestingly, assisted reproduction technology was a favorable predictor for both exclusive maternal milk feedings and exclusive breastfeeding at discharge. Conversely, 2 other studies have reported lower breastfeeding rates among women at 6 weeks and 3 months, respectively, after conception with assisted reproductive technology (342,343). However, these were mothers of both term and preterm infants. In our study, mothers conceived by assisted reproduction technology may have been more motivated to breastfeed than the other mothers in the study, accomplishing breastfeeding success through their continuous access to counselling and support by neonatal personnel.

Maternal overweight was an unfavorable predictor of high maternal milk feedings during week 2 and of maternal milk feedings at discharge. Maternal overweight is an identified risk factor for failure to initiate and sustain breastfeeding (344,345) but to our knowledge, this relationship is not previously identified for mothers of EPIs. Overweight may by biological mechanisms reduce the maternal prolactin response to suckling, thereby causing a delay of lactogenesis II which render the predisposed preterm mothers at a greater risk for lactation failure (346).

Non-university education and non-Nordic origin were negatively associated to the proportion of MMFs the first 6 weeks of life. In a study by Hill et al., income was the only maternal demographic factor that was independently predicting milk output at week 6 in mothers of very preterm VLBW infants; income in turn was positively related to both maternal education and ethnicity (347). On the contrary, in a study investigating milk output in pump-dependent mothers of preterm infants < 31 weeks, maternal education was not related to milk volume at week 8 postpartum (244).

In our study, age was the only favorable maternal predictor for the provision of maternal milk feedings at discharge. Results of previous studies investigating the effect on maternal age on lactation duration in mothers of very preterm or VLBW infants are contradictory, some report an association (348,349) whereas other studies do not confirm any relationship (242,350,351).

4.3 TRANSMISSION OF CYTOMEGALOVIRUS TO EXTREMELY PRETERM INFANTS THROUGH BREAST MILK. (PAPER III)

This pilot study, including 10 EPIs and their 6 mothers, was performed in 2002 to evaluate the rate and clinical expression of breast milk induced CMV infection in EPIs at the Neonatal Unit, Astrid Lindgrens Children´s Hospital.

Five out of 6 (83%) mothers were CMV-seropositive. Of these, 4 (80%) excreted CMV-DNA and 2 (40%) mothers had a positive CMV culture. By comparison, in the pioneering study of Hamprecht et al. on CMV transmission to very preterm infants in 2001, 96% of seropositive mothers had detectable CMV- DNA in breast milk and 76% had positive virus isolation in breast milk (116).

Transmission of CMV occurred in 2 of 7 (29%) EPIs that were fed both fresh and freeze-thawed CMV-positive breast milk. At that time, as still today, the reported transmission rates of CMV to preterm infants through breast milk varied considerably between prospective studies depending on the study design and prevailing feeding protocols and human milk treatment at the study centers.

Thus, at that time, Vochem et al. from Germany reported a transmission rate of 59% to very preterm infants fed only fresh maternal milk with formula as supplemental feedings whereas Mussi Pinhata et al. from Brazil reported a transmission rate of 22% in infants < 34 weeks fed fresh maternal milk supplemented with donor milk (113,352). By comparison, Jim et al. from Taiwan reported a transmission rate of 15% in infants < 33 weeks that were only fed freeze-thawed milk supplemented with formula (311).

Contrariwise, Yasuda et al. from Japan and Doctor et al. from Canada only reported a transmission rate of 10% and 6% in infants < 34 weeks and < 1000 gram, respectively, that were fed predominantly freeze-thawed maternal milk with formula as supplement (353,354).

In this pilot study, only infants that were fed with breast milk with a positive viral culture were infected by CMV. Early excretion of CMV-DNA in breast milk and virolactia, the presence of infectious virus in breast milk, are both associated to increased risk of CMV transmission to the preterm infant (116,311). Also, some studies have found a higher load of CMV-DNA in breast milk of CMV transmitting mothers (355,356). In both the CMV infected infants, CMV-DNA was detected in breast milk sampled during the first 2 weeks postpartum. However, in the samples of milk fed to one of these infected infants, the amount of CMV- DNA was so low that it could not be detected with quantitative analysis. A discrepancy between CMV-DNA load and viral infectivity has been observed in other studies (353,357) and could be explained by the fact that some of the viral particles detected by DNA analysis are incomplete and thus replicate-incompetent virions (149).

None of the infected infants presented with overt clinical symptoms at the time of the CMV infection. However, one of the infected infant developed liver involvement with laboratory abnormalities. Early postnatal CMV transmission and low birth weight are both reported risk factors in premature infants for developing symptomatic postnatal CMV disease (292,358). Likewise, postnatal CMV infection may aggravate the course of a pre-existing hepatic condition in preterm infants (296). Indeed, the CMV infected infant with liver involvement was the most preterm of all EPIs, he had an earlier postnatal excretion of CMV in the urine compared to the other infected infant, and he had at that time an unrecognized cystic fibrosis that was later diagnosed.

4.4 CYTOMEGALOVIRUS INFECTION AND NEONATAL OUTCOME IN EXTREMELY PRETERM INFANTS AFTER FREEZING OF MATERNAL MILK. (PAPER IV)

This clinical trial, conducted from 2005 to 2009 at the Karolinska University Hospital and the Stockholm South General Hospital, evaluated the effect of routine freezing of maternal milk

on CMV transmission, symptomatic CMV infection and mortality and morbidity during neonatal stay in EPIs. One hundred and forty EPIs were randomized to be fed only with freeze-thawed maternal milk (intervention group) or with fresh and freeze-thawed milk according to existing clinical practice (control group).

This randomized trial is the first of its kind to evaluate the effect of routine maternal milk freezing on breast milk acquired CMV infection and neonatal outcomes in EPIs.

The strength of the study was the detailed documentation of the enteral feedings in all EPIs during their first 6 weeks of life demonstrating a well-functioning intervention procedure.

By baseline characteristics comparison we found that significantly more infants in the control group were born SGA and delivered by caesarean section. Likewise, pre-eclampsia was more common in mothers in the control group. Pre-eclampsia is a known risk factor for intrauterine growth retardation and SGA (359). For elective delivery of an intrauterine growth retarded foetus during the very preterm period, caesarean section is the method of choice (360). Indeed, in our study, 60% of mothers with pre-eclampsia delivered infants that were SGA compared to 11% of mothers without pre-eclampsia. Similarly, 96% of all infants with SGA were delivered with caesarean section.

In this clinical trial, the overall CMV transmission rate to infants fed CMV-positive breast milk was much lower than in the previous pilot study performed in our unit (7% vs. 29%). No significant difference in CMV transmission rates was found between the study groups. The predefined sample size for our study was based on the documented CMV transmission rate of the pilot study, that was similar to reported rates of postnatal CMV transmission at other neonatal centres using fresh maternal milk at that time (352,361) . As the CMV transmission rate proved to be lower than expected, the sample size estimation was insufficient to detect significant differences in CMV transmission rates and symptomatic CMV infection between the study groups.

The transmission rates in CMV exposed EPIs fed only freeze-thawed maternal milk or fresh and freeze-thawed maternal milk were similar; 8% and 6%, respectively. None of the infected EPIs developed clinical symptoms at the time of CMV transmission but one infected infant in each group developed transitory cholestasis. The highest rate of breast milk acquired CMV transmission with clinical CMV disease in very preterm/VLBW infants hitherto reported in a prospective study is by the research group from Tubingen, Germany (113). In their first report, 17/29 (59%) of very preterm infants fed milk from CMV-seropositive mothers excreting CMV in milk became infected by the virus; 10 of the infected infants developed clinical or laboratory abnormalities. Infants were only fed fresh maternal milk with formula feedings as supplement if needed. Another recent retrospective study including only infants with gestational ages 22-24 weeks reported symptomatic CMV infection in 11/17 (65%) of infants fed fresh untreated maternal milk from CMV seropositive mothers. In that study, the type of supplemental feeds used were not stated (114).

In our study, 2/3 of the enteral intake the first 6 weeks in both groups was maternal milk; in controls, 37% of maternal milk was fresh milk. Until week 34 of gestation, insufficient maternal milk feedings in infants were supplemented with donor milk, not with formula. Early feedings with formula may have unfavourable effects on the immunological protective mechanisms of the immature gut, affecting mucosal structure and function and the intestinal microbial environment (362). Therefore, EPIs fed untreated fresh maternal milk containing CMV in a combination with formula may become more susceptible to CMV infection. Contrariwise, human milk contains a large number bioactive factors and a variety of microbes that together are thought to interact to protect and promote the development of the mucosal immune system (2,202). Thus, the relatively low amount of fresh MM and the use of pasteurized donor milk instead of formula may thus have contributed to the low transmission rate and lack of symptomatic infection among infected infants in our study.

In our study, the rate of neonatal death was similar in the study groups; 6% in both groups by intention to treat analysis and 7% in the intervention group and 6% in the control group by per protocol (PP) analysis. All deaths occurred after the first week of postnatal life. Late onset septicaemia, a recognized contributor to mortality in infants born very preterm, was the most common cause of death, occurring in 5 of the 8 (63%) deceased infants. Similarly, septicaemia was the most frequent diagnosis among infants in the Swedish Express study in infants who died after the first week (164).

Using PP analysis, including infants who remained in the study throughout their neonatal stay, the overall incidence of late onset septicaemia was 51% (62/121); bacterial sepsis was observed in 45% (54/121) of infants and fungal sepsis in 7% (8/121) of infants. Correspondingly, in the Swedish express study the overall rate of culture proven septicaemia in infants surviving the first year of life was 41% and most of these were acquired nosocomially (164). In our study, fungal LOS only occurred in the control group with an observed frequency of 12% analysis compared to 0% in the intervention group using both analyses. All infections were caused by the Candida species and all occurred in the first half of the study period before fungal prophylaxis had been fully introduced in our neonatal units. A primary risk factor for neonatal Candidiasis is colonization of skin and mucosa with Candida (363). In our study, a potential source leading to Candida colonization may have been expressed breast milk contaminated by Candida from mother’s skin or from milk containers during the milk pumping process (364). As freezing of maternal milk deactivates yeast, while still preserving some important immunological components of the milk, the provision of freeze-thawed maternal milk could have some preventative advantages over fresh maternal milk feedings with respect to fungal infections during neonatal stay when fungal prophylaxis is not practised (149,365).

Other known risk factors for invasive Candida infections are prematurity, low birth weight, intubation, the use of central catheters, prolonged treatment with broad spectrum antibiotics and parenteral nutrition (363). Indeed, we found that the EPIs with Candida LOS were treated

significantly longer on ventilator and had longer treatments with antibiotics and parenteral nutrition compared to infants without Candida LOS.

According to PP analysis, the proportion of infants in our study with BPD, NEC and ROP in the study was 46%, 8% and 31%. Corresponding numbers among surviving infants in the Swedish express study were 73%, 6% and 34% (164). The occurrence of BPD, NEC and ROP tended to be higher among infants in the control group that were partly fed with fresh maternal milk although this did not reach statistical significance.

In this trial, 3 EPIs were diagnosed with congenital CMV infection by postnatal urine screening. As all infants were asymptomatic, the infection was only detected due to the CMV screening in the randomized study, and not by clinical testing upon indication. The proportion of congenital CMV infection among all EPIs initially screened in urine in our study was 2% and thus considerably higher than the previous reported frequency of congenital CMV infection of 0.5% among newborns in Malmö in 1977 to 1986 and of 0.2% among newborns in Stockholm in 2003 to 2004 (266,366). To our knowledge, no study has hitherto evaluated the prevalence of congenital CMV infection in a population of EPIs. It is possible that a variable activity of CMV in the society over several years may have affected the different results (367) or that our findings may be a matter of chance due to the small number of study subjects screened. However, congenital CMV infection is associated to both prematurity and low birth weight (368,369) and considering the increased risk of neurological sequelae after both congenital infection and among EPIs, more studies are needed to appraise the potential value of routine surveillance for congenital CMV in EPIs.

4.5 HIGH PREVALENCE OF CYTOMEGALOVIRUS INFECTION IN SURGICAL INTESTINAL SPECIMENS FROM INFANTS WITH NECROTIZING ENTEROCOLITIS AND SPONTANEOUS INTESTINAL PERFORATION; A RETROSPECTIVE OBSERVATIONAL STUDY. (PAPER V)

In this retrospective observational study, 70 intestinal samples from 61 infants that underwent surgery for NEC, SIP or related surgical conditions at the Karolinska University Hospital Solna and the Uppsala University Hospital were investigated for the presence of CMV infection. Ten intestinal specimens from autopsied infants without bowel disease were used as controls.

NEC is a feared disease in preterm infants due to its high mortality rates, surgical complications and increased risk of the comorbidities of prematurity (192). SIP, although not presenting as deleterious as NEC, is also associated to increased risk of death and long-term morbidity in the most preterm infants (370).

Up until now, no study has investigated the prevalence of CMV infection in a larger cohort of infants with these surgical disorders; still, several case reports and case series have been published where histopathologic findings compatible with CMV infection have been detected in the bowel of infants with NEC, intestinal perforation or intestinal stricture (300,324,371–

373).

By using an IHC technique with antibodies directed against two different CMV proteins, CMV-immediate early antigen (CMV-IEA) and CMV-late antigen (CMV-LA), we could detect CMV-IEA in 81% (57/70) and CMV-LA in 64% (45/70) of tissue samples. In contrast, only 2/10 (20%) control specimens were positive for CMV-IEA and CMV-LA by IHC.

In index specimens, the prevalence of CMV antigens was highest in specimens with the pathologic diagnosis NEC with intestinal perforation; 95% were positive for CMV-IEA and 79% were positive for CMV-LA. Still, CMV antigens were found in more than half of the specimens with the pathologic diagnoses NEC, SIP and post inflammatory changes after NEC or SIP. Thus, CMV proteins were prevalent in the intestinal specimens irrespective of pathologic diagnosis.

In order to further confirm the presence of CMV in the collected bowel samples, we performed complementary tests for detection of CMV nucleic acids in selected intestinal tissue specimens.
In 10 intestinal specimens positive for CMV-IEA by IHC, CMV-positive cells were collected by laser capture microdissection with subsequent analysis of CMV-DNA by Taqman PCR. CMV-DNA was detected in 4/10 (40%) specimens. A discrepancy in the number of cells positive for CMV-antigens and CMV-DNA have previously been described by our group in tissue specimens from glioblastoma patients (374) and may illustrate the presence of non-replicating viral protein expressing cells. These cells may contain viral nuclei acids delivered via defective virus particles such as dense bodies or exosomes to intestinal epithelial cells (375,376).
Likewise, CMV-DNA was detected by in situ hybridization in 13/13 (100%) intestinal tissue sections from infants with positive IHC- CMV-IEA staining; however 3/ 5 (60%) tissue sections from infants with IHC- CMV-IEA negative staining were also positive for CMV-DNA by ISH. These findings could reflect a latent CMV infection in the bowel of these infants, or this might be a result of non-serial sections of the intestinal specimens used for the different analyses.

In our study, all NEC samples were retrieved from infants who had undergone surgery due to advanced NEC, SIP or related surgical complications. CMV infection was prevalent regardless of diagnosis, also in SIP patients presenting earlier in life. We speculate that the high prevalence of CMV in the intestine of index cases may be explained by a frequent, but hitherto underestimated prenatal, perinatal or postnatal exposure to CMV through blood, amniotic fluid, genital secretions or breast milk.

In NEC, it is possible that CMV nucleic acids and proteins are delivered to the bowel mucosa by exosomes or defective CMV particles present in amniotic fluid or breast milk leading to viral protein expression, presentation of viral peptides to the immune system and an aggravated inflammation. The local non-permissive CMV infection resulting in expression of viral proteins or the sole presence of CMV antigens may cause a disturbance in cell functions

(279) and a disruption in the epithelial barrier (377) and mediate an inflammation by stimulating an immune response directed against CMV peptides; this may aggravate the underlying pathology of NEC and contribute to disease progression. Inflammation by itself would also provide a microenvironment conducive for reactivation of latent CMV aggravating a pre-existing inflammation (378).

Whereas hemorrhagic-ischemic necrosis, inflammation and bacterial overgrowth are distinguishing pathologic features of NEC, the histopathology of the bowel in SIP reveals focal perforation, usually in the ileum, sometimes associated with necrosis in the muscularis externa (379). So, is it possible that CMV through other ways can contribute to the pathogenesis of SIP? The virus is able to infect both epithelial cells and smooth muscle cells that could result in a lytic infection with necrosis and bowel perforation. Also, through down regulation of the essential gap junction component connexin 43, loss of gap junction function could mediate loss of barrier integrity leading to SIP (377).

The most common route of postnatal CMV transmission to the preterm infant is maternal milk. However, human milk intake is the single most important preventive measure against NEC in preterm infants. The exact protective components by which human milk exerts its effect are not fully known; however, human milk shapes the development of the intestinal microbiota that is thought to play an essential role in the development of NEC (202). In addition, human milk contains immune-related, anti-infective and immune-modulatory components conferring additive innate immunity to the gastrointestinal tract (49,380).

We arbitrarily collected 10 specimens from autopsied infants without bowel disease as controls in our study. Although these infants were all born preterm, their median gestational age and median birth weight did not differ significantly from that of the index infants. Unfortunately, the postnatal age of the control index infants at intestinal sampling was significantly lower than in index infants (3 days vs 20 days) making it an unrepresentative control sample for the study. However, despite these dissimilarities, we only detected CMV infection in 20% of the control samples compared to in 81% in samples from index infants. Thus, we reason that the post mortem samples also provide an important control for the IHC assays used to detect CMV proteins.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

It is becoming increasingly evident that human milk is the most optimal nutrition for the preterm infant and so far very preterm infants in Sweden are preferably fed human milk, either expressed mother's own milk or donor milk.

The goal of our work herein was to document how human milk is handled before its use for preterm infants in Sweden. We also wanted to investigate what maternal factors were predictive for lactation success or lactation insufficiency in mothers of EPIs. Likewise, we wanted to evaluate the effect of routine freezing of maternal milk on the rate of postnatal CMV infection and the morbidity and mortality outcomes in EPIs. Finally, we wanted to investigate the occurrence of CMV infection in intestinal specimens from infants with NEC, SIP and surgical conditions related to these diseases.

In paper I, we could confirm an established role of human milk banking in the care of preterm infants. However, none of the neonatal unit entirely followed the prevailing recommendations by the Swedish National Board of Health and Welfare and we could observe that donor screening and analysis and treatment of human milk varied among the neonatal units. In parallel with this survey, ambitious work was performed by a group of representatives from the Swedish Milknet network, revising issues concerning the treatment and use of human milk, which resulted in updated national recommendations on breast milk handling and breast milk use in 2008. These recommendations were later revised in 2011 and are currently under the process of a new revision. In addition, the Milknet network has held regular national meetings both for education and for exchange of knowledge in matters pertaining to breast milk use that have been well incorporated by neonatal personnel. It is our believe that today, owing to these cooperative national measures, practices pertaining to breast milk handling in the neonatal care in Sweden are improved and more uniform. However, a new national survey will be needed to confirm this.

In paper II, we found that the establishment of high maternal milk feedings in EPIs at second week was important for sustained maternal milk intake the first 6 weeks of life, which in turn was positively associated with the provision of exclusive maternal milk feedings at discharge. Thus, according to the results of our study, mothers of EPIs should try to establish high breast milk volumes shortly after delivery in order to promote lactation success.
Likewise, in our study, non-Nordic origin and non-university education were unfavorable predictors of maternal milk intake the first 6 weeks of life whereas young maternal age and overweight were unfavorable predictors of any maternal milk feedings at discharge. Accordingly, mothers of EPIs without university education or of non-Nordic origin, young mothers and overweight mothers may be mothers in need of special interventions to avoid lactation insufficiency.

Through this new knowledge, and the documentation that breastfeeding rates have decreased recently, it has become evident that interventions are necessary to improve overall breastmilk

provision by mothers of preterm infants. At the Karolinska University Hospital, the collaborative work to educate and instruct engaged neonatal and obstetrical personnel in supporting mothers of EPIs in order to optimize breast milk production has been improved. Likewise, at the Stockholm South General Hospital, a "Breastfeeding group" of personnel from the neonatal unit has been formed that is responsible to educate, support and guide parents and care givers of admitted infants in issues of lactation and breastfeeding.

In the pilot study of paper III, we observed a rather high postnatal CMV transmission rate (29%) in EPIs fed with fresh and freeze-thawed CMV-positive maternal milk in our unit, with an aggravated course of a pre-existing liver condition in one of the two infected infants. Consequently, we became concerned about the potential risks of feeding our most preterm infants with untreated maternal milk with CMV transmission in mind.

Pasteurization was not considered a treatment of choice because of the detrimental effect of the heating procedures on many important milk components. At that time, the Swedish National Board of Health and Welfare recommended freezing of milk of CMV-seropositive mothers to infants < 32 weeks but no clinical trial had been performed to prove the effect of freezing on CMV transmission or CMV disease in EPIs. Likewise, as studies had shown that early feedings with fresh maternal milk was beneficiary for the preterm gut and immunity, we were apprehensive about the possible effects of routine freezing of maternal milk on neonatal outcome.

For this dilemma, we performed the randomized study of paper IV, aiming to evaluate the effect of routine freezing of maternal milk on postnatal CMV infection and neonatal outcome in EPIs. In this much larger study however, including 140 EPIs randomized to be fed either only freeze thawed maternal milk (intervention group) or both fresh and freeze-thawed maternal milk (control group), the overall postnatal CMV transmission to infants fed CMV-positive breast milk turned out to be much lower than in the pilot study, or 7%. With this unexpected lower transmission rate than in the pilot study, and loss of the original projected power, we could not demonstrate that routine freezing affected the rate of CMV transmission. The postnatal CMV transmission rate was 8% in the intervention group and 6% in the control group. Of only five infected infants, none presented with clinical symptoms but one infant in each group had transitory cholestasis. Thus, breast milk acquired CMV transmission rate was low, not affected by routine freezing and CMV infection did not cause overt symptomatic disease in the infected infants.

In contrast, by our CMV screening, we found a prevalence of congenital CMV infection of 2 % in the EPIs, a rate ten times higher than that in a previous population based Swedish report. To date, routine screening of congenital CMV infection is not performed among newborn infants in Sweden. Still, considering that extreme prematurity and congenital CMV infection both can affect neurodevelopmental outcome, we believe that EPIs might be a target group for routine surveillance in the future, especially if other studies also confirm a higher prevalence of congenital CMV infection among EPIs.

In our clinical trial, routine freezing of all maternal milk did not affect mortality during neonatal stay. With respect to morbidity, infants in the group that were partly fed with fresh maternal milk tended to have more BPD, NEC, ROP and bacterial sepsis, but this was not statistically significant. Surprisingly, LOS by candida was only observed in infants that were partly fed with fresh maternal milk and became less frequent after fungal prophylaxis was implemented in our units. One possible explanation of this finding is that expressed maternal milk may have been contaminated by Candida but that the freezing procedure prevented against subsequent invasive infection by deactivation of the yeast. Therefore, fresh maternal milk might have constituted an increased risk for fungal sepsis in EPIs that was countered by prophylaxis administration.

With the demonstrated low transmission rate of postnatal CMV infection and the subtle clinical manifestations observed in the infected infants in our study, EPIs in our neonatal units in Stockholm are still fed with fresh maternal milk in a combination with freeze-thawed maternal milk. Since this clinical trial was completed, there has been to our knowledge two observed cases of severe postnatal CMV infection in Stockholm. With respect to the possible potential benefits of routine freezing of maternal milk on morbidity in EPIs, further studies are needed to confirm these findings before the implementation of routine maternal milk freezing can be considered in our unit. Likewise, although fungal infections seem to be less prevalent in EPIs in our units after the introduction of prophylaxis, there is a need to perform a review to revise these numbers. Last but not least, the potential long-term effects in our CMV infected infants has yet not been evaluated and may come to guide us further in the future on if or how to treat maternal milk in EPIs.

In paper V, we hypothesized that CMV through transfer to the intestinal mucosa could contribute to disease pathogenesis in NEC, SIP and related surgical conditions. For this purpose, we collected 70 intestinal specimens from 61 infants that underwent surgery for these causes; 10 intestinal specimens from autopsied infants without bowel disease were used as controls. Strikingly, and to our surprise, we could detect the presence of specific CMV proteins by IHC staining in a majority of these specimens; 81% were positive for CMV-IEA and 64% were positive for CMV-LA. The occurrences were highest in infants with the most advanced NEC with bowel perforation where 95% of specimens were positive for IEA and 89% positive for LA. On the other hand, CMV antigens were only detected in 20% of the control samples. Furthermore, by CMV-DNA analysis with ISH and Taqman PCR after laser capture microdissection in selected intestinal specimens, we could further confirm the occurrence of CMV infection in several infants.

We reason that maternal CMV transmission either could occur to the fetal gut in utero or to the infant gut at delivery or postnatally through breast milk. In the preterm infant the gut is functionally immature and in a susceptible immunologic state due to an immature adaptive immune system making it more vulnerable to the exposure of microorganisms and foreign proteins. CMV is an opportunistic virus, known to cause colitis in the immunocompromised adult patient and is also proposed to act as a co-pathogen in adults with inflammatory bowel

disease. By interacting with the dysfunctional immune system in these infants, it could readily take advantage of the exposed immature gut of the preterm infant, inducing or aggravating a pre-existing inflammation in the mucosa, affecting mucosal permeability or even by lytic infection induce a local bowel perforation.

To conclude, our findings may indicate a possible role of CMV in the pathogenesis of severe NEC, SIP and related surgical conditions, aggravating the course of these diseases, leading to surgery. Progress in the treatment and prevention of NEC in the past decades has been almost none, and we hope our results might open up for new therapeutic possibilities for affected infants in the future. We suggest that routine screening for CMV should be undertaken in infants with these surgical manifestations, however, as these infants may not be viremic or have viruria due to a compartmentalised CMV infection in the bowel, new screening methods or potential biomarker need to be developed. Future studies should thus aim to explore reliable screening methods to confirm the high prevalence of CMV in NEC/SIP found in our study, to understand the pathogenic relevance of CMV in these diseases and to evaluate whether CMV targeted therapies may be of benefit for some of these patients.

6 SVENSK SAMMANFATTNING

Forskning visar att moderns egen bröstmjölk är mycket betydelsefull för det nyfödda barnets hälsa och utveckling. När ett barn föds för tidigt anpassas modersmjölkens biologiska sammansättning för att bättre tillgodose barnets särskilda behov. De allra mest för tidigt födda barnen (< 32 veckor) kan till en början ha svårt att amma och tillmatas då med urpumpad modersmjölk via sond. Mödrar till för tidigt födda barn kan ha svårt att få igång sin mjölkproduktion och att upprätthålla mjölkproduktionen under tiden barnet äter via sond. Därtill kan övergången från sondmatning till amning vara motig när barnet väl är moget att ta bröstet. I Sverige finns en lång tradition inom neonatalvården att föda upp för tidigt födda barn med bröstmjölk. I första hand ges moderns egen mjölk men om modern ej har tillräckliga mängder egen mjölk används givarmjölk. Med anledning av detta har ett flertal mjölkbanker etablerats runt om i landet, ofta i anslutning till neonatalvården.

De allra flesta människor i världen bär på ett virus som tillhör virusfamiljen herpes och heter cytomegalovirus, förkortat CMV. Viruset smittar via kroppsvätskor, till exempel blod, urin och modersmjölk. CMV är den vanligaste orsaken till medfödd infektion hos det nyfödda barnet. De flesta smittas dock av viruset under sin uppväxt. CMV är ett virus som kan orsaka allvarliga sjukdomstillstånd hos människa om immunförsvaret är försvagat. Hos de flesta individer med välfungerande immunförsvar orsakar CMV infektion inte sjukdom. När CMV infekterat en människa existerar det kvar i kroppen i en slumrande fas men kan i vissa fall reaktiveras och då utsöndras i våra kroppsvätskor. Studier har visat att de allra flesta kvinnor som bär på viruset utsöndrar det i sin bröstmjölk efter att de fött barn och att det ammande barnet kan smittas av viruset. Om barnet är fullgånget skyddas det av antikroppar från modern som går över till fostret via moderkakan i slutet av graviditeten. Fullgångna barn brukar därför inte bli sjuka i samband med bröstmjölksöverförd CMV smitta. De barn som föds för tidigt har dock ett omoget immunförsvar och saknar de skyddande antikropparna från modern och är därför mer infektionskänsliga. Kliniska studier har visat att för tidigt födda barn kan utveckla allvarliga sjukdomssymptom i samband med bröstmjölksöverförd CMV smitta med allmänpåverkan, lunginflammation, tarminflammation, leverpåverkan och påverkan på blodbild.

För att undvika smittöverföring av CMV via modersmjölk finns det sätt att behandla mjölken på innan det ges till det för tidigt födda barnet. Ett sätt är att värmebehandla mjölken, eller pastörisera den, vilket avdödar viruset. Nackdelen är att pastörisering förstör många viktiga näringsämnen och biologiska komponenter i mjölken som är viktiga för barnets utveckling och för att stärka barnets försvar mot infektioner. Ett annat sätt att behandla mjölken på är infrysning under några dagar vilket visat sig minska virusmängden i mjölken utan en lika skadlig effekt på modersmjölkens beståndsdelar. Rutinmässig infrysning medför däremot att tillförseln av den allra första modersmjölken till det för tidigt födda barnet blir fördröjd och det kan ha en stor betydelse för tarmslemhinnans utveckling och för uppkomsten av en gynnsam bakterieflora i tarmen. Internationellt pågår en omfattande debatt om det finns skäl att behandla moderns egen bröstmjölk innan den ges till de allra mest för tidigt födda barnen

och trots att ett flertal studier har utförts för att kartlägga konsekvenserna av bröstmjölksöverförd smitta i denna patientgrupp så föreligger inget enhetligt konsensus.

Mot denna bakgrund var syftet med denna avhandling:

Att i en nationell tvärsnittsstudie utforska gällande rutiner för hantering av bröstmjölk till för tidigt födda barn i Sverige (Studie I).

Att utforska maternella prediktorer för intag av modersmjölk hos extremt för tidigt födda barn under nyföddhetsperioden och vid utskrivning från nyföddhetsavdelning (Studie II).

Att i en pilotstudie utvärdera frekvens och kliniskt uttryck av bröstmjölksöverförd CMV infektion hos extremt underburna barn vid Astrid Lindgens barnsjukhus (Studie III).

Att bedöma om rutinmässig infrysning av modersmjölk minskar smittöverföring av CMV och symptom vid CMV infektion hos extremt underburna barn eller påverkar dödlighet och sjuklighet hos denna patientgrupp under vårdtiden på sjukhus (Studie IV).

Att undersöka förekomst av CMV infektion i tarmvävnadsprover från barn med nekrotiserande enterokolit, spontan tarmperforation och relaterade kirurgiska tillstånd (Studie V).

Baserat på resultaten i avhandlingen är våra slutsatser och framtida perspektiv:

Studie I.

Bröstmjölkshantering skilde sig åt bland de 36 neonatalenheterna i Sverige. Uppdatering av nationella riktlinjer kan bidra till att standardisera den praktiska bröstmjölkshanteringen vilket leder till förbättrad nutrition och mindre risk för bröstmjölksöverförda infektioner.

Studie II.

Gynnsamma prediktorer för intag av modersmjölk de första 6 levnadsveckorna var ett högt (>90%) intag av modersmjölk hos barnet under andra levnadsveckan samt universitetsutbildning och nordiskt ursprung hos modern. Ett högt modersmjölksintag de 6 första levnadsveckorna hos barnet och maternell ålder var positivt associerade till uppfödning med modersmjölk vid utskrivningen medan maternell övervikt var en ogynnsam prediktor. Mödrar till extremt för tidigt födda barn bör uppmuntras tidigt till adekvat urpumpning för att främja barnets tillgång till egen mjölk under och efter nyföddhetsperioden. Särskilda insatser bör riktas till lågutbildade unga överviktiga mödrar med icke nordiskt ursprung.

Studie III.

Frekvensen av bröstmjölksöverförd CMV infektion till extremt underburna barn som erhöll CMV innehållande mjölk var ansenlig (29%) med påvisbar leverpåverkan i ett av två infekterade barn. Fler studier behövs för att närmare belysa den kliniska betydelsen av

bröstmjölksöverförd CMV infektion hos det extremt underburna barnet och för att bedöma risk/nytta förhållandet för att ge modersmjölk till denna patientgrupp.

Studie IV.

Frekvensen av CMV överföring till extremt för tidigt födda barn som erhöll CMV innehållande mjölk var oväntat låg (7%) och samtliga CMV infekterade barn var symptomfria. Infrysning av modersmjölk påverkade inte smittöverföringen av CMV. Andelen barn med medfödd CMV infektion var relativt hög i studien (2%). Infrysning av modersmjölk påverkade inte dödlighet men minskade frekvensen av sen svampsepsis före införandet av rutinmässig profylaxbehandling mot svampinfektion. Ytterligare studier är nödvändiga för att avgöra om infrysning av modersmjölk skyddar mot uppkomsten av sen svampsepsis och om rutinmässig screening av medfödd CMV infektion är indicerad hos extremt underburna barn.

Studie V.

Två olika CMV specifika proteiner (CMV-IEA och CMV-LA) kunde påvisas med immunohistokemi i en hög andel (81% respektive 64%) av tarmvävnadsprover från barn med nekrotiserande enterokolit, spontan tarmperforation och relaterade kirurgiska tillstånd. Förekomsten av dessa CMV specifika proteiner i kontrolltarmvävnadsprover från tarmfriska obducerade barn var endast 20%. Kompletterande CMV-DNA analyser i utvalda CMV-IEA positiva tarmvävnadsprover för att bekräfta förekomst av viruset var positiva i 13/13 (100%) tarmvävnadsprover med in situ hybridisering och i 4/10 (40%) prover med Taqman PCR analys efter laser capture microdissection av CMV-positiva celler. Våra resultat antyder att CMV kan ha en bidragande roll i uppkomsten av nekrotiserande enterokolit, spontan tarmperforation och relaterade kirurgiska tillstånd och möjligtvis påverka utgång. Framtida studier bör riktas mot att hitta adekvat metod för screening av CMV orsakad nekrotiserande enterokolit och spontan tarmperforation, för att bättre utforska virusets roll i sjukdomsuppkomsten av dessa åkommor och för att utvärdera om CMV inriktad behandling kan vara indicerad i vissa fall av dessa sjukdomstillstånd.

7 ACKNOWLEDGEMENTS

Throughout my research and while writing this thesis I have been fortunate to be guided, helped and encouraged by many people whom I would like to thank.

At Menntaskolinn i Reykjavik (high-school in Iceland), we read the poems of Hávamál, old Norse poems from the Viking age presenting advice for living, proper conduct and wisdom.

One of the poems describes the importance of companions in life:

Young and alone on a long road,
Once I lost my way:
Rich I felt when I found another;
Man rejoices in man,

(Gestaþáttr, Hávamál, Poetic Edda)

Against this spoken wisdom, I would like to thank all participating study persons, the parents of the participating infants, as well as the personnel working at the neonatal departments at the time these studies took place, the members of Cecilia Söderberg Nauclér's research group, my colleagues and co-workers at the Astrid Lindgrens Children's hospital, my friends and my family. Without your contribution and engagement this research had not been practicable.

In particular, I would like to thank my supervisors:

Cecilia Söderberg Nauclér, my main supervisor, for your inventiveness, enthusiasm and everlasting confidence in me as a researcher. Thank you for your true inspiration and for introducing me to the stimulating atmosphere of your research group. Last but not least, thank you for helping me through the dissertation process; you have truly been the wind beneath my wings.

Mireille Vanpée, my co-supervisor, for guiding me into the field of neonatal clinical research, making these clinical projects happen. Thank you for the sharing of your expertise, for your thorough reviews of my work and for your approachability throughout the years.

Afsar Rahbar, my co-supervisor, for your warmth and wisdom and for your patience while teaching me how to perform laboratory work.

Charlotte Casper, my co-supervisor, for your true engagement in the projects and all the constructive feedback.

Hugo Lagercrantz, my co-supervisor, for inspiring me to believe in research.

In addition I would like to thank my co-authors:

My co-workers at the Department of Clinical Microbiology, Karolinska University Hospital; **Benita Zweygberg-Wirgart** and **Lena Grillner**. Thank you for your professional assistance with the randomized CMV study.

Lena Legnevall for your invaluable effort with the clinical projects throughout the years, for your friendship and for helping me out in times of trouble.

Frida Gustafsson for your good spirits and the many hours of work spent with the neonatal data base.

Lars Navér for your assistance and for your valuable advice.

Anna Adling for the hard work collecting data and for your excellent proof-reading.

Anna Karin Edstedt Bonamy for the always immediate feedback.

Mesfin Tessma for the thorough statistical advice.

Margret Agnarsdottir for the support in this whole process and for your help with the NEC project.

Staffan Polberger, Agneta Åkerlund and **Abiel Orrego** for your contributions to this work.

Of the neonatal personnel, I specifically would like to thank:

The personnel in the milk kitchens; **Inger, Anne** and **Elisabeth** at Karolinska Solna, **Inger** at Karolinska Huddinge and **Lena** at Danderyd as well as all the other nurses and assistant nurses that contributed to these studies. Without your invaluable help the projects of this thesis would never been performable.

My colleagues **Marco Bartocci** and **Mats Blennow** and our former colleague **Charlotte Carlsson** for the help with recruitment of patients to the study.

Marie-Louise at Karolinska Solna, **Freja** at Sachsska, **Elisabeth** at Akademiska and all the other administrators at the neonatal units for your good will and valuable administrative help.

Baldvin Jonsson, Mikael Norman, Björn Westrup and **Ihsan Sarman**, directors at the NICU´s in Stockholm at the time, and **Erik Normann**, director at the NICU in Uppsala, for all support with the studies.

I would like to thank the research group of Cecilia Nauclér at CMM:

Menzur for always helping me out and for the generous sharing of your skills. **Ling** for letting me take part in your exciting project. **KC, Chato** and **Aleem** – for answering all my questions and for guiding me in the laboratory. **Belghis** for your kindness and meticulously performed laboratory work. **Jessica** for helping me out with both paper work and laboratory

work! **Lotta** and **Sari** for teaching me the of basics of laboratory work. **All the other members of the group** for your kindness, support and constructive feedback.

Also, I would like to thank my co-workers of still ongoing projects: Giulia Aqilano, Jonas Teng, Björn Fischler, Antal Nemeth and Kajsa Bohlin.

Likewise, I would like to thank my colleagues and co-workers at the Rheumatology Department:

Eva Weidenhielm Broström and **Stefan Hagelberg**, for your supportiveness towards my research and for allowing me to take time off to complete my thesis. **Bo Magnusson**, for inspiring me to think "out of the box". **AnnaCarin Horne**, for your enthusiasm and for being a dear friend. **Erik Sundberg**, for your kindness and positiveness. **Karin Palmblad** for your encouragement. **Anna V, Anna P, Karina, Farideh, Mia, Elina** and **Malin** and the rest of the rheumatology team for making the work in the clinic enjoyable!

In addition I would like to thank:

My colleague, **Elisabeth Skantz** and **Caroline Printz** for their contributions to the projects.
Ann Hellström for your ever helpful attitude and for all assistance with administration.
Kerstin Selin Wikström, laboratory assistant at the Department of Clinical Microbiology, for all the help with administration connected to the laboratory analyses.
Fredrik Hansson, statistician @ norma, for the helpful assistance with statistical analysis.

My au pairs: Especially **Asdis, Margret**, and **Lisa** for helping me out and taking care of my children evenings and weekends when I had to urge to the NICU to recruit study subjects.

My friends:
Halldora and Freyr for your true friendship, advice and help throughout the years.
Loa for your encouragement and for the good times with your family.
My everlasting friends from the Icelandic medical school and their families – **Bessy and Einar**, **Kristin and Hallgrimur**, **Magga (again!) and Helgi**, **Agusta, Lilja and Einar**, **Magga and Össi.**
Aida and **Pernilla**, for the counselling during our "girl dinners" and of course the good food!
Håkan och **Nadine** for your support and friendship in good times and in bad times.
Gunilla och **Massanori**, our dear former neighbours, for your engagement in my family.

My family:
Ragnheidur and **Omar**, my beloved parents, for always believing in me and for making me who I am.

Saga, my favourite sister, for your love and never-ending optimism. My supporting big brother **Robert**, my trustworthy younger brother **Sturla**, and my joyful youngest brother **Kjartan** for always standing up for me.

Steingrimur, Thorhildur, Sonja and **Ragnhildu**r for your generosity and open-heartedness.

Sjöfn for being there for us when urgently needed.

Kari, Sigga, mormor and **Ljufust** for your love and support throughout the years.

Gudjon, my husband and partner in life, for always being there for me (and for the graphical help). My wonderful children: **Omar, Iris and Sara** for enriching my life and for your unconditional love.

Lastly, I would like to thank the **Karolinska Institutet** for giving me the opportunity to study at the Research school for clinicians in epidemiology and for the grant of KID funding during my doctoral education.

The papers included in this thesis were partly supported by grants from:

Mjölkdroppen Foundation, HRH Crown Princess Lovisa's Foundation, The Queen Silvia Jubilee Fund, Samariten Foundation, The Society Barnavård, The BILTEMA Foundation, Anna Britas and Bo Castegrens Memory Foundation, The Pediatric Research Foundation of Astrid Lindgrens Children's Hospital, Spädbarnsfonden, Stichting af Jochnick Foundation, Sten A Olsson Foundation for Culture and Research, Erling Persson Family Foundation, RATOS, Torsten and Ragnar Söderberg Research Foundations (MF14/10), the Swedish Research Council (10350 and K2007-56X-12615-10-3) and Swedish Research Council Framework Grant in Infections and Antibiotics (K2014-99X-22627-01-4), the Swedish Cancer Foundation (5044-B05-01XAB), Jane and Dan Olsson Foundation, Swedish Society for Medical Research, The Royal Swedish Academy of Sciences, Goljes Memory Foundation, Swedish Society for Medical Research, Nxt2b and Almänna BB:s Memory Foundation and the KID funding from the Karolinska Institute.

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