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BREAST-FEEDING: EARLY INFLUENCES ON LATER HEALTH

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Springer

Breast-Feeding: Early Influences on Later Health

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Preface

The theme of ISRHML 2004, the 12th Symposium of the International Society for Research in Human Milk and Lactation was ***Breast-Feeding: Early Influences on Later Health.*** The conference attracted almost 200 delegates from 32 different countries and included speakers from five continents. The meeting drew upon areas of research that have been championed in the UK for more than 50 years - early fetal programming of adult health. We extended this concept and examined the extent to which differences in infant feeding practices can also lay an indelible imprint on metabolism and behaviour, and hence affect later function and risk of disease. To the best of our knowledge this was the first symposium to focus its attention purely on the post-natal period in terms of early life programming. It should be recognised that there is much less information currently available than there is for fetal programming, and we anticipated that many new questions would be raised and numerous areas where further research is needed highlighted.

ISRHML 2004 was held at Queens' College, Cambridge. Queens' was founded in 1448 by Margaret of Anjou and in 1465 by Elizabeth Woodville, queens of Henry VI and Edward IV respectively. Delegates were able to enjoy many 15th and 16th century buildings, still more or less the same as when first built, although the conference facilities were built a little more recently! The famous 'Mathematical' Bridge, originally built in 1749 and subsequently rebuilt to the same design in 1866 and 1905, provided the link between scientific sessions, accommodation, and social events.

The ***core scientific programme*** was designed in three sessions, and the order of the chapters in this book reflects this. Chapters 1-4 lay down some of the basic biology of early life development; Chapters 5-9 examine how

breast-milk and breast-feeding might 'programme' these processes by acting as modulators of development; Chapters 10-17 examine the epidemiological evidence that such effects do indeed exist. In addition to the core programme, ISRHML 2004 included a special public health update session on ***HIV and Breast-Feeding*** (Chapters 20-22) in which we were fortunate in attracting some of the major researchers in this important field that has such a critical impact on child health and survival in many countries. Dr Dan Sellen gave a special lecture on the ***evolution of human lactation and complementary feeding*** (Chapter 18). The ***Macy-György Prize Lecture*** 'My Milky Way' was given by Lars 'Nenne' Hanson (Chapter 19). Also included in the mix were two workshops on ***Early breastfeeding cessation and infant mortality in low-income countries*** and ***Measuring trace immune factors in human milk.*** Summaries can be found in Chapters 23 and 24 respectively. The abstracts from the ***multi-disciplinary poster sessions*** can be found in Chapter 25.

The International Society for Research in Human Milk and Lactation thanks the sponsors named below for their generous support of ISRHML 2004, that, among other things, enabled us to award over £20,000 of travel awards to 16 scientists and health workers, from low-income countries. These individuals are identified in Chapter 25. **Major Sponsors:** Great Ormond Street Hospital Trust, London, UK; Institut de Recherche pour le Développement, Montpellier, France; Medical Research Council Human Nutrition Research, Cambridge, UK; Medical Research Council/London School of Hygiene & Tropical Medicine, UK; Unilever Health Institute; The Wellcome Trust. **Sponsors:** Kelloggs, McNeil Consumer Nutritionals Ltd, Medela AG, Switzerland, Tanita UK Ltd.

Finally we would like to record our thanks for all their hard work before, during and after the conference, to the ISRHML 2004 Conference Administrator, Kate Homan, and her team of students and staff from Human Nutrition Research in Cambridge – Emily Smith, Helen Mulholland, David Lee, Pippa Eyre and Suzanne Hartley.

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Chapter 1

GROWTH AND ORGAN DEVELOPMENT

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1. INTRODUCTION

The study of human growth conventionally focuses on the whole body, notably the linear and volumetric measures of height and weight, plus the derived ratio body mass index (weight/height^2). Body volume and body surface area are also important for some aspects of nutrition, either measured directly or estimated from weight and height. By contrast linear dimensions of body parts are studied for particular purposes, e.g. head circumference, sitting height or triceps skinfold thickness, but weights or volumes of body parts, particularly organs, are not routinely considered in the same way. The reason why is obvious – they are difficult to measure non-invasively, and before the relatively recent technological advances that have occurred in ultrasound and imaging they could only be measured at post-mortem. For this reason paediatric pathology has in the past tended to be the discipline most knowledgeable about organ development.

But times change. Developments in ultrasound in recent decades have made the assessment of fetal organ development routine, insofar as organ size can be inferred from two-dimensional ultrasound images. Brain size for example is highly correlated with head size, as measured *in utero* by biparietal diameter or head circumference, while abdominal circumference is a proxy for central organ development. With this ready access to measures of fetal organ size has come an increased interest in organ development, in health and disease, considering both short-term outcomes in early life and later child and adult outcomes.

G. R. Goldberg et al. (eds.), Breast-Feeding: Early Influences on Later Health,

The aims of this chapter are threefold: a) to describe the process of normal organ growth in the embryo, fetus and child, showing how it relates to growth in the whole organism; b) to consider some factors that affect the susceptibility of growth to external insults, particularly plasticity and critical periods of growth, and c) to summarise the common patterns of abnormal fetal growth as they impact on organ development.

2. THE FOCUS OF GROWTH

The brain is known to be a critically important organ for *homo sapiens*. Bogin[1] compared average organ weights in adult humans and higher primates, adjusting for body size, and showed a strikingly larger brain and smaller gut in humans, while the other organs were broadly comparable across species. This emphasis on brain development is also seen in the human pattern of brain growth, the brain being relatively enormous in the embryo and becoming progressively smaller compared to body weight through gestation, infancy and childhood. Brain growth effectively stops before puberty, in contrast to the other organs which continue increasing until adulthood. Brain-sparing, the preferential protection of the brain when organ growth is under threat, is important for a proper understanding of the process of organ development.

2.1 The processes of cell proliferation and expansion

Organ growth proceeds in three stages. The first is cell differentiation, the process of organogenesis where organs are first assembled, and this takes place while the fetus is still an embryo, 3 to 8 weeks after ovulation.

The second stage is cell proliferation or hyperplasia, when the number of cells in the organ increases sharply, and this takes place typically in the first half of fetal life after the embryonic stage. For most organs cell number stabilises during the latter half of pregnancy and thereafter increases little. Clear exceptions to this are fat mass and muscle mass, where cell number increases throughout childhood and stabilises after adolescence.

The third stage is cell expansion or hypertrophy, when the size of cells increases. This stage occurs throughout pregnancy and into childhood, but is more important in terms of its contribution to organ growth once cell number has stabilised.

Winick distinguishes three phases of cell growth – increasing cell number alone, increasing cell number and cell size, and increasing cell size alone. [2, 3] These distinctions are important for later discussions about critical periods.

2.2 Normal organ growth

The first stage of prenatal life is the embryo. This appears about three weeks after ovulation, or five weeks after the last menstrual period. The age of the embryo is conventionally measured in weeks post-ovulation, so that post-ovulatory weeks are two less than gestation weeks. The growth of the embryo is defined in terms of Carnegie stages, where stage 10 corresponds to day 22 and stage 23 corresponds to day 56. During this period the embryo increases in size from 2 to 20 mm crown-rump length, and the weight is less than 10 gm. With ultrasound the gestation sac is first visible at 5 weeks, and the crown-rump length can be measured from 6 weeks. The embryonic phase is the main period of organogenesis, so that most organ systems are in place by the end of this time, albeit in an undeveloped state. Figure 1-1 shows the growth pattern of some embryonic dimensions.[4]

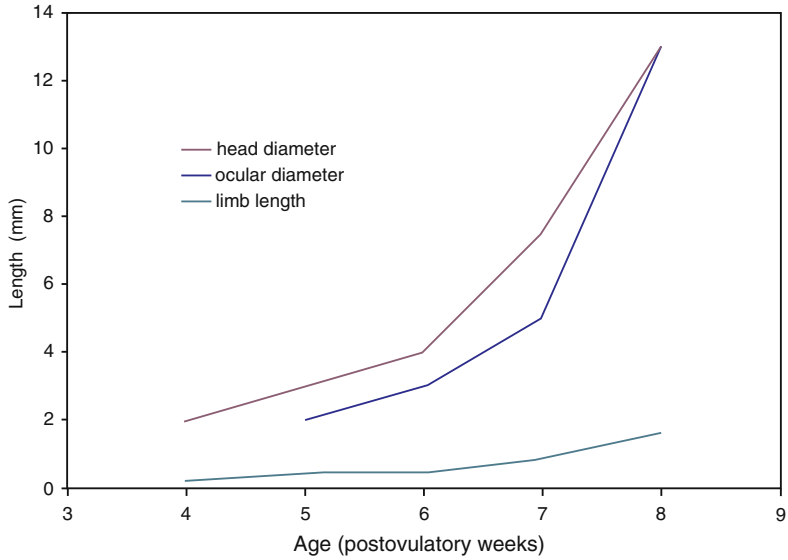

Figure 1-1. Growth of the embryonic head, limbs and eyes

The fetal phase starts at 8 post-ovulatory weeks (10 weeks of gestation), when organs are increasing primarily by cell proliferation (increasing cell number). Later in gestation cell proliferation switches to cell hypertrophy, and the overall rate of cell growth decreases. Growth in the linear dimensions of the fetus has been measurable with ultrasound for some 30 years, and ultrasound scanning is now routine both for dating the fetus and for monitoring its growth. Fetal growth centile reference charts now exist for

several dimensions,[5] and they have two features in common. The median curve is fairly linear but curves downwards in later pregnancy, and the outer centiles tend to diverge from the median over time. This suggests that the variability expressed as a percentage of the median, i.e. the coefficient of variation, is fairly constant.

Figure 1-2 provides cross-sectional data on organ growth between 12 and 26 weeks of gestation from Hansen *et al*,[6] based on measurements of 597 fetuses and early neonatal deaths. The weights are shown on a common log scale covering the range 0.01 g to 1000 g, a range of 100,000 to 1. Constant proportional growth appears as a straight line on this scale.

The legend to Figure 1-2 ranks the organs in order of decreasing size, with body weight at the top - this identifies the individual curves. Throughout this stage the brain is the largest organ, followed by the liver, lungs, kidneys and heart. There are two obvious features of the graph: the first is that they are curves not lines, so that the proportional growth rate is slowing throughout the period. The second impression is that the individual curves are broadly the same shape, so that the spacings between them do not change much throughout the second trimester.

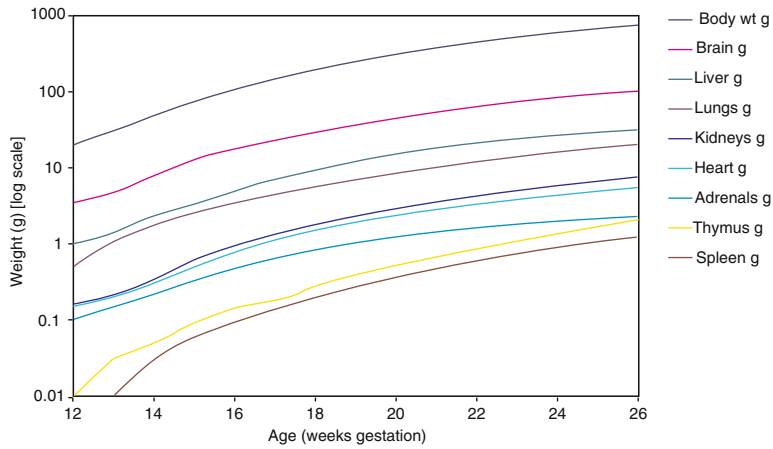

Figure 1-2. Growth of eight organs during weeks 12 to 26 of pregnancy

The relative size of different organs of the body, and their relation to the whole, is covered by the term *allometry*. The allometric equation $OrganSize \propto BodySize^{p}$ relates the two via the allometric constant p. The special case when $p = 1$ is called *isometry*, when organ size is a constant proportion of body size. If the curves in Figure 1-2 were exactly parallel then this would indicate isometric growth.

The way to test this visually is to replot the organ weights in Figure 1-2 as percentages of body weight, and see if the resulting curves are constant over time. Figure 1-3 shows that the larger organs do indeed grow isometrically, apart from slight deviations before 16 weeks, but the three smallest, the adrenals, thymus and spleen, do not. The adrenals slow down while the thymus and spleen accelerate. The effects may seem small, but the thymus and spleen for example grow 5 times and 3 times faster respectively than weight between 12 and 26 weeks.

Hansen *et al* [6] also provide information on linear dimensions such as crown-rump length. They also show isometry with body weight, but only when expressed as length. [3] So in this sense the fetus grows rather like a sphere, with most of the organ weights remaining in proportion to the whole, and the linear dimensions proportional to the cube root of weight.

Singer *et al* [7] provide similar data for the gestation range 20 to 42 weeks, which show broadly the same patterns as Figures 1-2 and 1-3. Such data are valuable for tracking organ growth, but their one weakness is that they are based on stillbirths or neonatal deaths, and so are of uncertain relevance for normal growth.

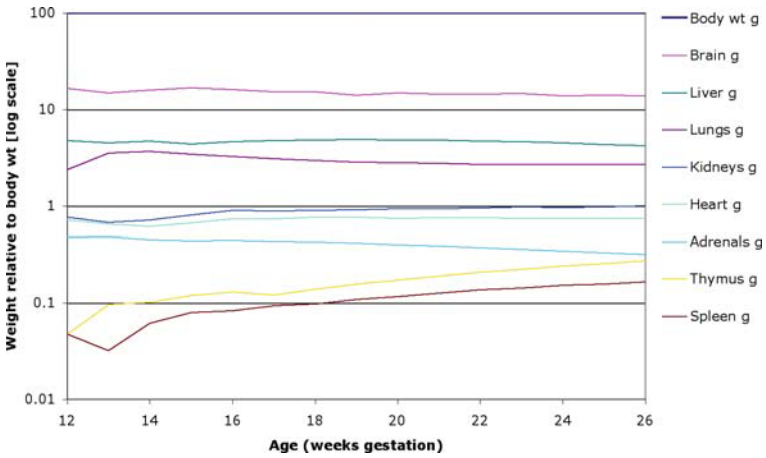

Figure 1-3. Weights of the organs in Figure 1-2 expressed as percentages of body weight

Recent developments in 3D ultrasound, computed tomography and magnetic resonance imaging are starting to make non-invasive organ assessment feasible,[8-11] though the required equipment is currently prohibitively expensive for routine use. These methods will inevitably take over from post-mortem studies, which are becoming more and more difficult to do with recent changes in the ethical climate.

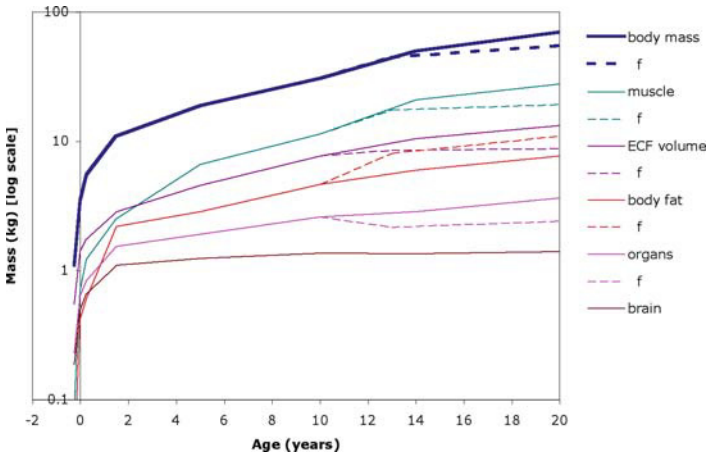

Figure 1-4. Postnatal growth of certain body compartments. The solid lines are for boys and pre-pubertal girls, the dotted lines for post-pubertal girls.

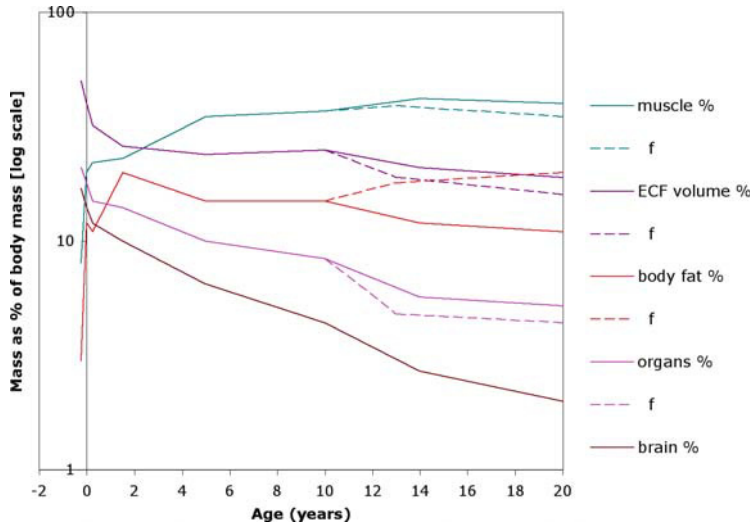

Figure 1-5. The compartments of Figure 1-4 expressed as percentages of body weight. The solid lines are for boys and pre-pubertal girls, the dotted lines for post-pubertal girls.

The literature on postnatal organ growth is tiny compared to that for the prenatal period, reflecting two things: most interesting organ growth occurs prenatally, and mortality is much lower postnatally than prenatally. Holliday[12] tabulated the weights of various body compartments through childhood, see Figure 1-4. The brain was the only organ treated in isolation, all the others being grouped together. But Figure 1-4 also shows the growth

of the large muscle, extra-cellular fluid and fat compartments, particularly the sexual dimorphism that occurs at puberty.

Note that brain growth has essentially stopped by 10 years. Figure 1-5 expresses the compartment masses as percentages of body mass, and shows that the brain falls from 14% of body mass at birth to only 2% in adulthood. Muscle is the only compartment to increase in relative terms after birth, while fat mass remains broadly the same at around 10% in males and 20% in females.

2.3 What factors lead to abnormal growth

Fetal growth is routinely monitored with ultrasound and plotted on fetal growth charts. But the degree of growth tracking is relatively low compared to that seen in the postnatal period, so that serial ultrasound measures predict birth size only poorly,[13] and periods of prenatal growth faltering are hard to identify. This leads to two obvious questions: are some organs particularly susceptible to growth faltering, and if so, at what stage(s) of development?

2.3.1 Plasticity – how to measure it?

Plasticity is a measure of an organism's ability to recover from an insult. In growth terms it indicates how much the organism can "catch-up" following a period of impaired growth. There is no standard way of measuring plasticity, but three possible approaches suggest themselves.

With cross-sectional data the population variability indicates how tightly growth is controlled, so that for example the variability of weight postnatally is three times that of height, as measured by the coefficient of variation (CV = standard deviation / mean).[14] This suggests that weight is more plastic than height, and responds more quickly to an insult and any subsequent opportunity for recovery.

Figure 1-6 revisits the second trimester organ data of Figure 1-3, expressing the variability of the organs by gestation in terms of their CV. The legend ranks the organs by size as in Figure 1-3, though the individual curves are difficult to identify. First impressions from Figure 1-6 are that variability falls rapidly with increasing gestation, and that the degree of variability is substantial, exceeding 20% for most organs. Curiously the variability is inversely related to size. The upper trace for example is the spleen, the smallest of the organs, while the lowest two traces are brain and fetal weight, the largest. The variability of the other organs is also inversely proportional to their size. This, plus the gestation effect, strongly implies that measurement error is an important source of variability, in that smaller organs are harder to measure.

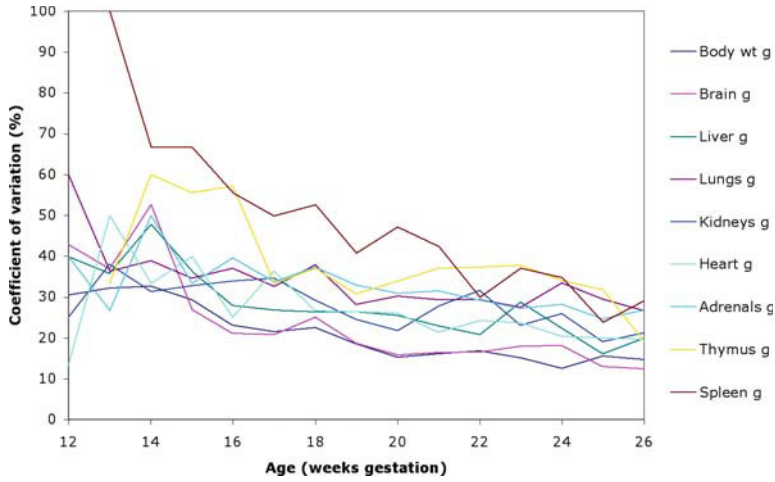

Figure 1-6. Variability of organ mass by gestation. Coefficient of variation (SD/mean x 100) for body mass and 8 organs.

The one exception to this is the brain, which is no more variable than fetal weight yet is only one-seventh the size (from Figure 1-3). This shows that the underlying variability of brain weight is less than for fetal weight or the other organs, clearly suggestive of brain sparing.

Two related measures of plasticity are available for organ size measured longitudinally: the correlation between measures at two different gestations and the variability of the organ growth rate over time. The higher the correlation the lower the growth rate variability, so the two measures are closely inversely related. Guihard-Costa *et al* [15] for example measured over 6-week periods the fetal growth rates of biparietal diameter (BPD or skull diameter), abdominal transverse diameter (ATD) and femur length (FL). They found that the growth rate variability increased markedly with gestation for ATD and FL, while for BPD it was relatively constant. They were also struck by the high variability overall, suggesting that conventional growth charts are misleading in suggesting a high level of tracking. The low variability of brain growth suggests again an element of brain sparing, particularly in later gestation, so that fluctuations in growth rate during the third trimester are seen more in other organs than the brain.

2.3.2 Critical periods – what are they?

In addition to plasticity, critical periods of organ growth have a potentially important effect on the developmental environment. Scott[16] has established certain principles about critical periods of growth: (i) a critical period is a time when an irrevocable developmental decision is made; (ii) critical periods have known time boundaries, and (iii) a process is most sensitive when changing most rapidly. What this means is that during a critical period developmental changes can occur which later cannot be undone; that the critical period has a start time and an end time, and outside this window of opportunity it does not operate, and rapid growth is more sensitive than slow growth to such periods.

There are two obvious critical periods of growth which operate largely in fetal life, and which arise from the pattern of growth in cell number and cell size. The first is the embryonic period, when organs are assembled from scratch in a complex pattern which relies on each stage of development being reached at the right time. Clearly, on the occasions when this process is disrupted the embryo has less chance of surviving, so the infants who survive it are a selected group.

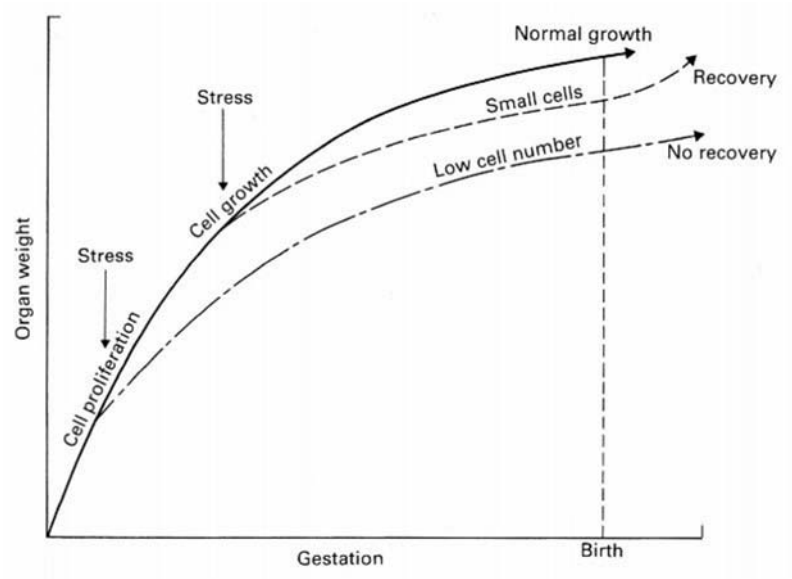

Figure 1-7. Cell number (proliferation) and cell size (growth) as they relate to critical periods of growth (reproduced with permission of Blackwells Ltd).

The second critical period occurs in fetal life after organogenesis has been completed, when cell number increases rapidly. Increases in cell

number and cell size are distinguished by the pattern of increase in DNA in the organ.[17] The amount of DNA per diploid cell is fixed, so that total DNA is a direct measure of cell number – after a certain stage DNA stops increasing, showing that cell number has stabilised. This satisfies Scott's principle of a window of opportunity, in that the period of increasing cell number extends from 10 weeks gestation or so to the age when cell number stops increasing.

Figure 1-7 shows why this period of cell proliferation is a critical period. If a stress occurs early on and reduces cell number, there is no opportunity once the period has passed to compensate for it. The organ will remain small. The same is not true for a later stress that reduces cell size, where the malnourished organ can catch up postnatally. In both cases the fetus shows intrauterine growth retardation, but the difference in timing affects the opportunity for catch-up.

Winick [2,3] showed that brain DNA stopped increasing soon after birth, whereas placental DNA stopped earlier, at around 36 weeks of gestation. Widdowson [18] found that in the kidney and heart, cell number doubled every three weeks or so until 30 weeks gestation, at which point it stabilised. However a review by Brasel and Gruen [19] argued that the story was not so simple, in that cell number for several organs including the brain continued to increase through postnatal life, with DNA content in adults appreciably greater than in infancy. The liver differs from other organs is its ability to regenerate, so that even in adult animals a portion of liver can be surgically removed and it grows again within days. Thus the liver does not have a critical growth period.

In principle the concept of cell number stabilising in early life neatly predicts the existence of critical periods for organ growth at that time. But the fact that cell number for several organs continues to increase through childhood, and that certain organs such as the liver do not have a critical period as such, suggests that the story may not be so simple.

A third critical period can be identified which, though not directly related to organ growth, is particularly relevant to lactation. The period of time immediately after birth, when enteral feeding starts, is important for infants programmed to alter their growth trajectory compared to prenatally. Recent work indicates that infants who grow rapidly at this time, showing "catch up" in weight following a period of intrauterine constraint,[20] are at increased risk of later obesity,[21-23] and that breastfeeding may protect against it.[24] Longitudinal studies of growth and milk intake show that intake correlates with weight gain for the first 2-3 months of life but not thereafter, whereas the association between intake and weight is maintained throughout lactation.[25] This suggests that for the first 3 months there is a dialogue between mother and child about how much milk is required; for example a

mother producing less milk than the child wants tries to down-regulate the child's appetite whereas the child tries to up-regulate the supply. Conversely when the supply is abundant the child takes less and the supply is reduced. Either way the child's growth rate reflects the milk intake. But after 3 months the period of negotiation ceases and the milk supply is effectively fixed. Thereafter fluctuations over time in the amount of milk have relatively little effect on weight gain in the individual, but infants who are receiving more milk tend to be heavier than those receiving less.

This has the two key properties of a critical period – a start point and end point, and a permanent effect. The reason why breast-feeding may protect against child obesity is that the child, during the critical period, has the opportunity to provide feedback about their energy requirement, whereas the formula-fed child has much less say about the amount of milk they are offered.

2.4 Abnormal organ growth

Among neonates who have grown abnormally *in utero*, clues to the nature of the growth abnormality can be found in the relative sizes of the infant's organs. Singer and colleagues[7] provide a useful summary of the different patterns that are seen. They distinguish between symmetric and asymmetric growth, where a symmetric growth pattern means that the organs have grown in proportion with each other, whereas with asymmetric growth they are disproportionate, usually with the brain showing sparing.

In symmetric growth retardation, the organs are relatively small for the infant's gestation, but in proportion, while maturation is appropriate for gestation. Congenital rubella is an example. Organ cell number in such infants is reduced, implying that the growth failure occurred relatively early in pregnancy during the hyperplastic growth phase. This in turn suggests some form of defect arising during organogenesis disrupting growth.

Growth retardation is more commonly asymmetric than symmetric, where typically the brain is of normal size but the liver, lungs and thymus are small. Weight for length is often reduced too, as is organ cell size. This implies an insult relatively late in pregnancy, during the hypertrophic growth phase, often related to a failure of the placenta in the third trimester.

Symmetric and asymmetric growth retardation have obvious analogies with "stunting" and "wasting" in malnutrition, not only in terms of their appearance but also in terms of timescale – stunting reflects long-term malnutrition whereas wasting occurs more in the short-term.

The same distinction between symmetry and asymmetry applies to overgrowth, the opposite end of the growth spectrum to growth retardation. With symmetric overgrowth the sizes of the organs remain in proportion,

and maturity is appropriate for gestation. This pattern is seen with tall and multiparous mothers, and reflects normal growth writ large. By contrast asymmetric overgrowth is seen in infants of diabetic mothers, where the liver, heart, adrenals and fat stores are overgrown, and the child is relatively fat. Brain growth may be largely unaffected, leading to a disproportionately small brain.

3. CONCLUSIONS

This brief review of organ growth has shown it to be mainly an embryonic and fetal process, and during fetal life organ growth is with some exceptions near-isometric. However the growth rate varies considerably over time in the individual, so that ultrasound tracking of linear measurements is poor and the variability is high, due to growth plasticity and/or measurement error. Plasticity is measured in terms of population organ size variability, or alternatively of organ growth variability over time. Brain sparing shows itself as low plasticity.

There are two critical periods of organ growth, during embryonic and early fetal life, relating to organogenesis and cell hyperplasia. A third (non-organ) critical period applies during the establishment of lactation.

Abnormal fetal growth can affect organs differentially, leading to a pattern of asymmetric growth. This implies a growth insult occurring late in pregnancy. A better understanding of fetal organ growth disorders will require application of new technologies such as 3D ultrasound or echo-plane magnetic resonance imaging, particularly as consent to use post-mortem material for research purposes becomes harder to obtain.

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Chapter 2

NUTRIENT TRANSFER: MAMMARY GLAND REGULATION

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1. INTRODUCTION

Breast-milk is arguably the ultimate functional food providing the nursing infant with basic nutrition as well as a complex mixture of immunomodulatory components, bioactive compounds and a vast array of hormones[1]. Having been breast-fed as an infant has been associated with enhanced cognitive development[2] and may also provide protection against cardiovascular disease[3, 4], obesity[5] and type 1[6] and type 2 diabetes[7] later in life. Appropriate trace element intake is essential for optimal growth and development and as such may play a role in some of the positive outcomes associated with breastfeeding. Breast-fed infants are entirely dependent upon the mother to provide an appropriate trace element supply and evidence indicates that trace element requirements of term infants are generally met by exclusive breast-feeding through about the first 6 months of life[8]. After 6 months of age, introduction of complementary foods with adequate trace element content is essential to meet the nutritional needs of the growing infant. This is due in part to milk iron (Fe), zinc (Zn) and copper (Cu) concentrations declining throughout lactation[9]. Furthermore, milk Fe, Zn and Cu concentrations are relatively refractory to maternal trace mineral status[10], even when the maternal diet varies considerably[11]. There is currently little information regarding the mechanisms through which the mammary gland regulates milk trace element concentrations. Similarities between humans

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and rodents[12] allow us to use rodent models to examine the regulation of mammary gland mineral transport. Recently, several transporters for Fe, Zn and Cu have been found to control trace element uptake and efflux in various cell types. We have utilised the lactating rat to determine changes in mammary gland Fe, Cu and Zn transporter expression and localisation that occur throughout lactation and in response to maternal trace mineral deficiency in hopes of elucidating some of the changes which may be occurring in lactating women.

2. MAMMARY GLAND IRON TRANSPORT

Adequate Fe intake is essential for optimal growth, hematopoiesis and cognitive development during infancy. Iron deficiency anemia is the most common nutrient deficiency and is estimated to affect 1 to 2 billion people worldwide.[13] While maternal Fe deficiency has not been associated with neonatal Fe deficiency anemia *per se*, neonatal Fe stores are decreased[14] leaving the newborn at increased risk for Fe deficiency if Fe intake is inadequate. Milk Fe concentration in humans and rats normally declines throughout the course of lactation.[9,15] However, little correlation between maternal Fe status and milk Fe concentration in lactating women[11] or between marginal Fe deficiency and milk Fe concentration in rats has been observed[16], indicating that mammary gland Fe transport is a tightly regulated process thus ensuring appropriate Fe transfer to the neonate.

Cellular Fe transport is tightly regulated and consists of Fe uptake across the plasma membrane, the partitioning of Fe into specific intracellular pools and Fe export across the plasma membrane in some cell types such as the secretory mammary epithelial cell. Iron uptake into the mammary gland is facilitated by transferrin receptor (TfR). However, no correlation between milk Fe concentration and TfR expression has been observed, suggesting that the control of milk Fe level occurs following Fe uptake into the mammary gland.[17] Once diferric transferrin binds to TfR at the cell surface[17], the transferrin–TfR complex is internalised in clathrin-coated vesicles that fuse with acidic endosomes. The acidic environment facilitates the release of Fe from the transferrin–TfR complex within the endosomal vesicle. Iron is transported out of the endosome by divalent metal transporter 1 (DMT1).[18-19]

While we have determined that DMT1 is expressed in the mammary gland[16], its localisation and the role it plays in mammary gland Fe metabolism have not yet been characterised. Once Fe has entered the mammary epithelial cell it may partition into a chelatable Fe pool or participate in a multitude of cellular processes such as sequestration into

ferritin (Ft) for storage, incorporation into Fe containing proteins in the endoplasmic reticulum (ER) or export across the luminal membrane into milk. How Fe secretion into milk is facilitated is currently unknown. However, ferroportin (FPN) or IREG1 is localised to the endoplasmic reticulum in reticuloendothelial cells where it is assumed to facilitate Fe transport into an intracellular vesicle prior to secretion.[20] We have determined that FPN is expressed in the mammary gland [16] and speculate that mammary gland FPN similarly transports Fe into secretory vesicles destined for export into milk.

To address questions regarding the regulation of mammary gland Fe transport we have used the lactating rat as a model to localise DMT1 and FPN and determine changes in mammary gland Fe transporter expression throughout lactation and in response to maternal Fe deficiency. Using immunohistochemistry, we have determined that both DMT1 and FPN are localised to intracellular vesicles and this cellular localisation in combination with predicated membrane topology suggests that DMT1 may play a role in endosomal Fe export while FPN may indirectly participate in the secretion of Fe into milk.[21] As mentioned previously, milk Fe concentration declines throughout lactation and this decline is associated with declining levels in TfR and FPN expression. In contrast, mammary gland Fe concentration and DMT1 expression both remain constant throughout lactation further suggesting that DMT1 may play a role in mediating cellular Fe pools.[16] These results taken together suggest that the decline in milk Fe concentration that occurs throughout normal lactation results from decreased Fe uptake and secretion from the mammary gland into milk and not from tissue Fe depletion and may partially reflect the improvement in maternal Fe status that occurs during the postnatal period.[22] Maternal Fe deficiency in lactating rats reduced mammary gland Fe levels during lactation and similar to observations in lactating women, milk Fe concentration was not affected. The maintenance of milk Fe level was associated with decreased mammary gland ferritin and DMT1 expression while TfR and FPN expression were not affected. These results further suggest that the primary regulators of milk Fe secretion may be TfR and FPN and indicate that milk Fe levels are maintained during Fe deficiency due to an uncoupling of the “normal” tissue Fe-responsive regulatory mechanisms in the mammary gland.

3. MAMMARY GLAND COPPER TRANSPORT

Copper (Cu) plays an essential role as a cofactor for enzymes that generate cellular energy, cross-link connective tissue and mobilise cellular iron.[23] A large amount of Cu is accreted by the fetal liver [24] and is effectively

mobilised during early neonatal life[25]. However, studies in rodents indicate that total body Cu content increases during suckling suggesting that Cu must be absorbed from their diet as well[26]. During lactation, milk provides the sole source of Cu to the offspring; however, milk Cu concentration decreases as lactation progresses in both rodents[12] and humans[27]. Currently, the mechanisms in the mammary gland which facilitate the decrease in milk Cu concentration are not understood.

Three mammalian Cu-specific transport proteins have been identified in the mammary gland[28-30]. The Menkes Cu ATPase (ATP7A) belongs to the P-type ATPase family of transmembrane proteins, and mutations in the ATP7A gene are associated with impaired cellular Cu export[31]. ATP7A expression is ubiquitous and its gene product is localised to both a perinuclear and vesicular compartment in mammary glands of mice and humans in the non-lactating state[29, 32]. However, during lactation mammary gland ATP7A expression is increased and ATP7A protein re-localises to the plasma membrane[29] suggesting that mammary gland ATP7A plays an active role in mammary gland Cu transport during lactation. The Wilson Cu ATPase (ATP7B) also belongs to the P-type ATPase family and is homologous to ATP7A[30]. Individuals with Wilson disease have mutations in the ATP7B gene which eliminates the ability of ATP7B protein to appropriately localise to an intracellular compartment in the liver, resulting in impaired biliary Cu excretion and subsequent hepatotoxicity[31]. In the rat mammary gland during mid-lactation, ATP7B is localised to an intracellular compartment and to the luminal membrane of secretory mammary epithelial cells[28]. Similar to observations in patients with Wilson disease, a murine mutation in ATP7B (toxic milk, *tx*) results in defective ATP7B translocation in the mammary gland thus impairing Cu export into milk (~20 % of normal). This mis-localisation of ATP7B in the mammary gland results in neonatal death from Cu deficiency suggesting it plays a major role in mammary gland Cu export into milk. [30] Prior to export into milk, Cu must be imported into the mammary gland; however, the mechanisms the mammary gland uses to accomplish Cu import are not well understood. In the circulation, Cu is tightly complexed with ceruloplasmin (Cp), associated with albumin and, to a lesser degree, small molecular weight ligands such as amino acids[25]. Recently Ctr1, an essential Cu import protein, has been identified and found to be expressed in all tissues examined[33, 34] including the mammary gland[28]. Studies in transfected cell models indicate that Ctr1 imports Cu^{+1} with high affinity[33,35] and import is believed to require multimerisation of several Ctr1 proteins[36], possibly forming a channel[37]. Additionally, recent evidence indicates that Ctr1 is vesicular and is endocytosed and degraded in response to physiological levels of extracellular Cu[38], presumably providing a rapid method of modulating Cu

import. Similar to what has been observed in numerous cell types[37], we have determined that Ctr1 in the mammary gland is localised to both the plasma membrane and intracellular vesicles[28].

We used a lactating rat model and characterised changes in Cu transporter expression and localisation during lactation. Similar to observations in humans[9], milk and plasma Cu concentration declined through lactation as did mammary gland Cu levels. The decrease observed in milk Cu level as lactation progresses may be primarily a result of the internalisation of Ctr1 from the serosal membrane as lactation progresses in combination with reduced Cu availability from maternal circulation due to decreasing plasma Cu concentration. These changes may facilitate the depletion of mammary gland Cu levels. Furthermore, the protein level and localisation of ATP7B is maintained throughout lactation while the amount of ATP7A protein is higher during early compared to late lactation. Although the role of ATP7A in mammary gland Cu export is currently unknown, high expression of ATP7A during early lactation may facilitate enhanced Cu secretion into milk during this period, while the longitudinal decrease in ATP7A level may reduce the ability of the mammary gland to secrete Cu into milk as lactation proceeds. However, the possibility that ATP7A plays a yet unknown role in mammary gland Cu transport cannot be excluded.

While Cu deficiency is uncommon, marginal Zn intake is very common and Zn deficiency during pregnancy and lactation has been associated with secondary affects on Cu metabolism in the offspring. Research from our group has recently demonstrated an inverse correlation between maternal Zn status and milk Cu concentration[28]; however, the underlying mechanisms are unknown. We used the lactating rat as a model and determined that marginal maternal Zn intake similarly resulted in increased milk Cu concentration and Cp activity. Furthermore, Zn deficient rats had increased mammary gland Ctr1, ATP7A and ATP7B expression and also relocalised ATP7A to larger vesicles in the mammary gland, potentially increasing Cu secretion into secretory vesicles. Thus, suboptimal maternal status of one trace element may affect the milk concentration of other essential trace elements, emphasising the need for adequate maternal nutrition of multiple trace elements to ensure optimal trace element transfer to the nursing infant.

4. MAMMARY GLAND ZINC TRANSPORT

Zinc is a nutrient required for many proteins involved in DNA synthesis, protein synthesis, mitosis and cell division. Adequate Zn supply is particularly important during the periods of rapid neonatal growth and

development as illustrated by observations of early neonatal death associated with low milk Zn levels in lethal milk (*lm*) mice.[39] During lactation, a substantial amount of Zn is taken up by the human mammary gland and secreted into milk (0.5-1 mg/d), facilitating the movement of almost twice the amount of Zn that is transferred daily across the placenta to the fetus during pregnancy,[40] which illustrates the extraordinary activity of mammary gland Zn transport. Furthermore, milk Zn concentration is maintained over a wide range of dietary Zn intake,[41-42] which suggests that mammary gland Zn import and export are tightly coordinated in order to provide adequate Zn to the nursing infant. Interestingly, although plasma Zn concentration increases, milk Zn concentration decreases throughout the normal course of lactation in both rodents and humans;[12] however, the transport mechanisms that regulate this longitudinal decrease are not well understood.[43]

Recently, a number of mammalian proteins have been described which participate in Zn trafficking from the cytosol across membranes.[44] These are divided into two distinct families. The ZnT family of Zn transporters is a member of the larger cation diffusion facilitator family (CDF) and currently contains 7 members (ZnT-1 through ZnT-7). With the exception of ZnT-5[45], they are structurally similar having six transmembrane domains and a histidine-rich domain that is believed to play a key role in Zn binding; however, the specific mechanisms these transporters utilise to transport Zn remain unknown. The importance of optimal mammary gland Zn transfer is recognised by the early death from severe Zn deficiency of pups suckled from dams exhibiting a nonsense mutation in the Zn transporter ZnT-4, known as the lethal milk (*lm*) mouse.[39] Although this suggests that ZnT-4 plays an important role in facilitating milk Zn secretion, observations that milk from these mice still contains measurable amounts of Zn (~50% of normal)[46] and that pup survival can be improved by maternal Zn supplementation, indicate that the mammary gland can utilise other Zn transport mechanisms to facilitate the export of Zn into milk. Thus far, ZnT-1 is the only Zn transporter that has been implicated in cellular Zn export.[47] ZnT-1 may therefore export Zn across both the serosal and luminal membranes of the mammary epithelial cell, the cell-type responsible for the secretion of milk components during lactation.[48] ZnT-2 is expressed in the mammary gland[48] and is primarily associated with the luminal membrane, possibly exporting Zn from the cytosol into secretory vesicles.[49-50] However, the physiological significance of this vesicular Zn sequestration remains obscure.

The initial step in milk Zn secretion is Zn import from the maternal circulation into the mammary gland. Although Zip1 expression is ubiquitous, abundant expression of Zip2, Zip3 and Zip4 is tissue-specific.[51] The expression of Zip3 is restricted to tissues with an unusually high requirement

for Zn such as brain, eye, pancreas and thymus. Additionally, we have detected Zip3 expression in the mammary gland and like other Zip family members,[52-55] Zip3 is localised to the plasma membrane in mammary epithelial cells. Taken together, these data suggest that Zip3 may play a unique regulatory role in mammary gland Zn import and thus ultimately in milk Zn secretion.

We used the lactating rat as a model and determined that, similar to observations in humans,[56] the plasma Zn concentration of lactating rats increases to pre-pregnancy levels as lactation progresses. Concurrent with the increasing plasma Zn level, mammary gland Zn concentration, ZnT-1 and ZnT-2 expression increase while ZnT-4 and Zip3 expression peaks during early lactation and then declines, but remains significantly higher than during early lactation.[48] While ZnT-1 expression increases throughout lactation, the formation of two distinct ZnT-1 complexes of different size may help to explain differential cellular localisation. Interestingly, the intensity of luminal-associated ZnT-1 staining is particularly high during early lactation and declines as lactation continues. This suggests that ZnT-1 may play a significant role in mediating the transfer of Zn into milk during early lactation and that its contribution diminishes as lactation progresses. While the expression of ZnT-2 slightly increases throughout lactation and the staining intensity of ZnT-2 at the serosal membrane remains constant, the intensity of ZnT-2 staining at the luminal membrane decreases through lactation. This decline in luminal staining as lactation proceeds provides an additional mechanistic explanation for the decline in milk Zn concentration. ZnT-4 in the mammary gland is also localised to both serosal and luminal mammary cell compartments; however, its relative distribution shifts from the luminal membrane during early lactation to a more even intracellular distribution during late lactation possibly reducing its overall contribution to milk Zn secretion. The peak in ZnT-4 and Zip3 expression during early lactation also suggests that mammary gland Zn uptake and milk Zn secretion are enhanced via these transporters during early lactation and further provides a mechanistic explanation behind the decline in milk Zn levels that has been observed.

Milk Zn level is maintained over a wide range of dietary Zn intake and most studies have failed to show a positive effect of Zn supplementation on milk Zn level, despite increased plasma Zn levels. This indicates that the regulation of milk Zn secretion is tightly controlled. Some studies have observed an inverse relationship between milk Zn (which is high) and plasma Zn (which is low) in women from developing countries; however, the mechanisms the mammary gland uses to facilitate this regulation is unknown. Using the lactating rat as a model, we determined effects of low Zn intake on mammary gland Zn transporter expression at mid-lactation and

found that similar to observations in humans, although plasma Zn levels are reduced, milk Zn concentration is maintained during marginal Zn intake. Furthermore, we speculate that milk Zn level may be homeostatically maintained via decreased Zn export back across the serosal membrane into maternal circulation, as ZnT-1 expression is decreased, and increased Zn secretion into milk, as ZnT-4 expression is increased. However, this effect is dependent upon the severity of Zn deficiency as once Zn intake is further compromised, milk Zn level decreases and is associated with decreased expression of Zip3, ZnT-1, ZnT-2 and ZnT-4, suggesting a threshold to which the mammary gland can respond to adequately maintain milk Zn levels.

4.1 Regulation of Zip3 and ZnT-4 by prolactin

Within the mammary gland, the highly specialised, secretory mammary epithelial cell is responsible for the secretion of milk components and thus facilitates the transport of large amounts of Zn from the maternal circulation into milk. Differentiation of proliferating mammary epithelial cells into a fully functional, secretory cell-type is hormonally regulated and essential for preparing these cells for secretion.[57] Furthermore, once differentiated, secreting mammary epithelial cells require episodic hormonal stimulation in order to maintain the expression, production and secretion of many milk components[58] similar to the requirements for galactopoeisis[59,60]. During lactation, prolactin (PRL), primarily secreted by the anterior pituitary gland[61-63], is responsible for regulating milk protein synthesis and maintaining lactation,[64-65] and circulating PRL levels decline as lactation progresses.

As we have characterised changes in Zn transporters that occur during lactation, we aim to further understand the regulatory mechanisms which facilitate these changes. The redundancy in the mammary gland Zn transport system has led us to question the unique role each Zn transporter plays in this process. The use of gene silencing techniques has greatly aided the understanding of many complex biological processes[66] and is becoming an increasingly common tool in evaluating protein functionality and essentiality in specific cell types. Using gene silencing we reduced Zip3 expression in cultured mouse mammary epithelial cells by ~80% and subsequently decreased Zn uptake, demonstrating that Zip3 facilitates Zn import into mammary epithelial cells. Furthermore, decreased cell viability following Zip3 knock-down illustrates the biological essentiality of Zip3 in mammary epithelial cells and may reflect the unique requirement for enhanced Zn transport via Zip3 in this highly specialised cell type. As mentioned previously, PRL secretion is episodic and circulating PRL level declines

throughout lactation, and thus we speculate that PRL may play a role in mediating changes in milk Zn (as well as Fe and Cu) concentrations. To investigate the mechanisms through which PRL affects Zip3 and ZnT-4 we used cultured mouse mammary epithelial cells and observed that PRL exposure transiently stimulated both serosal Zn uptake and luminal Zn export in these cells. However, this increase in Zn transport was associated with increased ZnT-4 expression but decreased Zip3 expression indicating that increased Zn transporter protein levels was not the only explanation for the observed increase in Zn transport. Using confocal microscopy we have determined effects of PRL on Zip3 and ZnT-4 localisation in mouse mammary epithelial cells. Similar to the localisation of Zip3 in lactating rat mammary gland[48], Zip3 was localised to the plasma membrane and to a vesicular compartment of mammary epithelial cells, indicating that Zip3 may episodically facilitate mammary epithelial cell Zn import[52]. Furthermore, PRL exposure facilitates the movement of Zip3-associated vesicles towards the plasma membrane presumably increasing Zn uptake into the cell. ZnT-4 on the other hand, generally stains throughout the entire mammary epithelial cell, but stains in very tight association with a perinuclear compartment following PRL exposure.

One important question that arises is: how does PRL mediate these transcriptional, translational and post-translational effects on Zip3 and ZnT-4? PRL binds to PRL receptor and through a series of phosphorylation events can stimulate the JAK2/STAT5[67-68] and MAP kinase[69] pathways, ultimately resulting in increased nutrient transport into the mammary gland[70] and stimulated milk protein production and secretion.[71-72] Preliminary evidence indicates that PRL stimulates both Zip3 and ZnT-4 mRNA expression, although changes in ZnT-4 expression appear to be transient, and inhibition of either JAK/STAT or MAPK signalling pathways using chemical antagonists results in decreased expression suggesting that these pathways somehow participate in the regulatory control of Zn transport mechanisms. A more convoluted question is: how does PRL stimulation result in the movement of Zip3 and ZnT-4 from one cellular location to another? We have preliminary evidence that indicates that both Zip3 and ZnT4 are themselves phosphorylated and studies are currently underway to determine if this phosphorylation is altered by PRL exposure.

5. CONCLUSION

In summary, using the lactating rat as a model we have determined that milk Zn, Cu and Fe levels are regulated temporally through coordinated changes in gene expression, protein levels and localisation of mineral-

specific transporters. While milk Zn, Cu and Fe levels remain somewhat refractory to maternal trace mineral status, maternal malnutrition may have unique effects on mammary gland mineral transporters through secondary effects on hormonal signalling in the mammary gland.

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Chapter 3

MICRONUTRIENT TRANSFER: INFANT ABSORPTION

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1. INTRODUCTION

Our knowledge regarding the newborn infant's capacity to adapt when exposed to deficiency or excess of micronutrients is very limited. Infants may be born with low stores of micronutrients, due to maternal deficiency during pregnancy, and may further be exposed to a low intake of micronutrients, either from breast-milk or from weaning foods low in micronutrients or with low bioavailability. On the other side of the spectrum, infants may be exposed to micronutrient supplements, provided in an effort to counteract perceived deficiencies. In adults, homeostatic regulation of intestinal absorption of micronutrients, such as iron (Fe), copper (Cu) and zinc (Zn), is well developed and up- and down-regulation of absorption occurs. Whether such homeostatic regulation occurs in newborn infants is not known, or, if absent at birth, when it develops.

We believe that in order to understand homeostatic regulation of intestinal absorption in infants, observational studies in infants need to be coupled to studies in animal models and human intestinal cells in culture. Animal models are needed to explore, at a molecular level, how various absorptive and excretory mechanisms respond to micronutrient deficiency and excess, and how they develop with age. The animal models used need to be validated, i.e. do the newborn animals respond in a way similar to that of

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human infants with regard to absorption (which can be evaluated in both species)? In animal models, gene and protein expression can be measured at the tissue level, and localisation of transporters in response to micronutrient deficiency and excess can be determined by immunohistochemistry. Such studies cannot be done in human infants. Cell studies can complement animal studies in several ways: a) human cells (eliminating species-specific differences) can be used, b) localisation and movement of transporters can be followed in live cells by confocal microscopy (which is difficult, if not impossible in animal models), and c) cellular responses, rather than tissue or whole body responses, can be studied, allowing the investigator the opportunity to observe, at the cellular level, how various mechanisms respond to nutrient deficiency and excess. These three types of studies, in combination, are likely to advance our knowledge about the development of trace element homeostasis in infants and also about the long-term consequences with regard to programming of mechanisms involved in up- and down-regulation of trace element absorption and excretion.

2. IRON

The use of animal models to explain observations made in human infants can be exemplified by two recent sets of studies.[1-4, 5-6] Breast-fed infants in Honduras and Sweden who were given iron supplements (1 mg/kg/d) from 4 months of age showed an increase in haemoglobin concentration at 6 months of age, which occurred regardless of initial iron status (Hb > 115 g/L or < 115 g/L at 4 months). However, at 9 months of age, there was a significant and robust increase in haemoglobin in infants who had low haemoglobin at 6 months of age, whereas there was no change in infants having normal haemoglobin levels. This suggested that iron homeostasis was immature in young infants (4-6 months of age), whereas older infants showed an appropriate homeostatic response to iron supplements. By using stable isotopes of iron, we could determine that iron absorption from both ferrous sulfate and breast-milk was similar in iron-supplemented and unsupplemented breast-fed infants at 6 months of age, while iron absorption at 9 months was considerably higher in the unsupplemented infants as compared to supplemented infants (45 % vs 21 %). Thus, the capacity of the infant to regulate iron absorption was not present at 6 months of age.

The inability of the young infant to regulate iron homeostasis can also lead to adverse effects. We found that Swedish infants given iron supplements had significantly lower length gain than unsupplemented infants, whereas this was not observed in Honduran infants. However, when the Honduran infants were divided into those who originally had adequate

iron status and those who did not, iron supplementation had a significant negative effect on length gain in those who originally were iron sufficient. Furthermore, iron supplemented infants at both sites showed significantly lower copper (Cu) status as assessed by the activity of red blood cell Cu, Zn-superoxide dismutase (SOD). Lower copper status may in itself cause potential adverse effects, but the lower Cu, Zn-SOD activity may also reflect a decreased capacity to respond to free radical mediated events, possibly induced by excessive iron intake. Taken together, it is obvious that iron supplements given to iron replete infants may cause adverse effects due to the immaturity in iron homeostasis.

The mechanisms responsible for the differences in iron homeostasis observed at different ages were explored in a rat pup model. Similar to human infants, exclusively breast-fed rat pups were given daily iron supplements at levels (on a body weight basis) similar to those given to human infants. We found that iron absorption and tissue iron uptake were similar in control and iron supplemented rat pups at day 10 ("young infants") and higher than at day 20 ("older infants"). This higher absorption also led to higher intestine and liver iron (which we could not assess in human infants) and haemoglobin, illustrating the inability of young rat pups to regulate iron absorption and the similarity to observations in human infants. The primary iron transporters regulating iron absorption in the small intestine are divalent metal transporter 1 (DMT-1) and ferroportin (FPN) (Figure 3-1).

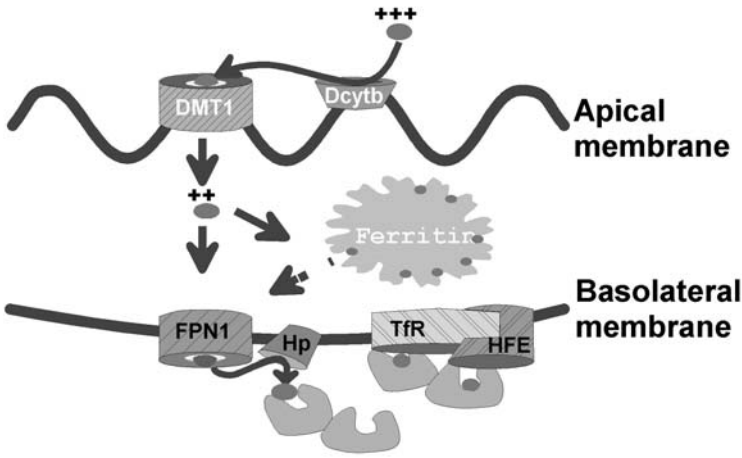

Figure 3-1. Schematic picture of factors regulating iron metabolism in intestinal cells. (DMT-1: divalent metal transporter 1; FPN1: ferroportin-1; Dcytb: D cytochrome b; Hp: hephaestin; TfR: transferrin receptor; HFE: haemochromatosis gene product).

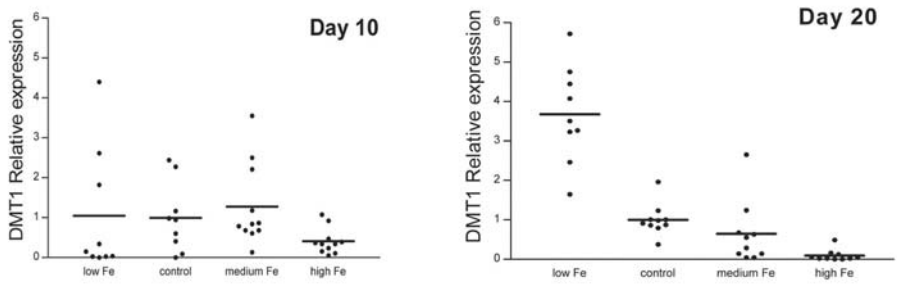

Figure 3-2. Expression of DMT-1 in small intestine from iron-deficient rat pups and pups given daily iron supplements (adapted from refs. 5 and 6).

We measured DMT-1 and FPN mRNA and protein expression in the small intestine of the rat pups and found no significant difference on day 10, whereas both DMT-1 and FPN were down-regulated in iron-supplemented pups at day 20 (data for DMT-1 shown in Figure 3-2).

In adult animal models (and in cells in culture) it has been documented that iron supplementation down-regulates DMT-1 and FPN expression and it thus appears that young infants lack this capacity. Similarly, we found no differences in DMT-1 and FPN expression in rat pups that were iron deficient (due to maternal iron deficiency) at day 10, whereas expression of both DMT-1 and FPN was significantly up-regulated at day 20. This shows that iron homeostasis is immature in young rats and that this is due to an inability of the small intestine to adequately respond to iron deficiency or excess. It is therefore likely that young infants are unable to appropriately respond to iron deficiency and excess, respectively, by up- and down-regulating intestinal absorptive mechanisms for iron.

Breast-fed infants may also acquire iron via an additional mechanism. A significant proportion of iron in breast-milk is bound to lactoferrin[7], an iron-binding protein capable of binding two atoms of ferric iron per molecule. Lactoferrin is remarkably resistant against proteolytic digestion and has been found in the faeces of breast-fed infants.[8] A specific receptor for lactoferrin has been found in the brush border membrane of human infant small intestine, and this receptor facilitates the uptake of iron by human intestinal cells (Caco-2) in culture.[9] Caco-2 cells cultured in low iron medium have been shown to have enhanced uptake of iron from human lactoferrin, suggesting homeostatic regulation. However, there have been no studies in human infants (or in animal models) on the development of iron absorption from lactoferrin with age or their ability to respond to infant iron status at different stages of development.

3. COPPER

Newborn infants can be exposed to highly variable copper intake.[10] Breast-milk is relatively low in copper, 0.2-0.3 mg/L, while most infant formulas contain 0.4-0.6 mg/L. Formulas intended for prematurely born infants often contain even higher levels of copper, 1-2 mg/L, and powdered formulas (most common type world-wide) made up with water from copper pipes or water naturally high in copper can sometimes contain even higher copper levels. The ability of the infant to homeostatically adapt to such varying copper intakes is not known. While overt copper toxicity only has been reported in rare cases, the existence of subclinical toxicity or interactive effects on iron or zinc absorption and status cannot be ruled out. It is also possible that copper absorption can be down-regulated to protect against excessive accumulation of copper; however, the ability of infants to regulate copper absorption is not known.

Copper absorption is high during early infancy in both humans and rats.[10-13] In fact, copper absorption in young rat pups appears linear, while older mammals show saturable absorption, suggesting developmental regulation of absorptive mechanisms.[13] Several cellular copper transporters (Ctr1, ATP7A and ATP7B) have recently been characterised.[14-15] In the apical membrane of the small intestine enterocyte, Ctr1 (copper transporter 1) appears to be the major copper importer, whereas ATP7A (copper-pumping ATPase, "Menkes protein") is the predominant copper exporter in the basolateral membrane (Figure 3-3). Additionally, ATP7B (also a copper-pumping ATPase, "Wilson's protein") is localised to the apical membrane suggesting it plays a role in copper export back into the intestinal lumen. A genetic defect in ATP7A has been shown to cause Menkes disease, a disorder of severe copper deficiency resulting from lack of transport of copper from the small intestine to the liver. A genetic defect in ATP7B causes Wilson's disease, which is manifested by copper toxicity resulting from over-accumulation of copper in the liver and other tissues due to defective transport of copper into bile.

In rat pups, similar to human infants, serum copper and ceruloplasmin increase with age, whereas copper absorption is lower at 20 days of age than at day 10. We have shown that Ctr1 expression increases during infancy, whereas ATP7A increases sharply during early infancy and then decreases through weaning. ATP7B and metallothionein (MT) show relatively little developmental change in breast-fed (control) rat pups. [16]

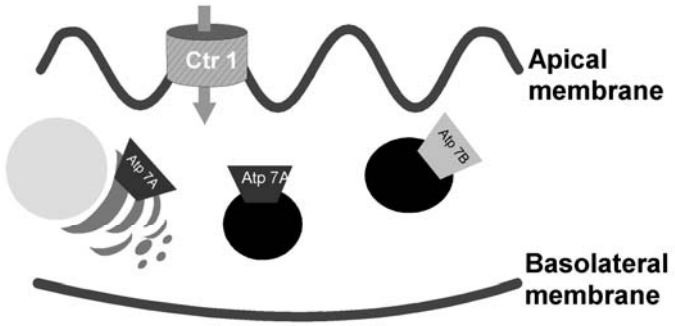

Figure 3-3. Schematic picture of factors regulating copper metabolism in intestinal cells.

When given modest doses of oral copper daily (similar on a body weight basis to levels that human infants may be exposed to), young infant rat pups (day 10) had higher small intestine copper compared to controls (Figure 3-4). While this was associated with increased MT expression, there was no change in the expression of the copper transporters Ctr1, ATP7A or ATP7B. In contrast, in older infant rat pups (day 20), copper-supplementation did not affect small intestine copper concentration; however, ATP7A expression increased considerably, suggesting that copper could now be exported from the small intestine.[17] Biliary excretion of copper is immature during early life in both human infants and rat pups and then increases as bile excretion matures. It is thus likely that the body can protect itself against copper excess in late infancy by absorbing less, and excreting more by reducing intestinal accumulation of copper. At younger ages, however, the body may attempt to protect itself by accumulating more copper in the small intestinal cell (for loss by sloughing). While this appears to be an appropriate protective response, we do not know whether this intestinal accumulation of copper, mediated by MT, can cause any adverse effects. High cellular copper has been shown to cause pro-oxidative events, but it is not known whether levels that infants can be exposed to may trigger such events. Caco-2 cells exposed to high copper levels reduced metabolically active cell number and increased apoptosis, whereas moderately elevated copper levels did not elicit these effects (unpublished observations). In our rat pup study, the pups were able to down-regulate intestinal copper at day 10 when given the moderately high level of copper, but not when the higher level was given, suggesting that there may be a threshold level, beyond which the protective mechanisms are inadequate.

Studies in human infants[18] have shown that chronic exposure to a copper level in water of 2 mg/L from 3 to 12 months of age did not cause any adverse or toxic effects (assessed by morbidity, serum biochemistry and liver function tests), suggesting an adaptive response to the higher copper intake.

However, this level (2 mg/L) is the World Health Organization Provisional Guideline Value for copper content of drinking water. Whether infants can adapt to higher intakes of copper is not yet known, although there certainly is a limit as evidenced by copper toxicosis in children resulting from copper contamination of milk from brass vessels.[32]

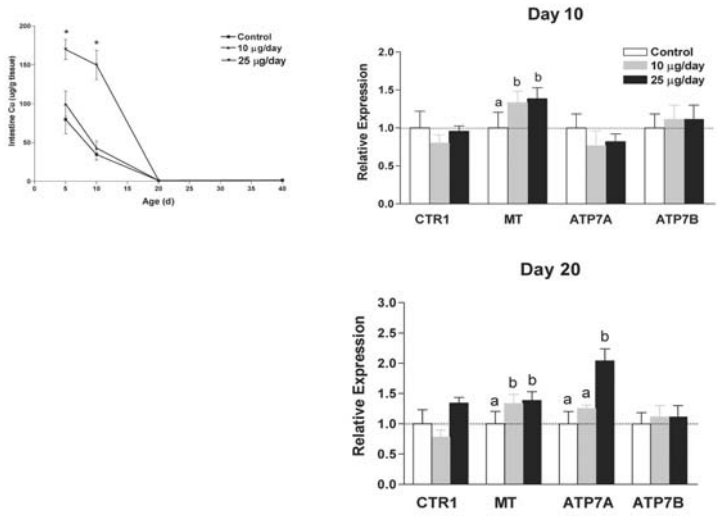

Figure 3-4. Small intestine copper concentration and expression of CTR1, MT, ATP7A and ATP7B in rat pups exposed to two levels of daily copper supplementation (different letters denote statistically significant difference, $p<0.05$).

4. ZINC

In adults, zinc absorption is negatively correlated to zinc intake[19], and presumably zinc status, although the latter is difficult to assess. In infants, however, zinc intake can also be variable, but zinc absorption appears to be positively correlated to zinc "status", and is primarily affected by zinc excretion (endogenous loss).[20-21] It has become increasingly recognised that zinc deficiency (or suboptimal status) is common in infants and children[22] and various programs for zinc supplementation and fortification have been instituted.[23,24] It is not known, however, how well infants can cope with increased zinc intake, particularly, in light of what we previously described for iron, infants originally having adequate zinc status. Several intestinal zinc transporters have been discovered recently, but their ability to respond to zinc deficiency and excess during infancy is largely unknown.

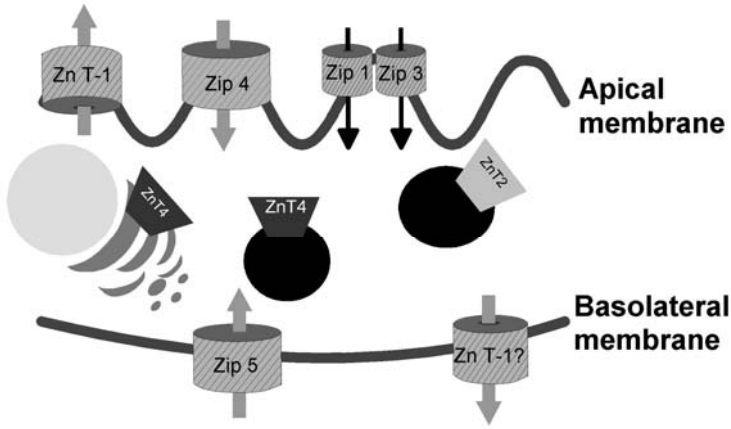

Figure 3-5. Schematic picture of factors regulating zinc metabolism in intestinal cells.

The major importer of zinc across the apical membrane into the small intestinal enterocyte is Zip4 [25-26], whereas ZnT1 is speculated to export zinc back across the apical membrane into the intestinal lumen (Figure 3-5).

ZnT2 and ZnT4 are primarily located intracellularly and are believed to transport zinc between and within intracellular vesicles.[27-28] However, as ZnT4 co-localises with transferrin receptor in the serosal membrane, ZnT4 may play a role in mediating Zn efflux into the circulation.[26] In newborn rats, Zip4 expression increases throughout early infancy (day 10) and then remains high into adolescence, whereas ZnT1 is absent during early infancy and is not appreciably expressed until weaning (day 20). ZnT4 peaks in early infancy, but rapidly decreases by weaning when it reaches adult levels. ZnT2 increases throughout infancy and continues to rise through adolescence.

Zinc deficiency in rat pups (induced by moderate or mild maternal zinc deficiency during pregnancy and lactation) does not affect intestine, plasma or liver zinc levels, suggesting that the rat pup can homeostatically adjust to mild zinc deficiency. ZnT1, which is only appreciably present at day 20, decreased in pups born to zinc deficient dams, possibly in an attempt to reduce zinc excretion/endogenous losses. Zip4 was markedly increased in rat pups born to zinc deficient dams at day 5 and 10, most likely to enhance the amount of zinc taken up by the enterocyte. ZnT4 was moderately increased during mid-infancy (day 10), possibly to ensure adequate zinc transfer across the serosal membrane into circulation, while it was decreased during weaning (day 20) when Zn intake from solid foods starts to increase. ZnT2 was unaffected by zinc deficiency illustrating its role in zinc sequestration only during zinc excess.

Zinc supplementation of control pups and pups born to dams exposed to mild or moderate zinc deficiency had unique yet pronounced effects. During mid-infancy (day 10), zinc supplementation regardless of initial zinc status increased small intestine, plasma and liver zinc, whereas at late infancy (day 20), only pups born to zinc deficient dams had higher intestine, plasma and liver zinc concentrations, suggesting an inappropriately strong response in zinc homeostatic mechanisms. The expression of several zinc transporters was dramatically affected and their cellular localisation also changed in response to zinc supplementation (Figure 3-6 – ZnT4 presented as an example).

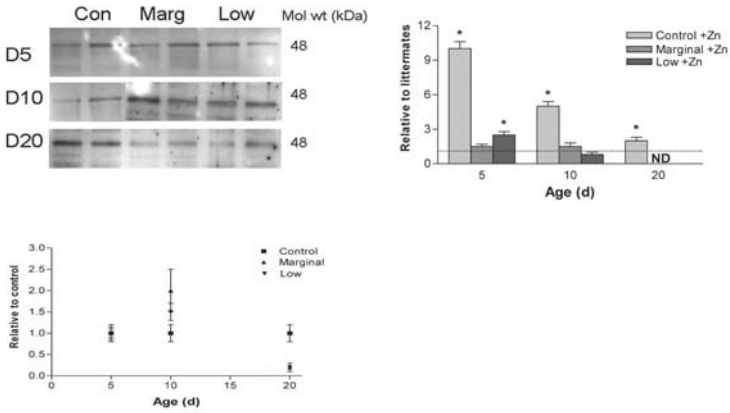

Figure 3-6. Expression of ZnT4 in rat pup small intestine during zinc deficiency (marginal and low – left panels) and following zinc supplementation (right panel)

In newborn rat pups, zinc supplementation decreased Zip4 expression in control pups but not in previously zinc-deficient pups. During mid- and late infancy, Zip4 regulation appears to be post-translational in that a large proportion of Zip4 was endocytosed in zinc-supplemented rat pups suggesting that zinc import was decreased. As mentioned above, ZnT1, the major zinc exporter in the small intestine is not expressed in young rat pups, possibly putting them at risk for zinc toxicity. Zinc supplementation increased ZnT1 in control pups at day 20, possibly in an attempt to protect against excess zinc. However, this did not occur in pups born to zinc deficient mothers, perhaps minimising zinc excretory mechanisms during zinc deficiency. Using immunohistochemistry we observed that zinc supplementation concentrated ZnT4 to punctate intracellular vesicles. This in combination with increased (~10-fold during early infancy and ~5-fold during mid-infancy) ZnT4 expression in control pups, may function to sequester zinc within the intestine and avoid further transport to the liver.

This effect was not observed in pups that were born to zinc deficient dams. Similarly, zinc supplementation increased ZnT2 expression in all groups, although the increase was much higher in control pups. Similar to ZnT4, the localisation of ZnT2 was concentrated to very large punctate vesicles in zinc supplemented rat pups. Thus, it appears that zinc import via Zip4 is decreased by zinc supplementation during early life, and that this occurs until excretory mechanisms (ZnT1) develop. Zinc sequestration (via ZnT2 and ZnT4) occurs during excess zinc intake, particularly in infants that are previously not zinc deficient.

It is not yet known how zinc interacts with iron at the intestinal level, but it has been shown that zinc can inhibit iron absorption when the two micronutrients are given together in water solution.[29] Similarly, when Indonesian infants were given oral supplements with zinc and iron for 6 months, the beneficial effects of iron on haematology and behaviour were eliminated.[30-31] Since zinc and iron are not likely to cross the apical membrane via the same transporters (DMT-1 and Zip4, respectively), it is possible that the intestinal sequestration of zinc (discussed above) may interfere with intracellular trafficking of iron. Further research on factors regulating intestinal iron and zinc metabolism is clearly needed.

5. CONCLUSION

Based on our studies on human infants, rat pups and cells in culture, it appears that some caution is warranted with regard to trace element supplementation of infants. Young infants seem to have difficulties responding appropriately to iron status and may therefore be at risk for iron toxicity. It appears that infants can tolerate moderate levels of copper excess, but higher levels may induce copper toxicity. We do not yet know at what level of copper excess this may occur. While we know less about the consequences of zinc supplementation of human infants, our rat studies suggest that the intestine may have inadequately regulated zinc homeostasis, particularly if their zinc status has been compromised during early life. Further, although elaborate mechanisms are present in infants to regulate zinc homeostasis, they may be inadequate during long-term deficiency. Finally, trace element intake and status can have profound effects on trace element transporters, which may also last long after the deficiency or excessive intake occurred.

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Chapter 4

DEVELOPMENTAL ASPECTS OF THE MUCOSAL IMMUNE SYSTEM: ROLE OF EXTERNAL ENVIRONMENT, MUCOSAL MICROFLORA AND MILK

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1. INTRODUCTION

The mucosal surfaces of the upper airway, the lower respiratory, and the gastrointestinal tracts are constantly exposed to an overwhelming and often changing spectrum of environmental components, including, microbial organisms, dietary constituents, and a variety of chemical and other toxic macromolecules. However, a symbiotic relationship between mammalian mucosal surfaces and the external environment has evolved over millions of years of biological, and more recent societal and cultural evolution in an elegant manner, which is by and large still conducive to the survival of the mammalian species, and at the same time designed to retain the integrity of the environmental ecosystem.

This report will briefly review the recent developments in immunological aspects of host mucosal surfaces, and their interaction with environmental factors, notably, microflora, dietary components and maternal products of lactation delivered to the mammalian infant via the process of breast-feeding. The important components of mucosal immunologic defence are listed in Table 4-1.

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Table 4-1. Components of mechanisms of defence in mammalian mucosal sites

A. Innate Immunity
Pathogen recognition receptors
secreted
endocytic
signalling
Antimicrobial peptides
Phagocytic cells
Neutrophils
Alternate pathway of complement activation
Epithelial barrier proteins
B. Adaptive Immunity
Mucosal immune system
T and B cells and their subsets
Chemokines and cytokines
Mucosal microflora and mucosal microenvironment
Commensals
Pathogens
Other macromolecules

2. THE HOST

2.1 Mucosal immunological development

The complex organisation of the mucosal epithelium and the lymphoid structures associated with respiratory, gastrointestinal and genital tracts, the ocular tissues, the mammary glands, and the products of lactation is generally referred to as the mammalian common mucosal immune system. The epithelial surfaces of many mucosal sites have evolved unique features in order to function as an anatomical barrier as well as a distinct site for specific immunological defences. The major immunological mechanisms operating at mucosal sites include the structural and functional elements of the innate and adaptive immune systems.

2.2 Innate mucosal immunity

The effector mechanisms involved in the expression of innate immunity include several antimicrobial peptides, phagocytic cells, neutrophils, and alternative pathway of complement activation. The innate immune responses represent the first line of host interaction with mucosal microflora and other environmental pathogens. It should be recognised that the mucosal

microflora is extremely heterogeneous representing many species of bacterial, viral, fungal and parasitic agents. Many bacterial organisms can mutate at much higher rates than the mammalian host cells. As a result, the host mucosa does not recognise every possible antigen, but only a few, highly conserved cellular or soluble components of microorganisms, known as pathogen-associated molecule patterns (PAMP). Microbial products such as bacterial lipopolysaccharides (LPS), lipoteichoic acid (LTA), peptidoglycan, mannans, bacterial DNA, and double stranded RNA represent important PAMP, identified to date. PAMP are only produced by the microorganism, not by their host. They are essential for the survival of the microorganisms and represent structures shared by an entire class or a family of microbial agents.[1]

The host structures of the innate immune system which recognise the PAMP are generally referred to as pathogen-recognition receptors (PRR). They are genetically predetermined (germ-line encoded) receptors which appear to have evolved by natural selection with defined specificities for infectious microorganisms. It has been proposed that the total number of PRR is relatively low (in hundreds). These receptors are expressed on many mucosal effector cells especially on the antigen-presenting cells, the macrophages, dendritic cells and B-cells.[1] The PRR belong to several structural protein families. These include leucin-rich repeat domain, calcium-dependent lectin domain and scavenger-receptor protein domains which are selectively involved in microbial pattern recognition.[2] The PRR have been classified, based on their functional characteristics as secreted, endocytic and signalling. The secreted PRR include mannan-binding lectins and function as opsonins and initiate the lectin pathway of complement activation. The endocytic PRR are present on phagocytic cell surface and mediate the uptake and delivery of microbial pathogens into the lysosome with eventual lysis of the pathogen. Such PRR are also important in antigen processing and presentation by major histocompatibility complex (MHC) molecules on the surface of macrophages. The signalling PRR recognise specific PAMP and activate signal transduction pathways important in the induction of several immune response genes. The best characterised members of this group include the family of Toll–like receptors (TLR). It has been recently demonstrated that the sequence of the extracellular domain of the Toll protein is remarkably similar to the cytoplasmic domain of the interleukin-1 receptor (IL-1R). Both Toll proteins and IL-IR induce signal transduction leading to the activation of transcription factors in the NF-Kβ family.[2] To date, at least 10 toll-like receptors have been identified in man. TLR-4 and TLR-2 have been studied extensively. The TLR-4 complex involved in the recognition of lipopolysaccharide PAMP appear to have at

least 3 components CD14, MD-2 and TLR-4. CD14 is a receptor on macrophage and B cells and is possibly recruited after LPS binding. MD-2 is consistently associated with TLR-4 and is required for TLR-4 mediated LPS recognition.

In addition to its activation by PAMP, recent studies have demonstrated the activation of TLR-4 by respiratory syncytial virus as well as by other pathogens. It is now clear that several members of TLR family especially, TLR-4 and TLR-2 are intimately involved in the regulation of proinflammatory and immunoglobulin cytokine gene expression. These include IL-1β, IL-6, TNF-α, IL-8, and GM-CSF. A variety of TLR are expressed on macrophages, dendritic cells, epithelial cell, corneal epithelium, mast cells, lungs and intestine. TLR-2 and 4 are widely expressed in many mammalian tissues. Recently, it has been shown that expression of CD80 and CD86 molecules on the surface of antigen presenting cells is also controlled by the innate immune system. Receptors such as TLR induce these molecules on antigen presenting cells when they recognise PAMP. Recognition of an antigen presenting cell in the absence of CD80 or CD86 molecules appears to result in apoptosis or inactivation of the T cell.[2-5]

Available evidence suggests a strong role for innate immunity in the mechanism of mucosal defence against pathogenic bacteria, release of inflammatory cytokines and development of adaptive immune responses. Thus innate immune mechanisms, especially TLR appear to provide the host mucosa with unique abilities to discriminate between different pathogen-associated molecular patterns, and at the same time contribute to the development of antigen-specific T and B cell responses.

2.3 Adaptive mucosal immunity

The organised lymphoid follicles in the gastrointestinal and bronchial subepithelial regions are considered to be the principal inductive sites of mucosal immune responses. It is also clear that under certain circumstances, nasopharyngeal tonsils, appendix, peritoneal precursor lymphoid cells and rectal lymphoepithelial tissue (rectal tonsils) may also serve as inductive sites of local immune responses.[6-7]

The common features of all inductive mucosal sites include an epithelial surface containing M cells overlying organised lymphoid follicles. Their ultrastructural and functional characteristics were extensively defined in the early 1970s. Mucosal epithelium is a unique structure and in addition to M cells, it contains mucin producing glandular cells, lymphocytes and plasma cells, dendritic cells and macrophages. The mucosal epithelial cells express polymeric immunoglobulin receptor (PigR) and secretory component, MHC

class I and II molecules, other adhesion molecules, and a variety of cytokines and chemokines. The dendritic cells are present both in the organised lymphoid tissues and the mucosal epithelium. These cells have been strongly associated with potentiation of the immune response, enhanced antigen capture via induction of TLR, and development of active immunity. Other studies have suggested that dendritic cells may also enhance the induction of mucosal tolerance in *in vivo* settings. Recent observations have suggested that dendritic cells are most potent antigen-presenting cells (APC) and are critical in initiating primary immune responses, graft rejection, autoimmune diseases, and generation of T cell dependent-B cell responses. The APC function is attributed in part to their ability to express co-stimulatory molecules (CD80, CD86), and TLR, and other accessory ligands necessary for induction of immune response or induction of tolerance.[2, 4-5]

The M cells are important in luminal uptake, transport, processing and to a smaller extent in the presentation of mucosally introduced antigens. The M cells appear to be critical in transport of luminal antigens, and entry of organisms such as reovirus, poliovirus, rotavirus and salmonella into the human host. M cell-mediated antigen uptake is characteristically associated with the development of secretory IgA responses (S-IgA).

The luminal appearance of S-IgA in mucosal secretions results from transcytosis of polymeric IgA (pIgA) across mucosal epithelia via binding to the pIgR Receptor (pIgR). The receptor is eventually cleaved resulting in association of pIgA with a substantial part of the pIgR receptor. The complex of IgA and pIgR is generally referred to as S-IgA.

Following exposure to an antigen and its uptake via the M cells, there is a variable degree of activation of T cells, dendritic cells, and B cells, especially of the IgA isotype. The interaction of lymphocytes with mucosal epithelia is important in differentiation of some segments of mucosal epithelium into M cells. Activation of T cells results in the release of a number of distinct cytokines or chemokines from different T cell subsets, and recognition of antigenic epitopes involving MHC class 1 or 2 molecules. Both T cell activation, and release of specific cytokines are involved in the eventual process of B-cell activation, isotype switch, and specific integrin expression on antigen-sensitised B-cells. Both Th1 and Th2 cells appear to benefit the development of S-IgA responses. Th2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13) are thought to be of significant help in antibody production. Secretory IgA antibody response is also enhanced by immunologic adjuvants such as cholera toxin which results in polarised Th2 cell response.[8] Secretory IgA antibody response may also be induced

through Th1 cytokines (IL-2, IFNγ) as shown with studies on intracellular pathogens such as salmonella.

It appears that the process of isotype switching of B cells to polymeric IgA producing plasma cells begins in the mucosal inductive sites. Such switching requires specific signals by co-stimulating molecules including cytokines and T helper cells. However, Th1 and Th2 type cytokines appear to contribute only minimally to the switching to surface IgA positive B cells. Such switching is greatly enhanced by TGF-β. Following activation and acquisition of antigen specificity, the IgA producing cells migrate to the lamina propria of the effector sites in the mucosal tissues, regardless of the site of initial antigen exposure. There is, however, a preponderance of homing to the original site of antigenic exposure. The migration of antigen-sensitised cells is preferentially determined by the concurrent expression of integrins and homing-specific adhesion molecules in the tissue endothelium, especially mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and the specific receptors (integrins) expressed on the activated lymphoid cells. Oral (intestinal) mucosal exposure to antigen seems to favour expression of α4β7 integrins, and intranasal immunisation has been shown to induce expression of L-selectin as well as α4β7 integrins. However, systemic immunisation is generally restricted to the expression of L-selectin. The antigen-sensitised B cells undergo terminal differentiation in the mucosal lamina propria to IgA-producing plasma cells. Such differentiation involves interaction with a variety of cytokines and T cell subsets.[6-7] Recently, an alternative pathway has been proposed for the switch of IgM B cells. It has been suggested that IgM B cell in the lamina propria can switch to production of IgA isotype without the need for T cell help. [9]

Locally produced IgA consists mainly of J chain-containing dimers and the larger polymeric-IgA that is selectively transported through epithelial cells by the polymeric Ig receptor. The resulting secretory IgA molecules are designed to participate in immune exclusion and other immunological functions at the mucosal surface. IgG also contributes to such surface defence. It often reaches the secretions by passive diffusion from the bloodstream and, less frequently, by local synthesis. However, its proinflammatory properties render IgG antibodies of potential immunopathological importance when IgA-mediated mucosal elimination of antigens is unsuccessful. T helper cells activated locally mainly, by a Th2 cytokine profile, promote persistent mucosal inflammation with extravasation and priming of inflammatory cells, including eosinophils. This development may be considered as ‘a pathologic enhancement of local defences’. It appears to be part of the late phase allergic reaction, perhaps initially driven by interleukin 4 (IL-4) released from mast cells subjected to

IgE-mediated or other types of degranulation, and subsequently maintained by further Th2 cell stimulation. Eosinophils are potentially tissue-damaging, particularly after priming with IL-5. Various cytokines up-regulate adhesion molecules on endothelial and epithelial cells, thereby enhancing accumulation of eosinophils and, in addition, resulting in aberrant immune regulation within the epithelium.[6-7] It would seem that soluble antigens available at the epithelial surfaces normally appear to induce various immunosuppressive mechanisms, but such homeostasis seems to be less potent in the airways than the induction of systemic hyporesposiveness to dietary antigens operating in the gastrointestinal tract. Numerous cytokines and chemokines have been shown to be intimately involved in the induction and maintenance of mucosal immune responses and the level of mucosal inflammation during infections and exposure to environmental agents. [6-7]

3. THE ENVIRONMENT

3.1 Microbes, diet and lactation

Many life forms including all mammalian species are permanently colonised by other microbial organisms. All human beings virtually live on or within a "sea of other microbes." The magnitude of mammalian host-environmental microbe interaction is best illustrated by the following observations. It is estimated that about 4600 species of prokaryotic microorganisms reside in each gram of natural soil, and microorganisms represent about 700-7000 grams of biomass/per cubic feet of soil. The overall density of microbes on earth is estimated to be in the range of 10^{29} organisms. It is remarkable to note that the mucosal surface of the adult human gastrointestinal tract alone is believed to contain over 100 trillion organisms, representing over 400 different species. On the other hand, the adult human is composed of only about 10 trillion human cells, representing about 200 human cell types.[10] It is apparent that for every human cell, there may be as many as 10 non human, (mostly bacterial) cells residing in or on a normal adult subject throughout its lifespan (Table 4-2). The longstanding physical relationship established between bacteria and the mammalian species have significantly contributed to the evolution of the mammalian, (including the human) genome. This is reflected by the fact that significant homology exist between bacterial (but not eukaryotic) proteins and the 223 proteins encoded by the human genome. It has been proposed that bulk of

the genetic information in man may have been acquired thorough horizontal transfer of bacterial genes.[11]

Table 4-2. Cellular composition of a human adult

Cellular Components	Number
Total no. of cells	>110 trillion
No. human cells	~10 trillion
No. cell type	~200
No. non-human (bacterial cells)	>100 trillion
No. cell types	>500
Ratio of bacterial vs. human cells in human ecosystem	10:1

Most bacterial species residing in human mucosal surfaces co-exist peacefully with their host and remain harmless, with little or no evidence of systemic translocation or disease. Over 90% of the total bacterial load in human gut is comprised of 30-40 bacterial species, with anaerobic bacteria being the most predominant. At birth, the neonate is exposed initially to the flora of maternal gastrointestinal and genital tracts, with initial colonisation by members of enterobacteriacae. By the end of the first week of life, breast-fed infants acquire a flora comprised primarily of bifidobacteria which may exceed other organisms by a ratio of 1000:1. On the other hand, formula-fed infants remain colonised by enteric bacteria and additionally, acquire bacteroides, clostridia, lactobacillus and occasional bifidobacterial species. The load of bifidobacteria is often less than 10% of the colonisation observed in breast-fed infants. During the first year, after the introduction of solid foods, the microbial flora of the gut is predominantly made up of anaerobic gram negative organisms especially bacteroides. By the second year of life, mucosal microbial profile changes to more adult-like flora, with increased load of bacteriodes, other anaerobic and gram negative organisms, and a decreased load of coliforms, clostridia and streptococcal species.[12] The resident and more stable (permanent) microbial flora can be replaced by transient and often pathogenic microflora acquired from external environment at any age. The factors associated with alteration in the permanent resident flora in the human mucosal surfaces include infections, local inflammation, malnutrition, immunosuppression, use of antibiotics, chemotherapy and radiotherapy, stress, and significant alterations in dietary habits. Such transient microflora can be successfully eradicated by recolonisation by the permanent flora.[10-12]

The relationship between mucosal microflora, diet and mucosal immunological homeostasis is currently an area of extensive investigation. The association of breast-feeding with the development of gut flora characterised by the heavy colonisation with bifidobacteria has generated considerable interest in the use of probiotics. These microorganisms detected

initially in fermented food, include species of bifidobacteria, lactobacilli, non pathogenic yeasts, saccharomyces boulardi, and some enterococcal species. [13-14]

Dietary components have been shown to play an important role in the nature and acquisition of mucosal microflora. Certain classes of foods have been termed as protective nutrients and may function to stimulate the growth of probiotics and enhance development of the mucosal immune response. These include glutamine, arginine, zinc, vitamin A, and several non-digestible food ingredients collectively referred to as prebiotics. The inulin, and fructose oligosaccharides of human milk, fructo-oligosaccharides from plants, and lactose-based galactooligosaccharides resembling those of human milk have been studied recently.[13] Their mechanisms of action appear to be multifaceted. Limited data available to date suggest that such nutritional supplements may significantly influence the growth of probiotic microorganisms in the human mucosal surfaces and may provide a protective influence on the expression of allergic disorders in later life. [14-15]

Recent investigations have examined the possible role of maternal diet on breast-feeding infants, relative to intrauterine similization, or sensitisation via breast-feeding on the outcome of food allergy in infancy. Some beneficial effects after maternal elimination diets during lactation have been observed for atopic eczema and food allergies. However, other investigations have failed to demonstrate any significant benefits from maternal dietary interventions during pregnancy.[16]

The biological characteristics, and development and function of milk and other products of lactation; the primary focus of this conference, have been reviewed in other chapters of this volume, and will not be discussed here. However, it is important to emphasise the role of the common mucosal immune system in the development of immunity in the mammary glands and milk. Human colostrum and milk derive most of their secretory IgA antibody activity, and possibly other immunoglobulin, as well as T cell reactivity, secondary to exposure to infectious and other environmental agents in the maternal mucosal inductive sites. It is generally felt that maternal milk delivers the entire spectrum of mucosal immunological experiences of the mother to the neonate via the process of breast-feeding. [17]

3.2 Host mucosa-environmental interactions

A large number of innate and adaptive immune mechanisms, as well as environmental factors are critical in the development or lack of adequate immune response after exposure to an antigen, as outlined in Table 4-3.

Based on the information summarised in the preceding sections of this chapter, it is apparent that the human immune system has evolved with significant input from external environmental factors, including microbial and other environmental antigens, and is able to recognise any antigenic structure. The human genome contains about 10^5 genes and most have little or no role in immune recognition. On the other hand, it is estimated that there are approximately 10^{14} immunoglobulin and about 10^{18} T cell receptors.[2, 11, 18] These receptors are generated somatically and are encoded in the germ line. Thus, they are not destined to recognise any one antigen, and a diverse spectrum of B and T cell receptors is generated randomly and expanded clonally after exposure to an antigen. These receptors interact with bacterial, viral, other environmental agents, as well as self antigens. In contrast, the receptor repertoire in the innate immune system is limited to the recognition of only select components of infectious agents, and to signal the presence of an infectious agent. Such signals in turn control, via the induction of co-stimulatory molecules, cytokines and chemokines, the activation of antigen specific T and B cell-mediated adaptive immune responses. Thus innate immunity at the mucosal surfaces plays a critical role in maintaining competence of the mucosal immune system and at the same time in down-regulating expression of inflammatory cytokines associated with mucosal inflammation, asthma, and other autoimmune disease processes.[2] Recent observations of increased susceptibility to several bacterial infections associated with mutational inactivation of certain components of the TLR and IL-IR receptors, following mutations in macrophage-mannan receptors, and mannan binding lectin, respectively in human and experimentally induced settings, support the role of such innate mechanisms in the natural history and outcome of mucosal disease.[2] Clearly, the mucosal microflora in the first year of life is highly influential in determining the outcome of subsequent innate and adaptive immune responses. It has been suggested that probiotic (commensal) flora of the gut interact with gut epithelium and fortify the mucosal barrier via induction of decay accelerating factor (DAF), complement reactive protein and ductin, a factor which facilitates repair. The IgA response to pathogens is in general T cell dependent, but to the probiotic commensals the development of secretory IgA is thought to be T independent.[11] This may allow the host to respond to a shift towards colonisation by commensal flora, without specific immune responses. Recently it has been shown that intestinal commensals remain confined to the gut lumen without translocation to the bloodstream, largely because of a specific IgA mediated mucosal response. This immune response is uniquely induced by the presentation of the commensal microbial antigen to the organised lymphoid tissue in the gut by intestinal dendritic cells (DC). These studies have shown that the DC carrying the commensal

microbial load do not cross the lymphoid tissues, thus preventing systemic infection and limiting the development of response to the commensal in the mucosal one.[19-20] However, if commensal bacteria escape from the DC, they seem to be rapidly coated by specific mucosal IgA, taken up and destroyed by the FC / bearing phagocytic cells. It appears that mesenteric lymph nodes provide a barrier function which prevents mucosal microflora from reaching the bloodstream and at the same time, may act as an inductive site for the mucosal IgA response. Mucosal microflora appear to influence a number of other cellular functions. Studies in germ-free mice after colonisation with B. *thetaiotamicron*, an anaerobic commensal have suggested that such colonisation is important in expression of host genes regulating post-natal maturation, nutrient uptake and metabolism of the tissue, processing of xenobiotics, and angiogenesis. Of particular importance are the observations suggesting that early acquisition of commensals is a prerequisite for induction of tolerance to self, dietary and other luminal antigens. Immune response to OVA was easily elicited in germ free mice. However, restitution of gut flora was associated with development of specific tolerance. Commensal bacteria are more often associated with tolerance to dietary antigens than pathogenic organisms (Table 4-3). This phenomenon appears to be related to the lack of inflammatory signals and low expression of co-stimulating molecules necessary for the induction of the active immune response. [19-21]

Table 4-3. Regulation of the systemic immune response after mucosal exposure to antigens

	Development of immune response	
Antigenic Condition	Yes (Active immunity)	No (Mucosal tolerance)
Soluble proteins	-	++
Dietary antigens	-	++
Microbial protein (inactivated)	++	±
Pathogenic (live) organisms	+++	-
Commensals		
Own	-	++
Other human	++	-
Other species	+++	-
APC+ inflammatory signal	++	-
APC, no inflammatory signal	-	++
Lack of costimulatory molecules (CD80, 86)	-	++
Germ free mucosa	+++	-
Multispecies mucosal flora	-	+++
Restoration with only one species	-	++

- no response, + to +++ minimal to strong response

A number of immunoregulatory cytokines and other cellular products induce or facilitate induction or persistence of tolerance. These include IL-2, IL-4/IL-10, TGF-β, cholera toxin-β subunit, LPS and IFN-β. On the other hand, substances which result in breakdown or non-induction of tolerance include IFN-γ, anti-MCP-1, intact cholera toxin molecule, anti-γδ TCR antibody, and a variety of immunosuppressive agents. Many of these cytokines also regulate activation of T cell subsets in health and disease. The immunological disorders associated with proinflammatory (Th1), and immunoregulatory (Th2), cytokine profiles are listed in Table 4-4. Current understanding of the mechanisms underlying the development of immunologically mediated diseases suggests a major role for different T-helper cell (Th) subsets.[21-22] Increased Th1 cytokine profile (IFN-γ, IL-2) has been associated with diseases such as experimental allergic encephalitis, multiple sclerosis, insulin-dependent diabetes mellitus, Crohn's disease, allograft rejection, and auto-immune thyroiditis. Diseases associated with increased Th2 cytokine profile (↑ IL-4, IL-5, IL-13) include many parasitic infestations, mycobacterium liprea, candida, toxoplasmosis, HIV and other viral infections, and autoimmune or allergic disorders such as asthma, atopic dermatitis and allergic rhinitis-conjunctivitis. [22]

Table 4-4. Clinical disorders associated with T-helper subset cytokine profile

Increased Th1 Activity	Increased Th2 Activity
Experimental allergic encephalitis	Leishmania
Multiple sclerosis	Leprosy
Type 1 diabetes mellitus	Candida
Crohn's disease,	Toxoplasmosis
Allograft regulation,	HIV and other viral infections
Auto-immune thyroiditis	Asthma
	Atopic dermatitis
	Allergic rhinitis
	Conjunctivitis

Both atopic and non-atopic subjects exhibit low Th1 activity at birth. However during the maturation of the immunological functions, in early childhood, most non-atopic subjects exhibit an increasing Th1 cytokine profile. By contrast, atopic subjects fail to shift to a Th1 profile. It appears that non-atopic subjects exhibit a high Th2 profile at birth which is replaced by a Th1 profile as the child grows. However atopic subjects fail to switch to a Th1 type of response and continue to exhibit high Th2 activity often associated with clinical expression of disease. Several environmental factors appear to be critical in modulating Th1 vs Th2 cytokine expression and development of allergic diseases.[23] Of these, the role of mucosal microflora appears to be of paramount importance. Based on large number of earlier and more recent investigations, it appears that the principle trigger for the

shift to a Th1 cytokine profile and resistance to allergic sensitisation comes from the commensal probiotic flora of the mucosal surface. These observations have lead to the formulation of the "extreme hygiene" hypothesis, which states that implementation of aggressive hygienic life style in early infancy may result in a reduced or altered pattern of intestinal commensal colonisation.[24] Such alterations result in the failure to induce or break down existing tolerance to otherwise benign food and inhaled antigens. These events predispose to increased allergic and immunological hyperactivity-mediated diseases in later life. This hypothesis is supported by a number of earlier and more recent observations. These include the observed reduction in the incidence of allergic disease after breast-feeding, oral supplementation of diet with lactobacillus, presence of dogs or cats in the family, attendance in day care settings or living on a farm. In contrast, introduction of sanitation, use of antibiotics, formula-feeding, and introduction of processed foods has been associated with increased incidence of atopic and autoimmune disease. [24-26]

Although the hygiene hypothesis has continued to remain an important concept underlying the emergence of allergic and autoimmune diseases, it is difficult to explain why there is also an increase in the incidence of diseases associated with ↑Th1 cytokine profile and why the risk of allergy in Th2-inducing helminth ingestion is low. It is has been proposed that most mammalian species, including humans have been infested with parasites since the very early days of evolution and Th2 responses may have evolved specifically to combat helminth infestations.[27-29] Th2 responses against otherwise harmless allergens may represent a negative exponent of antiparasitic Th2 responses.

The role of human milk and breast-feeding in modulating the mucosal immune response has been explored extensively during the past four decades. Its nutritional advantages and its impact in preventing mucosal infection are well established. Human milk is replete with: immunoglobulins, especially secretory IgA; specific antibody activity against most gastrointestinal and respiratory pathogens; T and B lymphocytes; and macrophages, prebiotic constituents, and microbial agents or environmental antigens acquired from the maternal gastrointestinal or respiratory tracts. Human milk also contains a wide spectrum of cytokines, including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFNγ, TNFα, and TGF-β representing all subsets of T-helper cells. The relationship between breast-feeding and development of atopic diseases is still open to further evaluation. It is also possible that the protective role of breast-feeding against mucosal infection may have an adverse impact on the development of allergic disorders in formerly breast-fed subjects.[30]

4. CONCLUSIONS

The human mucosal immune system is a unique evolutionary acquisition, designed to maintain a homeostatic balance between the complex array of microorganisms and other environmental macromolecules, and the human host. The innate immunological mechanisms associated with mucosal epithelium, dendritic cells and macrophages are designed specifically to identify pathogen associated molecular patterns (PAMP) through the expression of pattern recognition receptors (PRR), including Toll receptors. The response of mucosal epithelium to microbial PAMP depends on the expression of TLR-4 for LPS, TLR-2 for LTA and peptidoglycan and TLR-5 for bacterial flagyllin. Other TLR receptors such as TLR-3, TLR-5, and TLR-10 are also involved in interaction with bacterial PAMP. Activation of the innate immunity is thus the first event in a sequence of cell activation mechanisms in the mucosa following exposure to an infectious agent. The nature of innate immune response is determined by the nature of the mucosal microflora. Such responses are essential for the outcome and nature of subsequent proinflammatory (Th1), immunoregulatory (Th2) T cell or specific antibody responses to the infectious agents and to other environmental macromolecules. The permanent resident flora of human mucosal surfaces represent commensal bacteria that coexist peacefully with the host, generally remain harmless, and do not cross into the bloodstream. This unique relationship has evolved over a million years, and maintenance of this balance is essential to the maintenance of normal physiological functions in the host. A large number of recent societal developments have significantly altered this balance, associated with a significant increase in the incidence of allergic, autoimmune and other immunologically mediated disease processes. The practice of breast-feeding and increased knowledge of human milk as an important immunomodulating agent should serve as a reminder of the immense benefits provided by the commensal flora, and lactational products to the mechanisms of immunological homeostasis at mucosal surfaces.

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Chapter 5

INFANT FEEDING AND GROWTH

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1. INTRODUCTION

It is well documented that the growth patterns of breast-fed and formula-fed infants differ significantly. In industrialised countries, formula-fed infants generally grow more rapidly than breast-fed infants. In developing countries, the opposite may be observed, due to high rates of infection associated with bottle-feeding in environments with poor sanitation. This paper will focus on the former situation, where infant growth is largely unconstrained by environmental factors. The questions to be addressed are: a) how do growth patterns differ between breast-fed and formula-fed infants, b) how does energy intake differ between breast-fed and formula-fed infants, c) what are the potential explanations for differences in energy intake and growth by feeding mode d) what are the long-term consequences of differences in early growth?

2. GROWTH PATTERNS OF BREAST-FED AND FORMULA-FED INFANTS

Numerous studies have documented differences in the growth patterns of breast-fed and formula-fed infants, although there is variability across studies regarding the specific growth outcomes measured and the age intervals during which differences by feeding mode are evident.[1] Some of

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the variability in findings is due to the different criteria used to define feeding groups, in particular the duration and exclusivity of breast-feeding.

In industrialised countries, differences in growth by feeding mode are evident beginning in the first week of life. Macdonald *et al*[2] found that mean weight loss (as % of birthweight) was 6.6% among 420 breast-fed newborns and 3.5% among 396 formula-fed newborns ($p < 0.0001$). This is not surprising, given that it usually takes 2-3 days before full breast-milk production occurs. Once full milk production has been established, the weight gain of breast-fed and formula-fed infants is similar during the first 6 weeks of life. Nelson *et al*[3] reported a mean weight gain of 36.4 g/d in breastfed infants (n=419) and 36.6 g/d in formula-fed infants (n=720) from 1 to 6 weeks of age. However, during the subsequent interval, from 6 to 16 weeks, weight gain was significantly lower in the breastfed group (24.0 g/d) than in the formula-fed group (26.6 g/d). Similar findings have been reported by other investigators,[1,4] although the differences are not always significant, possibly due to small sample sizes and/or variability in the exclusivity of breast-feeding in the breastfed group.

Differences in weight gain between breast-fed and formula-fed infants are most evident from 3 to 12 months of age.[1,5] When breast-feeding duration is $\geq$ 12 months, the difference in average attained weight at 12 months is ~600-650 g.[1] The difference in length gain by feeding mode is smaller and not always statistically significant, depending on the study. As a result, weight-for-length is generally lower in breast-fed infants by 9-12 months. By contrast, growth in head circumference generally does not differ between breast-fed and formula-fed infants.

There have been mixed results regarding differences in fatness by feeding mode.[1] In the DARLING study, the sum of skinfold thickness at 5 sites was similar between breast-fed and formula-fed infants during the first 6 months of life, but between 9 and 17 months of age it was significantly greater in the latter group.[6] However, in a study by Butte *et al*[7] in which body composition was measured using a multi-compartment model, breast-fed infants were significantly fatter than formula-fed infants at 3 and 6 months (although the difference was significant only among boys at 6 months), and there were no significant differences between feeding groups at 9-24 months of age. The apparently conflicting results of these two studies may be explained by differences in the criteria for the breastfed groups. In both studies, these infants were exclusively breast-fed for at least 4 months, but in the DARLING study, all of the breastfed infants continued to receive breast-milk as the sole source of milk for $\geq$ 12 months, whereas in the study by Butte *et al*, only 38% of the breast-fed group were still breast-fed at 12 months, and most of those who did continue to breast-feed also received supplemental formula or cow's milk after 4 to 6 months. Thus, lower body

fatness after 6 months may only be apparent among infants who are still fully breast-fed. This conclusion is consistent with the results of 7 studies in North America and northern Europe that documented the growth of infants who were breast-fed for at least 12 months.[8] In all 7 studies, there was a declining trend in mean weight-for-length z-score between 3 and 12 months, suggesting that the breast-fed cohorts were leaner than the National Center for Health Statistics (NCHS) reference population (which was based on mostly bottle-fed infants).

These differences in growth patterns by feeding mode prompted the World Health Organization to develop a new international growth reference, which was based on growth of healthy infants breast-fed throughout the first year of life.[9] The new growth chart, based on a six-country study, was released in 2006. www.who.int/childgrowth/en.

3. ENERGY INTAKE OF BREAST-FED AND FORMULA-FED INFANTS

It is also well documented that energy intake differs between breast-fed and formula-fed infants. In the DARLING study, for example, the total energy intake of the formula-fed infants exceeded that of the breast-fed infants by 15% at 3 months, 23% at 6 months, 20% at 9 months and 18% at 12 months.[10] This difference is attributable to a greater volume of formula consumed, relative to the volume of breast-milk consumed by the breast-fed cohort. Even when energy intake is expressed per kg of body weight, the differences remained significant at 3, 6 and 9 months. It is noteworthy that differences in total energy intake by feeding mode persist even after complementary foods are introduced.

Several pieces of evidence indicate that these differences in energy intake are not attributable to insufficient breast-milk transfer from mother to infant, but instead reflect self-regulation of energy intake by breast-fed infants at a lower level than consumed by formula-fed infants. First, when given access to an increased supply of breast-milk (stimulated by the mothers' daily expression of extra milk for 2 weeks), breast-fed infants may increase their intake for a day or two, but they generally return to their usual level of intake after 1-2 weeks.[11] This indicates that they have the ability to self-regulate energy intake to meet their needs. Second, breast-fed infants typically leave behind some "residual" milk in the breast – averaging about 100 g/d – and this occurs even among infants who have a relatively low milk intake (< 650 g/d).[12] Third, once complementary foods have been introduced, breast-fed infants regularly leave unconsumed ~25-30% of the food offered to them,

even if their total energy intake is relatively low.[13] Thus, the lower energy intakes observed among breast-fed infants appear to be voluntary. The key question is why formula-fed infants consume more energy than breastfed infants.

4. POTENTIAL EXPLANATIONS FOR DIFFERENCES IN ENERGY INTAKE AND GROWTH BETWEEN BREAST-FED AND FORMULA-FED INFANTS

This section will explore several possible explanations for the differences in energy intake and growth described above. With regard to energy intake, one key finding is that the energy requirements of formula-fed infants are higher than those of breastfed infants. Using stable isotope methodology, Butte *et al*[14] determined that the total energy expenditure (TEE, which does not include the energy required for growth) of formula-fed infants was 10% higher at 3 months and 5% higher at 6 months of age, compared to the values for breast-fed infants. This was attributable to a difference in sleeping metabolic rate (similar to resting metabolic rate in adults), not to differences in physical activity. The amount of energy required for growth, based on careful measurements of body composition, did not differ significantly between feeding groups. This was because even though the breast-fed infants gained less weight, a higher percentage of their weight gain was fat (which requires more energy per g deposited). Because of the difference in TEE, total energy requirements (TEE plus energy deposition) of formula-fed infants, per kg of body weight, were 7% higher at 3 months and 9% higher at 6 months, compared to the requirements of breast-fed infants.

These differences in energy requirements can explain at least part of the difference in energy intake between breast-fed and formula-fed infants, but they do not explain the differences in growth. The growth difference observed (~ 3 g/d greater in formula-fed infants) represents only a small amount of energy (7-14 kcal/d, ~1-3% of total energy intake at 3-6 months). Thus, energetically speaking, it does not "cost" formula-fed infants very much to deposit more weight. Something else must be driving this difference in growth (and possibly also the higher TEE of formula-fed infants).

It has been suggested that the higher protein content of infant formulas, relative to human milk, could stimulate excess growth.[15] Other differences between infant formula and human milk that may play a role include protein quality, potential renal solute load (PRSL, which is higher in formulas and may contribute to greater thirst, thereby driving greater intake), and the

numerous bioactive substances in breast-milk that may influence metabolism. A double-blind randomised controlled trial was recently conducted to evaluate whether protein quantity or quality (i.e. whey:casein ratio) or the PRSL of infant formula influences intake or growth between 1 and 5 months of age.[4] The 84 formula-fed infants recruited were randomly assigned to one of 3 types of formula: control formula, modified protein quality formula, and low protein, low PRSL formula. The third formula was a 2-stage formula with a protein content of 12.1 g/L at 0-2 months and 10.7 g/L at 2-4 months. A comparison group of 73 exclusively breast-fed infants was also recruited. Growth was measured monthly, intake was measured at 1, 3 and 5 months, and blood samples were collected at 5 months. There were no significant differences among the 3 formula-fed groups with regard to intake or growth. As observed previously, the breast-fed group consumed a much lower volume of milk than the formula-fed groups and gained less weight. The breast-fed infants also had significantly lower plasma concentrations of insulin and insulin-releasing amino acids. These results suggest that the protein content and PRSL of formula do not explain the higher intakes and growth rates of formula-fed infants. The difference in insulin levels implies that there is a metabolic effect of breast- vs. formula-feeding, but whether this is a *cause* or an *effect* of higher energy intake is unknown.

Recent data from a very large infant feeding study (The PROBIT Study) in Belarus[16] confirm the observation that formula or animal milk consumption has a growth-stimulating effect. Data on 16,755 infants whose growth was measured during the first year of life were analysed with respect to the milk source (coded as breast-milk only, breast-milk + formula or other milk, or formula/other milk only) and consumption of other fluids or foods (water, juice/other liquids, cereals and other solid foods) at the beginning of each age interval examined (1-3, 3-6, 6-9 and 9-12 months). Controlling for initial size, consumption of formula or other milk was consistently associated with greater weight, length and weight-for-length from 3 months onwards, but not with greater head circumference. Cereal consumption during the 3-6 months age interval was strongly associated with *lower* weight, length and head circumference. The underlying mechanism for the growth-accelerating effect of formula merits further investigation.

It may be that the growth-accelerating effects of infant formula are due not to its composition, but rather to the way in which it is usually fed, i.e., by bottle. Breast-fed infants are able to self-regulate intake easily, by simply latching off when satiated. With bottle-feeding, however, the caregiver may not recognise the infant's satiety cues, or may even encourage the infant to finish the bottle. Furthermore, breast-fed infants who continue sucking for comfort once the breast is nearly empty will not get much more milk,

whereas bottle-fed infants who continue sucking – even if all they want is comfort – will get more formula unless the bottle is empty and not refilled. Data from the randomised controlled trial described above were analysed to examine whether certain bottle-feeding behaviours are associated with infant growth. Two behaviours were examined: 1) the tendency for "bottle-emptying", defined as the percentage of feeds at which $\leq$ 10 mL of formula remained unconsumed, and 2) the usual amount of formula prepared per feed (i.e. "bottle size"). For the first behaviour, mother-infant pairs were categorised in terms of whether they emptied the bottle frequently ($\geq$50% of feeds) or infrequently (<50% of feeds). Infants in the former group had greater body fatness at 5 months (sum of skinfold thickness, 69 vs 55 mm, $p<0.01$). For the second behaviour, mothers were categorised into those who usually prepared $\leq$ 6 oz (180 mL) per feed and those who usually prepared > 6 oz per feed at 3 months. The latter group of infants had significantly greater formula intake at 3 months (1008 vs 785 mL/d, $p=0.001$), even though there was no significant difference in intake in early infancy (at 1 month). There was also a significant difference in weight gain between 3 and 5 months (17.4 vs 21.0 g/d in the "smaller bottle" vs "larger bottle" groups, respectively, $p=0.03$).

The cut-off of 6 oz per feed for "bottle size" was chosen because it was observed that the breast-fed infants in that study almost never consumed more than that amount at a single feed. The median intake per feed was consistently higher among the formula-fed infants when compared with the breast-fed infants, with a difference of 49% at 1 month, 57% at 3 months, and 71% at 5 months. These results suggest that bottle-feeding practices may encourage excess intake, but in the analyses described above it is difficult to distinguish whether it is the caregiver's wishes or the infant's characteristics that are "driving" bottle size and bottle-emptying. Thus, a randomised controlled intervention trial to modify bottle-feeding behaviors by caregivers is needed to adequately test the hypothesis that these behaviours explain the difference in intake and growth between breast-fed and formula-fed infants.

5. LONG-TERM CONSEQUENCES OF DIFFERENCES IN EARLY GROWTH

There is increasing evidence that infant feeding practices and the rate of postnatal growth are linked with health outcomes later in life. This section will consider three such outcomes: adult height, child obesity and cardiovascular disease risk factors.

Although breast-fed infants may grow less rapidly in length than formula-fed infants during the first year of life, this does not lead to shorter

height in adulthood. Four recent studies have explored the relationship of breast-feeding to adult height.[17-20] In Brazil[18] and Israel[19], there was no significant relationship between the duration of breast-feeding and adult height. The study in Brazil controlled for numerous potentially confounding variables, such as socioeconomic status, maternal education and birthweight. Two studies in the UK[17,20] showed that in males, those who had been breast-fed were taller as adults than those who had not been breast-fed, whereas there was no significant association in females. These results suggest that the rate of linear growth exhibited by breast-fed infants does not have any long-term negative effects on attained height.

Recent evidence indicates that breast-feeding is associated with a 20-30% reduced risk of child obesity.[21-23] The relationship is most evident when the duration and exclusivity of breast-feeding are taken into account, and when the age at follow-up is 6-15 years. The influence of breastfeeding is probably small compared to other factors such as parental overweight, but in most of the studies that controlled for such potential confounders, there was still a significant relationship between breast-feeding and a lower risk of child obesity. It remains possible that residual confounding explains some of the relationship, but there is increasing evidence that metabolic programming associated with postnatal growth patterns may play a role. Several studies have demonstrated that a higher rate of weight gain during infancy is predictive of childhood obesity, independent of birthweight.[24-26] In a large cohort of children in the U.S., almost 20% of the obesity observed at age 7 was attributable to having a rate of weight gain in the top quintile during the first 4 months of life.[25] Unfortunately, these studies did not have adequate data on infant feeding practices to disentangle how much of this relationship is explained by breast-feeding vs formula-feeding, or whether there is an interaction effect with infant feeding mode (i.e., whether rapid weight gain during infancy is predictive of later obesity regardless of feeding mode, or is more evident in formula-fed infants than in breastfed infants).

One potential factor linking infant feeding, early growth patterns and later outcomes is plasma insulin levels. As mentioned in section 4, formula-fed infants have higher plasma insulin levels than breast-fed infants. Higher insulin levels stimulate greater adipose tissue deposition, and have been associated with subsequent increased weight gain and obesity in Pima Indian children[27]. Further research is needed to understand the long-term endocrinological impact of infant feeding practices and early growth patterns.

An intriguing set of studies by Singhal *et al* has prompted interest in what has been called the "growth acceleration hypothesis".[28] These investigators conducted two randomised controlled trials with preterm infants.[29-32] In one study, the infants were provided with either banked human milk or preterm

formula (n=502) and in the other, they were provided with either term formula or preterm formula (n=424). The preterm formula was enriched in protein and fat. The assigned diets were given until infant weight was ≥ 2000 g or the infant was discharged, which took an average of about 4 weeks. The infants were followed-up at various ages, with the most recent set of assessments being conducted when they were 13-16 years of age. Several outcomes related to risk of cardiovascular disease were assessed, including blood pressure, lipoprotein profiles, insulin resistance and endothelial function. Human milk feeding had a protective effect on blood pressure and LDL/HDL ratio and was marginally associated with lower insulin resistance. In regression analyses, the investigators determined that the rate of growth during the first two weeks of life, which was higher among infants fed preterm formula in both trials, was a significant predictor of several outcomes (LDL/HDL ratio, insulin resistance and endothelial function, but not blood pressure). These outcomes were less favourable among infants with more rapid early growth. For LDL/HDL ratio, the beneficial effect of human milk feeding was explained by the difference in early growth between feeding groups.

In a separate trial, the same research group investigated neurodevelopmental outcomes of term, small-for-gestational age infants who were randomised to receive a standard formula or a nutrient-enriched formula.[33,34] There was greater gain in length and head circumference during infancy in those fed the nutrient-enriched formula, especially among females, which the authors interpreted as a positive impact of the intervention.[33] However, in a subsequent report on the neurodevelopmental outcomes[34], the investigators reported that the group fed the nutrient-enriched formula had a *lower* developmental quotient at 9 months (especially among the females), though there was no significant difference at 18 months. This finding draws attention to the need to better understand the consequences of promoting rapid growth during early life.

Singhal and Lucas[28] have proposed that early growth acceleration has adverse effects across species, which may be due to programming of the hypothalamic-pituitary axis. They further suggest that some of the observations supporting the fetal origins hypothesis (linking poor nutrition *in utero* to adverse outcomes later in life) may be explained by early growth acceleration post-natally, as growth-retarded fetuses generally show a faster rate of postnatal growth than those born with normal birthweight.

Taken as a whole, the evidence to date supports the notion that the rate of growth exhibited by breast-fed infants, which is slower than observed in formula-fed infants, is optimal in terms of longer-term outcomes. Although much is still unknown about why this growth pattern appears to be

advantageous, there is no longer any doubt that the breast-fed infant should be considered the biological norm.

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Chapter 6

INTESTINAL FLORA

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1. INTRODUCTION

The intestinal colonisation with a balanced microflora is of major importance for an appropriate development of the immune system. In recent years, there has been increasing scientific and commercial interest in modifying the flora for health promotion. Dietary factors that are thought to be effective in that context are the so-called prebiotics, i.e., unabsorbable carbohydrates as microbial substrates, or probiotics defining specific live, digestion-resistant bacteria with potential health benefits.

As the gut at birth is sterile, it is an organ sensitive to environmental influences. Furthermore, there is an intensive cross talk between the microflora and intestinal epithelial cells. The mechanisms by which these cells perceive and respond to microbes, both pathogenic and commensal, are important to the understanding of the physiology of defence systems and the pathogenesis of inflammatory diseases. To better understand the distinct interactions, e.g. between Pattern Recognition Receptors (PRRs) such as toll like receptors (TLR) of the host and Pathogen Associated Molecular Patterns (PAMPs) of the microbes, more knowledge is necessary regarding factors which facilitate the growth of certain microbes that lead to very specific metabolic activities.

Current research on prebiotic oligosaccharides (PBO) is focussed on specific effects to achieve a flora dominated by lactobacilli. In this context

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the PBO effect is often considered to be the same as the bifidogenic effect of human milk oligosaccharides (HMO). Both carbohydrate groups may exert local functions either by stimulating microbial growth (PBO, HMO) or by preventing the adhesion and colonisation of pathogenic bacteria to the intestinal cell surface (shown for HMO). Further investigations are necessary with regard to particular oligosaccharides, their origin, i. e. milk or plants, their mechanisms and dose dependency especially in infant nutrition. Comparing the structures of HMO and PBO, neither the monosaccharide composition nor the specific linkages are equivalent. Hence, the underlying mechanism for observed functions is not expected to be the same.

Our current knowledge with regard to the metabolic activity of the intestinal flora is relatively fragmentary, but it is this aspect that probably offers the greatest potential for preventive and therapeutic exploitation.

2. THE GUT MICROFLORA IN INFANTS

The heterogeneous composition of the microflora in humans and factors influencing the development after birth have been reviewed elsewhere.[1-5] Comparing the flora of breast-fed and formula-fed infants, it is generally believed that there are high numbers of bifidobacteria and low population densities of potentially pathogenic microbes in the former group. This observation has been confirmed by several research groups (for review: see ref.1). However, the normal pattern of bacterial colonisation of the intestine may be more complex than previously reported. There are several potential reasons for the increasing number of studies not confirming a specific bifidus flora in breast-fed infants: a) methodological problems in identifying different bacterial species, e.g. microbiological assays versus gas chromatography or molecular biological techniques; b) environmental factors such as mode of delivery, e.g. vaginal delivery versus caesarean section; c) treatment with antibiotics; d) preterm delivery; e) collection and storage of fecal samples (considering the slower proliferation of apathogenic bifidobacteria); or f) the type of infant formula.

Compared to the relevance of bifidobacteria as a marker for the breast-fed infant's microflora, there are several other differences in the intestinal flora between human milk-fed and formula-fed infants which are better documented. As thoroughly discussed by Adlerberth,[3] it has been consistently observed that breast-fed infants have lower counts of clostridia and enterococci and higher numbers of staphylococci compared to formula-fed infants. In addition, it is noteworthy that the influence of breastfeeding on bacteroides and lactobacilli seems to be very weak if at all detectable.

As human milk is still the gold standard for the production of infant formula, new products like those with prebiotics are on the market which have been shown to increase the number of bifidobacteria; however, there are no data published with regard to the growth inhibition of pathogenic microorganisms.

This raises the important issue that it is not the mere presence of growth factors in milk that determines bacterial colonisation and leads to a higher number of bifidobacteria and lactobacilli, but that more specific mechanisms are responsible for the concomitant low number of pathogenic bacteria in breast-fed infants. As will be discussed below, certain complex oligosaccharides in human milk might be involved in these processes.

3. PREBIOTIC OLIGOSACCHARIDES VERSUS MILK OLIGOSACCHARIDES

PBO like inulin, oligofructose or galactosylated oligosaccharides are widely used in food technology for various reasons. Today, research activity is focused on specific effects of these carbohydrates on the development of a beneficial intestinal flora.[2,6-7] As HMO are often directly compared to PBO with regard to their potential benefit, the question, however, is whether there are similarities between these oligosaccharides other than the chemical class of compounds?

Both groups are considered to be non-digestible leading to their appearance in the lower gastrointestinal (GI) tract. It has been shown that about 1% of ingested HMO (5–10 g/d) is excreted via urine and absorbed by term as well as by preterm infants.[8-9] These compounds may therefore exert not only local functions within the GI tract but also systemic effects, e.g. on inflammatory processes in human milk-fed infants.[10]

For both carbohydrate groups, localised functions, e.g. stimulating the growth of a certain beneficial gut flora, preventing the attachment of pathogenic bacteria to cell membranes or influencing the absorption of minerals and trace elements, have been discussed but not yet proven.

Summarising the current literature, the reader must have the impression that PBO and HMO are very similar, and hence have the same biological functions. With regard to their structure, neither the monosaccharide composition nor the specific linkages of PBO and HMO are comparable (Table 6-1).

Table 6-1. Monosaccharide composition of human milk oligosaccharides and prebiotics

Compound	Human Milk	Prebiotics
Glucose	Traces	+
Galactose	+	+
N-Acetylglucosamine	+	-
Fucose	+	-
N-Acetylneuraminic acid	+	-
Fructose	-	+
Xylose	-	+
Arbabinose	-	+

Milk oligosaccharides are mainly composed of glucose, galactose, N-acetylglucosamine, fucose and N-acetylneuraminic acid (sialic acid) whereas most of the monosaccharides in prebiotics are not present in milk (e.g. fructose, xylose or arabinose). Even more important, there are no similarities in the specific monosaccharide linkages, which is a prerequisite for their functional acivity (Tables 6-2 and 6-3).

Table 6-2. Specific monosaccharide linkages in lactose-derived oligosaccharides

Lactose-derived oligosaccharides	Monosaccharide linkage
Fucosyl-Lactose	α1-3, ß1-4,
Lacto-N-Tetraose	ß1-3, ß1-4
Sialyl-Lactose	α 2-3, α 2-6, ß1-4
Sialyl-Lacto-N-Tetraose	α 2-3, α 2-6, ß1-4,ß1-3

Table 6-3. Specific linkages in prebiotics

Oligosaccharide	Monosaccharide linkage
Fructo-	ß1-2,
Galacto-	α1-2, α 1-4, α 1-6
Transgalacto-	and
Soybean-	other linkages

4. MILK OLIGOSACCHARIDES AND THE GUT

The decisive factor in the pathophysiology of infectious diseases such as diarrhea seems to be the ability of microorganisms to adhere to the mucosal surface, their subsequent spreading, colonisation and invasion (e.g. for *Escherichia coli*, *Helicobacter jejuni*, *Shigella* strains, *Vibrio cholerae* and *Salmonella* species).[11-12] Bacterial adhesion is a receptor-mediated interaction between structures on the bacterial surface and complementary (carbohydrate) ligands on the mucosal surface of the host. There are many

examples from *in vitro* studies demonstrating the high potential of milk oligosaccharides to interfere with these specific host-pathogen interactions. As it is still not possible to produce large amounts of complex milk-type oligosaccharides for clinical studies, *in vivo* data are still missing. However, some oligosaccharides have recently been tested as anti-adhesive drugs in animals. For example, an intranasal or intratracheal administration of either oligosaccharides or neoglycoproteins in rabbits and rat pups markedly reduced experimental pneumonia caused by *S. pneumoniae*.[13]

In another study, *H. pylori* positive Rhesus monkeys were treated with 3´sialyllactose alone or in combination with either one of the commonly used anti-ulcer drugs, bismuth subsalicylate (a gastric surface coating agent) and omeprazole (a proton pump inhibitor).[14] Of the six monkeys that were given milk oligosaccharides only, two were permanently cured, and a third animal was transiently cleared, while three of the animals remained persistently colonised. According to the authors, the anti-adhesive therapy is safe and can cure or reduce *H. pylori* colonisation in Rhesus monkeys.

Clinical experience with oligosaccharides as anti-adhesive drugs is still very limited. Ukkonen *et al.*[15] investigated the efficacy of 3'-sialyllacto-N-neotetraose, given intranasally for prophylaxis of acute otitis media.[15] In this randomised, double-blind placebo-controlled study, 507 healthy children were assigned either to the acidic milk oligosaccharide or placebo as intranasal sprays twice daily for 3 months. Although the study failed to reduce the incidence of nasopharyngeal colonisation with *Streptococcus pneumoniae* and *Hemophilus influenzae* as well as that of acute otitis media, this may be, for example, due to the fact that during natural infection, bacteria can express multiple lectins with diverse specificities, whose inhibition may require a cocktail of oligosaccharides.[16]

Besides their potential in pathophysiological situations such as inflammation, milk-type oligosaccharides might also exert specific functions in physiological events, e.g. in intestinal cell maturation. Present data suggest that the cell adhesion molecule MAdCAM-1 is involved in the homing of lymphocyte subsets to the intestinal tract.[17-18] It is strongly expressed in endothelial cells in Peyers' patches and in mesenteric but not in peripheral lymph nodes. In addition, it also contains a mucin-like domain which functions as a ligand for L-selectin which allows the rolling of leukocytes along a MAdCAM-1 coated surface. Therefore, MAdCAM-1 has the structural requirements to contribute to both the rolling (by low affinity binding) and firm adhesion (by very high affinity-binding) through its interactions with integrins. With regard to infant nutrition, it is intriguing to speculate that oligosaccharides may interfere with this process resulting in an increased leukocyte/lymphocyte accumulation in the mucosa, thus supporting phagocytosis or antibody production.

Recent observations indicate that HMO not only influence systemic processes such as leukocyte endothelial cell or leukocyte platelet interactions[19-20] but also induce intestinal cellular processes.[21] These oligosaccharides seem to affect the gut in at least two different ways: as growth factors influencing normal gut development and maturation, and as anti-inflammatory and immune components modulating the intestinal immune system.

5. EFFECTS OF PROBIOTICS AND PREBIOTICS WITHIN THE GUT

It is evident that there is an intensive cross talk between the microflora and epithelial cells. [22-25] The mechanisms by which epithelial cells perceive and respond to microbes, both pathogenic and commensal, is important to understand the physiology of defence systems and the pathogenesis of inflammatory diseases. Cells respond to microbes by distinct interactions, e.g. between Pattern Recognition Receptors (PRRs) such as Toll Like Receptors of the host and Pathogen Associated Molecular Patterns (PAMPs) of the microbes (Figure 6-1).

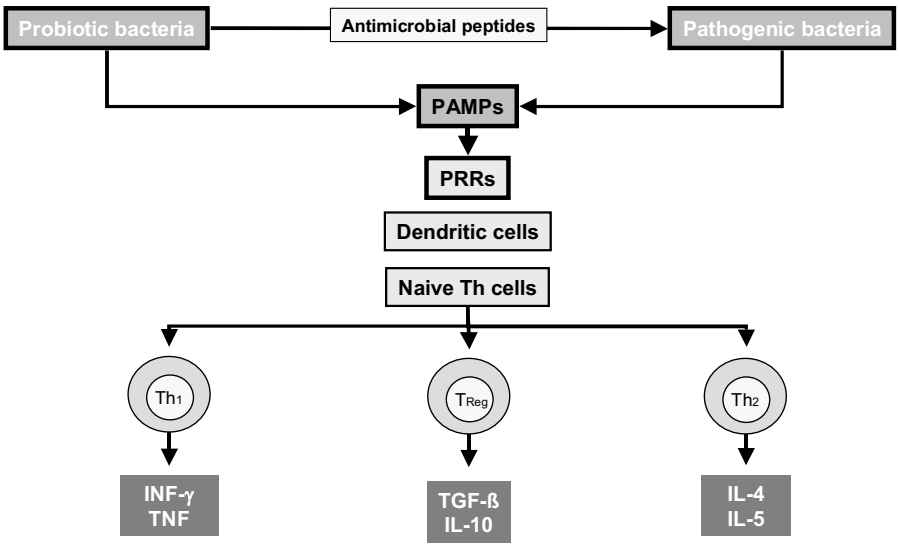

Figure 6-1. Recognition of Pathogen Associated Molecular Pattern (PAMPs) on microbes by Pattern Recognition Receptors (PRRs) and the effect on T cells (modified according to reference 29).

5.1 Host defence systems

The many proposed functions of probiotics and prebiotics and how the host may prevent a perturbation of its immune system have been summarised.[25] The key features are as follows:

1. The mucus layer and the glycocalyx is the first defence system characterised by a dense glycosylation layer. Both layers have been shown to demonstrate the importance of carbohydrate in the host defence system. Pathogens or antigenic material (antigens) have to pass these layers before they can activate specific pro-inflammatory systems.

2. After crossing the heavily glycosylated layers, antigens are taken up by the enterocytes. These cells are equipped with the whole antigen processing machinery and they are important in maintaining a normal barrier function, and in regulating the immuncompetent T cell, e.g. down regulate a Th1 immune response or excessive Th2.

3. Specialised enterocytes (M cells) internalise macromolecules and microbes through endocytosis and transport them into special areas with antigen presenting cells (AP cells). If induction of an immune response in the gut associated lymphoid tissue (GALT) or lymph nodes occurs, the effector and memory immune cells acquire a mucosal homing program (a4ß7 integrin) and return to mucosal effector sides.

4. Circulating monocytes are recruited and differentiate into dendritic cells. These cells open epithelial tight junctions, capture and internalise antigenic macromolecules and microorganisms through dendrites. They subsequently migrate into GALT or to mesenteric lymphnodes, where they process the antigen and present them to naïve lymphocytes, or they present the antigens to lamina propria B cells. Here, in the absence of T cells, these B cells produce antibodies mainly directed against commensal bacteria.

To understand the communication between commensals or pathogens and the intestinal cells, more information is necessary regarding specific processes within the enterocytic pathway, the M cells or the dendritic cells. Of particular importance is: How do the intestinal cells of the host differentiate between a commensal microbe and a pathogen?

5.2 Pathogen Associated Molecular Pattern (PAMPs) and Toll-Like Receptors (TLRs)

Intestinal epithelial cells play an essential role in innate immunity by activating pro-inflammatory gene expression upon detection of enteric pathogens (Figure 6-1).[23, 25] Such a gene expression promotes direct bacterial killing, e.g. by antibacterial peptides, and leads to recruitment of immune

cells that serve to clear the perturbing pathogen. These cells usually utilise TLRs due to their ability to recognise many different microbe-associated receptors.[26-28] However, many if not most TLR ligands (e.g. lipopolysaccharides, lipoteichonic acid) are common to both pathogens and commensal microbes (Table 6-4).

Table 6-4. Some Toll-Like Receptors and their ligands

Toll-Like Receptors	Ligands
TLR2	Lipoprotein/lipopeptides (a variety of pathogens) Peptidoglycan (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) Zymosan (fungi)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria) HSP60 (Chlamydia pneumoniae) HSP60 (host) Oligosaccharides of hyaluronic acid (host) Fragments of heparan sulfate (host) Fibrinogen (host)
TLR5	Flagellin (bacteria)

Therefore, it is important to understand how the organism is able not to have a constant activation of its proinflammatory signalling pathways.

Subcellular localisation of TLR may provide some answers.[26] TLR which are located at the basolateral surface will not be activated unless a pathogen invades or unless a perturbation in epithelial barrier function occurs. TLR4 and TLR2 appears to be apically located, whereas TLR9 seems to be intracellular. Hence, it should not be activated simply by cell contact with luminal contents.

The definitive answers regarding which TLR are actually expressed in the human intestine, their subcellular localisation and their functional status under standard and specialised physiological conditions are not yet available.[28] This information which should be available soon will help to better understand some of the questions raised above.

5.3 Proposed mechanisms of probiotics and prebiotics

Transduction of bacterial signals into host immune responses probably involves more than one pathway, but NF-κB and subsequently pro-inflammatory genes such as IL-8 has been established as central regulators of epithelial responses to invasive pathogens.[29-31] The counter-regulatory

factor of NF-κB is IκB, and some non-pathogenic components of the flora may attenuate pro-inflammatory responses by delaying its degradation. Probiotic bifido bacteria and Lactobacilli probably do not use the same mechanism but other signal trandsduction pathways are likely to emerge to account for their anti-inflammatory effects [36] (Figure 6-2).

One hypothesis regarding the effects of probiotics and prebiotics is that both can directly influence the intestinal immune system through active uptake by M cells and transfer to antigen presenting cells (AP cells).[32] This may shift the Th_1/Th_2 balance in favour of the former, leading to an increase in IgA producing B cells and a concomitant reduction of IgE producing cells. Of central importance seems to be the induction of a certain cytokine pattern, e.g. dominated by IL-2, IL-12, IL-18 and IFN-γ in opposition to the induction of IL-4, IL-10 and TGF-ß (Figure. 6-3).

Unfortunately, the direction in which a reaction develops can not always be predicted. It is important to recognise that among Lactobacilli different species of bacteria induce a distinct mucosal cytokine profile in the gut immune system. For example, in BALB/c mice, an increase in the Th_2 cytokines IL-10 and IL-4 was observed after feeding *Lactobacillus delbrueckii* subspecies *Bulgaricus* and *Lactobacillus casei.* With *Lactobacillus acidophilus*, however, a significant induction of the Th_1 cytokines IL-2 and IL-12 was observed. [7]

The mechanism of action of probiotics is uncertain, but is likely to vary with different strains, and more importantly depends on the clinical condition for which they are used. Thus, the role of probiotics in preventing or managing infections may depend on 1) mutual competitive metabolic interactions with potential pathogens; 2) production of antimicrobial peptides; or 3) inhibition of epithelial adherence and translocation by pathogens. For each of these protective effects, experimental evidence *in vitro* has been reported with different probiotics.[30] The proposed mechanisms of probiotics are summarised in Figure 6-4.

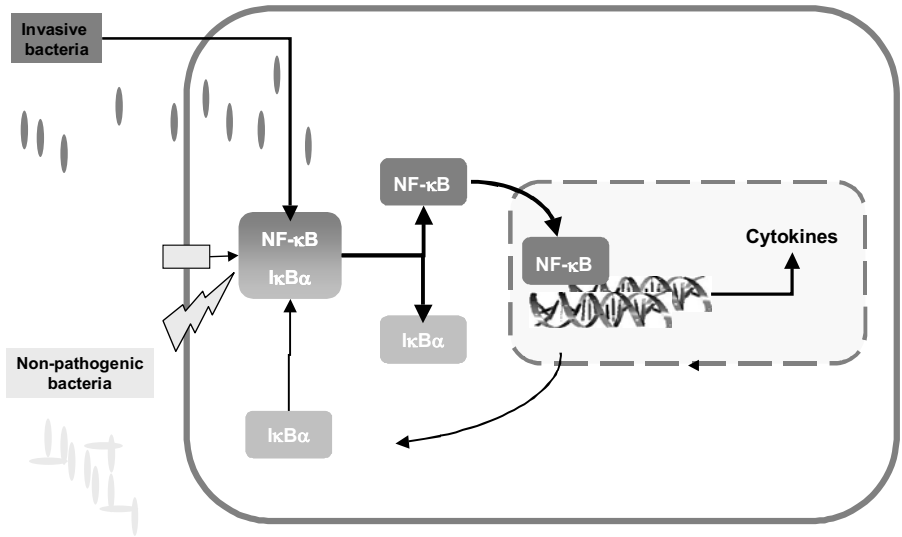

Figure 6-2. Suggested mechanism of microorganisms such as probiotics in the inactivation of the inflammatory effects of pathogens. The delayed degradation of IkB prevents a sudden increase of NF-kB and, hence, a pro-inflammatory situation (modified according to reference 33).

6. CONCLUSION

The development of novel methods to characterise and modify the gut microflora has opened up new perspectives regarding its role in health and disease. Numerous studies have suggested beneficial effects of lactobacilli on gut health. However, results have been inconsistent, which may be due to differences in strains, routes of administration, and investigative procedures used in these studies.

When discussing probiotics, a clear distinction has to be made between their application as a therapeutic or their use as a preventive measure. Common to both is the assumption that there is a "normal healthy microflora" which has not yet been defined.

Questions which need to be addressed regarding prebiotics are: What kind of new components may be suitable to promote the so-called bifidus flora in lower daily dosages than currently necessary (6 to 8 g/d in small children)? How can the high drop out rate of up to 30 % or even more due to discomfort be prevented? Important in the field of infant nutrition is not only the effects on the growth of bifidobacteria but on other major microbes. Although human milk oligosaccharides are considered to be the role model

for prebiotics in infant formula, their structural differences do not support a direct parallel functionality and thus, need to be further investigated.

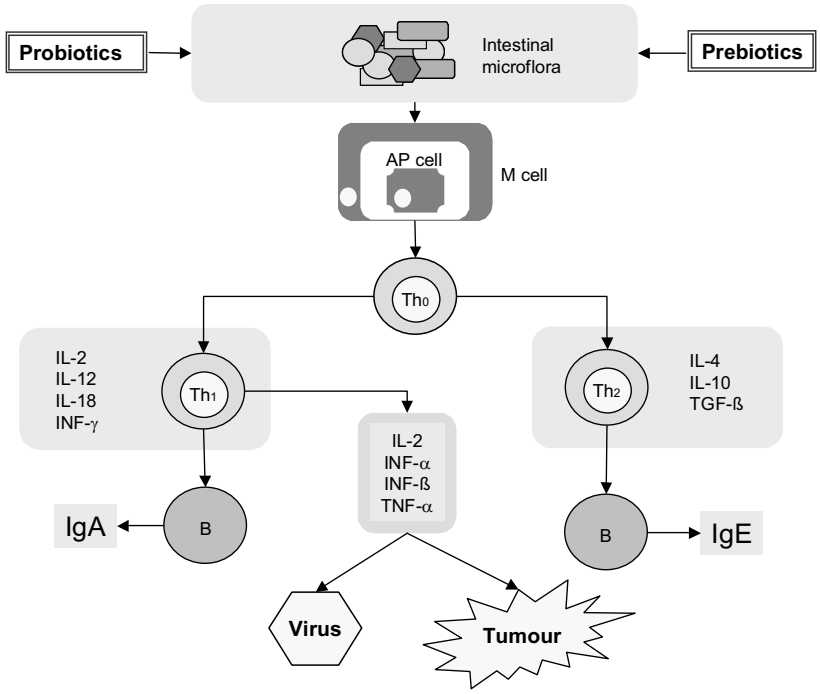

Figure 6-3. Influence on the intestinal immune system of probiotics and prebiotics. For details see text (modified according to reference 32).

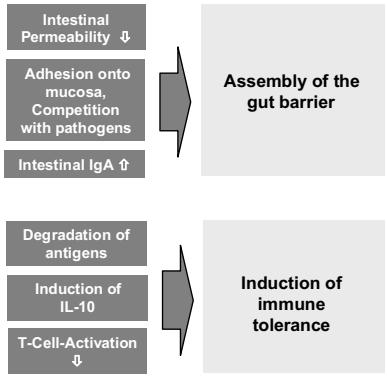

Figure 6-4. Probiotics-proposed mechanisms

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Chapter 7

TRANSENTERIC SIGNALLING: MAMMARY MESSAGES OR WHITE NOISE?

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1. INTRODUCTION

It has long been known that breast-feeding does more than simply nourish the baby. Human milk contains a large number of so-called non-nutritional substances. Those that have been most fully studied are protective factors, such as immunoglobulin A (IgA) and lactoferrin, which have been shown to defend the neonatal gut from the adverse affects of microorganisms and foreign food proteins. However, an abundance of other substances in milk, many 'bioactive' such as hormones and trophic factors, has raised the suggestion that some may be messages from mother, destined to play a role in infant metabolism. To make the journey from maternal breast to infant circulation such 'signals' must pass across the infant gut wall intact on their way to their site of action.

The gut is more than simply a conduit for the intake of food. While the microvillus membrane is furnished with transcellular and paracellular pathways for the transfer of small solutes and other substances, embedded within it are conductance pathways, enzymes, and receptors for a range of bioactive molecules. The microvillous membrane also has defence properties that have been well studied, and a sophisticated way of distinguishing between 'desirable' and 'hostile' substances and of processing them

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accordingly. During early life there are specialised endocytotic pathways for the intact transfer of some substances and the disposal of others.

To determine experimentally whether a substance in milk has a physiological effect in the infant, four conditions must be satisfied: an effect in the offspring must be obtained in response to exposure to the substance in milk; this effect must be abolished by removal of that substance in milk, and restored when it is returned; the substance must be present and active in milk; and it must retain its biological activity in the offspring to the site where it is postulated to act.[1] When considering transenteric signalling, satisfaction of the last condition implies that the substance will pass intact across the gut wall into the infant circulation. The evidence that this may occur is reviewed here.

2. MAMMALIAN MILKS

Milk is a complex, multi-phase fluid within which its various constituents - nutrients, non-nutrients, other chemicals and cells - are partitioned. Human milk contains a wealth of substances which may aid neonatal adaptation to oral feeding, by promoting mucosal defence and function, and by assisting digestion during a period when the infant's own physiological systems are underdeveloped. These factors include antimicrobial and anti-inflammatory agents, digestive enzymes, trophic factors and growth-modulators, cytokines and transport proteins. The chemical forms of these substances includes peptides, proteins, oligosaccharides, nucleotides and other macromolecules, and they are best classified by their known or supposed biologic actions.

The large number of defence factors in milk, and the complexity of the defence systems that protect the newborn gut from infection and assault by other toxic substances, is a reflection of the immunological naivety and immaturity of the newborn. The newborn is immature in other ways too. Several of its organ systems have been hardly used *in utero* – the tasks of the lungs, kidneys and liver are largely performed *in utero* by the placenta, and the nervous system has little but its autonomic function tested before birth. Birth represents a major challenge to the newborn, when these hitherto barely used organ systems must take independent responsibility for the growth and development of the newborn. It is in the mother's interests to assist in this process, which she does by nourishing her baby, not only with nutrients, but also with a range of other milk-borne substances that aid adaptation to life outside the womb.

Biologically active substances in milk are either transported (passively or actively) across the mammary epithelium from the maternal circulation, or are synthesised in the mammary gland. Many are present in concentrations

well above those in maternal blood and some are well recognised as true hormones and/or paracrines. Such non-nutritional bioactive substances in milk may therefore transmit signals from mother to young, which may initiate or modulate gastrointestinal function, may regulate metabolism and may stimulate the immune and endocrine systems of the infant. To reach the site of action these substances must cross the intestinal wall intact.

3. MORPHOLOGICAL DEVELOPMENT OF THE GUT

The intestinal epithelium forms a barrier that permits the selective passage of macromolecules between the gut lumen and portal circulation. The tight junctions between epithelial cells limit paracellular transport of macromolecules and pathogens. Macromolecules that cross this barrier do so via membrane-bound vesicles. The ability of the epithelial barrier to allow some macromolecules to cross whilst selectively excluding others (antigens or pathogens) is critical for normal health and function. During development the immature intestine goes through a phase in which its enterocytes are highly endocytotic.

3.1 Endocytosis

Endocytosis is the process by which surface-bound ligands and fluid-phase macromolecules are internalised by eukaryotic cells. The internalised macromolecules may participate in cellular metabolism or, in the case of some hormones or trophic factors, may regulate intracellular metabolic processes. The endocytotic process is mediated by specialised invaginations of the plasma membrane that pinch off to form vesicles. The best-characterised mechanism for uptake at plasma membranes is via clathrin-coated pits and vesicles. Caveolae are another type of vesicle involved, initially identified as flask-shaped membrane invaginations in endothelial cells and adipocytes. [2]

Sorting, recycling and targetting of endocytosed material are performed by a series of morphologically and functionally heterogeneous membrane-bound compartments known collectively as endosomes (Figure 7-1). Sorting endosomes located at the periphery of the cell are the first compartments entered by internalised ligands and receptors. After sorting has occurred the cisternal elements of sorting endosomes are thought to separate from the tubular elements and to undergo maturation into late endosomes, which involves the selective removal of some proteins and the importation of

others. Lysosomes are the terminal destination in endocytotic pathways. Late endosomes can fuse with pre-existing lysosomes. In contrast to the degradative pathway, recycling receptors and membrane lipids that have been concentrated in the tubular extensions of the sorting endosomes are routed to a second compartment (recycling endosomes) and subsequently returned to the cell surface. The process of internalising, sorting and packaging proteins and targeting them to their appropriate subcellular destination requires complex cellular machinery that is tightly regulated.

The developing gut of some mammals has the capacity for the endocytosis of macromolecules in swallowed fluids, and the absorptive enterocytes are highly specialised for the uptake and processing of these materials. Passive immunity is conferred upon the neonatal rat, for instance, by the selective binding of maternal IgG present in milk, to Fc receptors associated with B_2-macroglobulin in the enterocyte apical plasma membrane. In addition to specific transport of IgG across the epithelium, antigens from pathogens and dietary sources can cross the epithelium in significant amounts – this may be critical for the development of mucosal immunity and tolerance. An important route of antigen uptake is via microfold or M-cells that overlie lymphocytes in Peyer's patches. Transepithelial transport occurs in two directions. Transfer of IgG from the apical plasma membrane to the basolateral membrane forms the basis of passive immunity in some mammals. In contrast IgA (primarily found in mucosal secretions), must be transported from its site of synthesis in the lamina propria into mucosal secretions.

3.2 Endocytotic compartments in the developing intestine

The ontogeny of the gastrointestinal tract follows a common pattern among mammals. An embryological stage of infolding of the endodermal layer to form an alimentary tube is followed by organogenesis when the major organs of the digestive system are formed. There is then a stage of cytodifferentiation, when the cells types of the gut wall become distinct, followed by growth and maturation of the digestive system in preparation for its tasks if independently feeding the newborn. A sucking stage, and then weaning with transition from milk to non-milk diet and the cessation of breast-feeding succeed the neonatal stage of adaptation to milk.[3]

The majority of endocytotic activity occurs after conversion of the developing epithelium from a stratified to a simple columnar epithelium, when the enterocytes form an extensive endocytotic complex in the apical cytoplasm. This apical endocytotic complex is located just beneath the microvillous membrane and is composed of an extensive array of membrane

tubules and vesicles (Figure 7-2). It is found in the enterocytes of many species during development: in the pig, calf and sheep (precocial-born mammals) it is present for a significant time *in utero* and persists for a short time after birth. In the fetal human this complex is present from ten weeks after conception and persists until at least 22 weeks gestational age. In the rat it assembles shortly before birth and persists until weaning at 17-20 days after birth. There are considerable inter-species variations in the timing and duration of expression of such endocytotic systems. [4]

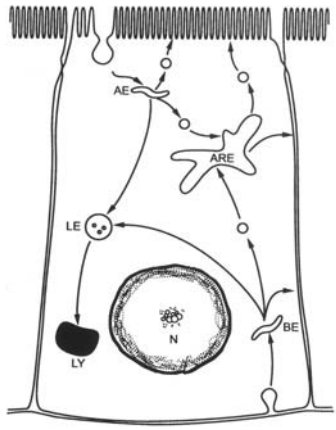

Figure 7-1. Endocytotic pathways in epithelial cell. Endocytosis occurs at apical (luminal) and basolateral (serosal) surfaces, after which vesicles fuse with apical endosomes (AE) or basolateral endosomes (BE). Macromolecules can be recycled to their respective plasma membrane domains or targeted to apical recycling endosomes (ARE) or late endosomes (LE). Macromolecules that are to be degraded are targeted to lysosomes (LY). Modified from reference 2.

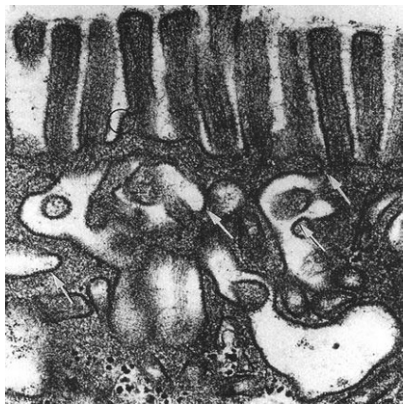

Figure 7-2. Photomicrograph of apex of enterocyte from 17-18 week human fetus showing specialised apical tubular system (ATS). Note the ordered array of particles on the inner membrane of some elements of the ATS (arrows) and on membrane at base of villus (rings). Reproduced from reference 3 with permission.

4. ALTRICIAL AND PREOCIAL MAMMALS

The significance of this apical vesicular-tubular system to the animal depends on the time that it is expressed in the course of gut ontogeny. The offspring of many mammals can be divided into altricial and precocial young[5]. Those with altricial young produce large litters, often in nests, that grow rapidly and reach sexual maturity early. Lifespan is short and brain size is relatively small. A high reproductive rate allows these species to take advantage of favourable conditions and periods of food abundance when rearing their young. The offspring are born in an 'embryological' state early in the course of gastrointestinal development, and they depend on milk not only for energy and nutrients, but also for immunoglobulins, and quite possibly trophic factors and other physiologically active substances that, in precocial newborn, are transferred from mother to young largely before birth via the placenta. [6]

Mammals that produce precocial young are usually large and those that are terrestrial are mainly herbivores. They have a low reproductive rate, long pregnancy and small litters. Their brains are relatively large and social behaviour is complex, especially in primates. Born after a relatively long gestation, milk is mainly a source of nutrients for precocial newborn mammals. In primates the lipids are vital for neurodevelopment. Rapid postnatal brain growth occurs *pari passu* with retention of the young with

mother, and transmission of knowledge and learned behaviour. Non-nutritional substances are transferred from mother to young largely prenatally, via the placenta *in utero*. Born later in the course of gut development, precocial young have a less vital need for milk-borne protective and trophic factors. [6]

4.1 Gastrointestinal tract of altricial and precocial newborn

In altricial newborn mammals the small intestine is adapted for the uptake and utilisation of non-nutritional substances in milk. It is vital for those species that depend on milk for the acquisition of protective and growth factors to have luminal digestive function that does not interfere with this process. Diminished gastric acidity and pancreatic protease secretion permit the survival of intact milk proteins during their passage through the stomach and small intestine. Selection of such macromolecules occurs at the microvillous surface, and further sorting occurs intracellularly. In the jejunum receptor-mediated IgG uptake occurs, and in the ileum, bulk transfer of trophic and other factors. [4] These processes operate both pre and postnatally (see section 5). Whereas some physiologically active molecules are transferred intact across the epithelium to the portal circulation, some are utilised in the cell, and others are digested in intracellular vacuoles.

The postnatal persistence of a sophisticated system for the recognition, uptake, sorting and transport of macromolecules during the suckling period, and the ability of the small intestine to distinguish between physiological and non-physiological molecules, are associated with birth early in the course of macromolecular development. They represent essential adaptation of altricial-born neonates to extrauterine life; for example, fetal rat intestine can transport macromolecules and the gastrointestinal tract retains the capacity to transfer them from milk to the systemic circulation until 'closure' at weaning. In precocial neonates non-nutrient factors are transferred from mother to young prenatally via the placenta *in utero*. The neonatal gut exhibits only a transitory period during which it is capable of intact macromolecular uptake. Systems for the macromolecular transfer operate before birth, but are of slight importance to perinatal adaptation. After birth intracellular sorting, with intraluminal digestion of milk proteins occurs.

In the human neonate an apical tubular system, characterised by invaginations and tubules, adjacent to cytoplasmic vesicles and vacuoles, undergoes considerable differentiation during the second trimester of fetal life, reaching its maximal development around the 20th week. It is replaced by relatively 'mature' enterocytes by the 25th week. [7]

5. EXPERIMENTAL EVIDENCE FOR INTACT TRANSFER OF TROPHIC FACTORS FROM MILK TO INFANT CIRCULATION

While the ontological changes in the morphology of the intestinal epithelium during early life have been well described, there is less research into the physiological effects of milk-borne bioactive substances on the newborn. Most experimental studies designed to define the pathways for the transfer of substances across the developing gut have been performed in the rat. The newborn rat is altricial, as summarised before, and exhibits an apical tubular system from shortly before birth until weaning at around 20 days, when 'intestinal closure' occurs. Trophic factors have many of the properties that suit them to the roles of chemical messengers that could transmit signals across the gut.

5.1 Epidermal growth factor

Epidermal growth factor (EGF) is a polypeptide of molecular weight 6400 kD made up of 53 amino acids, found in salivary and Bruner's glands, in colostrum, milk, amniotic fluid and urine. It can bind to a specific receptor on the cell membrane, where it activates tyrosine kinase, and through a series of intermediate reactions, stimulates DNA synthesis. It thereby has mitogenic, trophic, cytoprotective and antisecretory effects. These four biologic actions are particularly relevant during the perinatal period of adaptation from intrauterine to extrauterine life. EGF is found in murine milk, and it resists degradation by gastric acid and proteolytic enzymes. When given intraperiotneally to pregnant mice EGF accelerates maturation of brush-border enzymes. [8] It also stimulates DNA and protein synthesis when applied topically to organ cultures of fetal small intestine.[9] Postnatally EGF is trophic to the small intestinal mucosa when administered systemically, [10] but given orogastrically it is trophic only when co-administered with a protease inhibitor or a 'competitive substrate' such as casein. [11] These studies suggest that, although EGF can 'survive' in the gut mucosa and be active at the brush-border, it also traverses the gut wall, whence it can act systemically. Indeed it appears that some of its actions, including those on the gut mucosa, are mediated only if it first reaches the systemic circulation.

5.2 Animal studies

Newborn rat pups fed artificial formula containing EGF had a significant increase in hepatic incorporation of ^{3}H-thymidine into DNA compared with pups not fed EGF. Moreover, EGF-fed pups had heavier hearts and kidneys than pups fed no EGF. These data suggested that ingestion of EGF is associated with the growth of extra-intestinal organs. [12]

A number of studies suggest that the gastrointestinal tract of the rat is capable of taking up EGF both prenatally and postnatally, after which it may pass intact into the circulation and reach the liver and peripheral tissues. EGF was measured in amniotic fluid of pregnant rats by radioimmunoassay. It was infused into loops of proximal and distal fetal rat intestine, which were later removed and processed for electon microscopy. Using an anti-rat EGF antibody, followed by gold-labelled goat anti-rabbit immunoglobulin, EGF was found membrane-associated along the luminal surface of microvilli, within apical invaginations and endosomal compartments, free from the membrane in multivesicular bodies, within large clear vesicles, basal vesicles and in association with the basolateral membrane and beyond (Figure 7-3). [13]

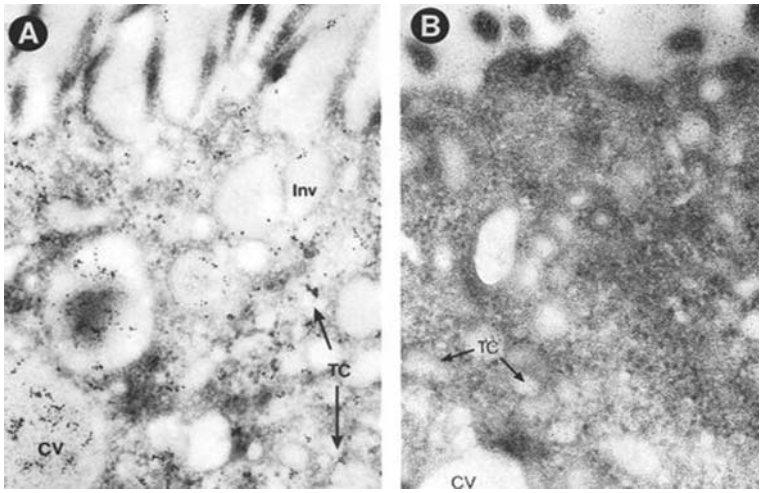

Figure 7-3. Electron micrograph of apical region of enterocyte of distal small intestine of fetal rat. A: localisation of EGF in LR gold-embedded tissue. EGF is membrane bound, along the microvilli, apical invaginations (Inv) and throughout the tubulocisternae (TC). EGF is detected free from the membrane in large clear vesicles (CV). B: EGF is not seen when non-immune serum is substituted for immune serum. (From reference 13 with permission).

To determine whether absorptive cells of suckling rat ileum selectively transport EGF, uptake of ^{125}I-EGF from ileal loops was studied by autoradiography. Specific binding sites for ^{125}I-EGF were localised by electron microscope on apical membranes of ileal epithelial sheets *in vitro*. Radiolabelled molecules were concentrated in apical endosomal compartments and were also associated with lysosomal vacuoles, basolateral cell surfaces, and lamina propria (Figure 7-4). All radiolabel in blood and liver represented breakdown products.[14] These two studies showed that EGF is transported across the epithelium of fetal rats by an endocytotic process in both the upper and lower small intestine, and is selectively transported across the ileal epithelium in suckling rats, but is modified during transport.

To investigate the distribution and the degradation of EGF, the rat bile duct was cannulated and ^{125}I-EGF injected into the femoral vein. High levels of radioactivity were found in the liver (57% of total administered) and small intestine (10%). 34%-70% of radioactivity in bile and liver and 20%-41% of radioactivity in the small intestinal wall and contents were capable of binding to EGF-specific receptors. The study suggested that systemic EGF is rapidly taken up by the liver and the gastrointestinal tract and secreted into the bile and intestinal luminal contents of suckling rats in form(s) capable of binding to anti-EGF antibody and EGF-specific receptors.[15] The stability and distribution of orally administered ^{125}I-EGF was examined in newborn and 5-day-old pigs. Over 95% of recovered radioactivity was found in the gastrointestinal tract, of which 78-86% was found in the luminal contents with the remaining found in the gastrointestinal wall. Substantial amounts of EGF recovered from the luminal contents (63-86%) and the gastrointestinal wall (42-81%) remained 'intact'. These results indicate that most of orally ingested EGF remained in the gut after oral ingestion, and significant amounts remained biologically active.[16]

Whether or not EGF also influences postnatal hepatic development by affecting nonparenchymal cells or by modifying intrahepatic blood is not clear. Suckling rats fed rat milk substitute (RMS) with and without EGF were compared to pups breast-fed for 14 days. The livers of anaesthetised pups were examined by *in vivo* microscopy to determine the numbers of sinusoids with flow (SCF) in each of 10 microscopic fields and the numbers of phagocytic Kupffer cells (KC) in the same fields following an intraportal injection of fluorescent 1-micron latex particles. In pups fed RMS without EGF, SCF and KC/SCF was 75 and 45%, respectively, of that in maternal-fed animals. The addition of EGF to the RMS restored SCF and KC/SCF nearly to the levels measured in breast-fed pups. The results suggest that milk-borne EGF plays a role in the development of KC phagocytic function

and affects the amount of blood that perfuses the sinusoidal bed in the suckling rat.[17]

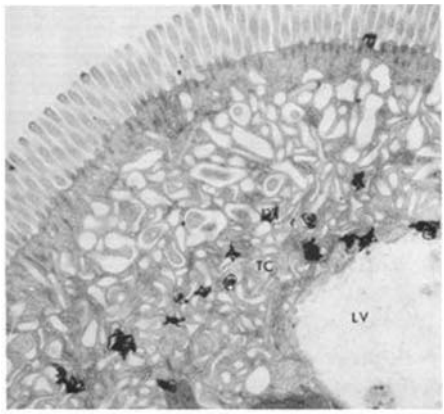

Figure 7-4. Electron microscope autoradiograph of apical region of ileal enterocyte of suckling rat. Labelled ^{125}I EGF is seen throughout endocytotis pathways, in lysosomal vacuole (LV), basolateral regions and apical tubulocysternae (TC). (From reference 14 with permission).

In summary, rat studies indicate that there appear to be two distinct intestinal epithelial endosomal compartments involved in the transfer of membrane-bound or fluid-phase macromolecules to the giant lysosome, and that these cells are capable of sorting internalised macromolecules. Growth factors, such as EGF, which are present in salivary secretions, milk and amniotic fluid, can be selectively internalised by jejunal and ileal cells, transported across the epithelium, and released intact into the circulation in biologically significant amounts. They may also have an effect on liver growth and hepatic function.

5.3 Human studies

Evidence for an active role of orally ingested EGF on systemic metabolism during early life in man is not strong. Human milk contains many hormones, hormone-like peptides and trophic factors.[18] The gastrointestinal tract of newborn infants exhibits lower proteolytic activity than in adults and higher 'permeability' for some substances.[19] However the endocytotic pathways that are present in early fetal life and characterise the suckling rat gut, are not present after term birth. EGF is present in human amniotic fluid,[20] colostrum and milk [21-22] in significant concentrations, and

there are EGF receptors on the epithelial cells of the fetal stomach, small intestine and colon.[23] Concentrations of EGF in colostrum and milk are many-fold higher than in serum. EGF resists degradation in the human gastrointestinal tract [24] and is found in greater concentrations in the urine of breast-fed than formula-fed infants.[25] There are no published studies of the effects of milk-borne EGF on the internal extra-intestinal organs of the human infant.

6. BREAST AND GUT: SIGNIFICANCE OF EXPERIMENTAL EVIDENCE IN BIOLOGICAL CONTEXT

All mammals have conserved lactation as an essential part of reproduction, and it has been adapted to serve a wide range of early life course strategies. The co-evolution of breast and neonatal gut has led to the multitude of ways in which mother's milk helps to protect the baby against infection, and assist in its adaptation to extrauterine life. The lactating mammary gland and neonatal digestive systems are complementary in structure and function. The epithelium of the breast is the site of the synthesis and secretion of the constituents of milk. Conversely the epithelium of the gut is the site of the digestion and absorption of substances in milk. The human baby is born relatively 'mature', in comparison with the young of other mammals. Its precocity means that it is supplied with defence factors in the form of IgG, for instance, in the womb, transplacentally, in contrast with the young of species that are altricial at birth, born early in the course of gastrointestinal development. They depend on a ready supply of protective factors from mother, after birth in milk.

During the course of human gastrointestinal development the fetal gut passes through a phase during which it appears to be adapted for the uptake and intact transfer of biologically active macromolecules. However this is transient, occurs well before term, and illustrates the significance of time of birth in the ontogeny of the digestive system on the biological importance and contribution of milk to infant development. The occurrence of birth later in the course of gastrointestinal development, in precocial as opposed to altricial newborn, is associated (Fig 7-5) with a restriction of the functions of milk feeding from a means of delivering to the newborn a wide range of bioactive substances (immunoproteins, trophic factors and other substances that compensate from immature neonatal organ function), to the provision of nutrients alone.[6] Care must be taken in assuming that a process occurring at a

particular stage in development in one species occurs at a comparable time and serves a homologous purpose in another.

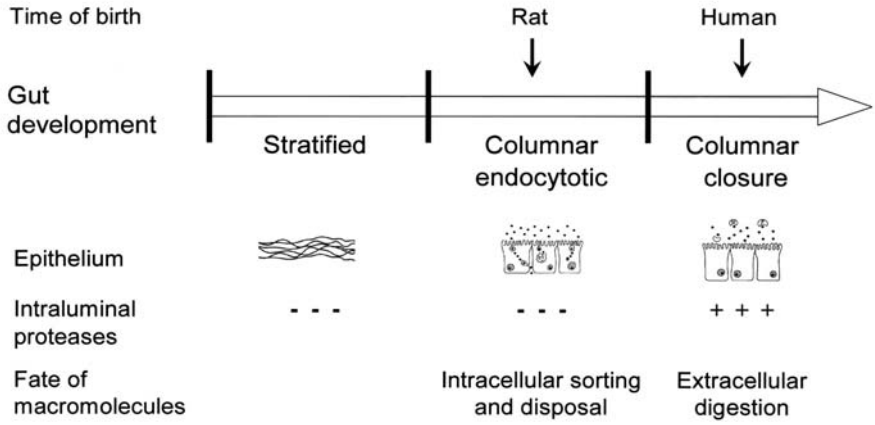

Figure 7-5. Time of birth in relation to course of intestinal epithelial differentiation.

Table 7-1. Evidence that milk-borne hormones and trophic peptides act as transenteric signals from mother to young in man

In support
Present in colostrum and milk in 'physiological' concentrations
Plausible biological role in neonate
Important in other (altricial-born) mammals
Some resist digestion in gut and may be found in urine
Not supportive
Human neonate is relatively precocial at birth
Major phases of gastrointestinal development undergone before birth
Human neonatal intestine lacks pathways for macromolecular transfer
Generation of infants reared on non-human milk formula

Table 7-1 summarises the evidence that bioactive substances in human milk may cross the neonatal gut and act as chemical messengers from mother to baby. Transenteric signalling may serve a biologic purpose in the reproductive strategies of some mammals, particularly those that bear altricial young. Just as hormones and trophic factors act as chemical messengers within the organism, to send signals between organs and effect changes at their targets, so too may they afford communication between mother and young during a critical period when the latter is dependent on the former. As summarised above, the gastrointestinal tract of many species,

endowed with a sophisticated system for the recognition, uptake, sorting and disposal of macromolecules, is adapted, during suckling, to allow the free and intact passage of some milk-borne substances across it. For EGF the animal studies summarised together provide data that fulfil the postulates proposed by Peaker and Neville[1]. In altricial young transenteric signalling may indeed allow mammary messages to pass from mother to its internal organs. In man, it is more likely that the abundant hormones and other bioactive peptides in breast-milk play an insignificant or minor role in neonatal adaptation to extrauterine life, and should be regarded as little more than 'white noise'.

ACKNOWLEDGEMENTS

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Chapter 8

IMMUNE FUNCTION

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1. INTRODUCTION

Human milk contains numerous components, which have been linked to immune functions. Initially it was easy to recognise such constituents as classical members of the immune system, for example antibodies and lymphocytes, but today the list is very long. This is due to the fact that we have learnt for one thing that certain nutrients, for instance polyunsaturated fatty acids, and immune components often cooperate and/or direct each other, as exemplified below. Another reason is that the central nervous system and the immune system, which developmentally have different origins, in fact are in several modes intertwined in their functions. This may be illustrated by the fact that hormones such as leptin and growth hormone have a cytokine structure and that certain cytokines such as the pro-inflammatory IL-1β, TNF-α and IL-6 activate the HPA stress axis and induce production of glucocorticosteroids[1], as well as leptin.[2]

Another aspect is based on observations that breast-feeding has several long-term effects on the infant child, such as defence against certain infections and immunological diseases such as allergy. The basis for such effects seems to be both nutrients and immune factors in the mother's milk. Some recent work showing examples of relations between milk components and short, as well as long term effects on the infant child's health are

G. R. Goldberg et al. (eds.), Breast-Feeding: Early Influences on Later Health,

described below. These studies also illustrate that the many and and various forms of host defence provided via human milk have one capacity in common: they defend without inducing inflammatory mechanisms, which via cytokine release and phagocyte activation cause clinical symptoms like pain, increased temperature, tissue damage, loss of appetite and increased energy consumption.[3] Some milk components even actively counteract inflammatory processes.

2. ESSENTIAL FATTY ACIDS, BREAST-FEEDING AND IMMUNOLOGICAL EFFECTS

There is much confusion as to the possible role of essential fatty acids (EFAs) in the maternal diet and the risk of the breast-fed offspring in developing allergic diseases. For instance a recent study from Australia did not show that higher levels of *n*-3 fatty acids in maternal milk decreased the risk of developing allergic disease in breast-fed infants.[4] Previously an increased risk of developing allergy has been linked to low levels of *n*-3 fatty acids in the milk.[5-7] It could be, however, that it is not the absolute levels of these EFAs which are crucial, but the ratio between the n-6/n-3 fatty acids. This was suggested by an initial experimental study where we gave various levels of EFAs to rat dams during late pregnancy and lactation and investigated the effect of this diet on the development of neonatal tolerance in the offspring to a foreign protein, ovalbumin (OVA), given perorally to the dams and reaching the rat pups via the milk. A diet deficient in EFAs (7% hydrogenated lard) enhanced development of neonatal tolerance, not seen with a diet sufficient in EFA (7% soybean oil). The tolerance seemed to be mediated by regulatory T cells producing TGF–β. Increased levels of RNA for this suppressive cytokine was found in the local lymph glands.[8] The study was repeated with diets with defined ratios of *n*-6/*n*-3 EFAs. A diet with the ratio of 0.4 resulted in neonatal tolerance against the OVA, but also against an unrelated antigen human serum albumin.[9] This agrees with effects of regulatory T cells functioning via TGF-β. The tolerance included cell-mediated immunity as well as IgM, IgG and IgE antibody responses. With a ratio of 9, in contrast, neonatal tolerance did not develop against the food antigen given.

It seems relevant to test whether or not the ratios of *n*-6:*n*-3 EFAs in the food given in early life to babies via the milk has effects on the risk of developing allergic disease. Formulas today have ratios of *n-6*:*n*-3 closer to 9 rather than 0.4. Likewise the food of breast-feeding mothers may need to be analysed further from this point of view, not just studying *n*-6, or *n*-3 fatty acids as often done.

3. ANTI-SECRETORY FACTOR

This protein was discovered because it prevents the secretory effects of cholera toxin in the gut.[10-11] It also seems to have anti-inflammatory effects in inflammatory bowel disease.[12-13] We have previously shown this hormone-like structure to be present in milk from Guatemalan and Pakistani mothers, but usually not from Swedish mothers.[14]

In a recent small study we investigated the possibility of inducing the anti-secretory factor in the milk of Swedish mothers using a specially treated cereal known to promote the production of anti-secretory factor appearing in serum. Even in the limited group studied we could show how effectively the factor could be induced. Furthermore it turned out to significantly protect against the appearance of mastitis in these lactating mothers.[15] It is of course of major interest to expand these studies to investigate if the anti-secretory factor in the milk may protect the offspring from e.g. diarrhoea. Ongoing studies in Pakistan giving children with diarrhoea egg yolk from hens fed the special cereal and therefore containing the factor, may show us if there is also a treatment effect from giving the ready made factor.

4. LACTOFERRIN

This major human milk protein has been much studied and is well known for its anti-microbial effects, although there are no clinical studies to prove its protective capacity in the breast-fed infant. Such studies would be difficult to do. In recent work we have defined the bactericidal capacity of the whole molecule and of various peptides based on the 12-15 aminoacid long surface-exposed αβ region at the N-terminal end of the molecule, which have such effects.[16] Using these peptides, as well as the whole molecule, we have studied their anti-infectious and anti-inflammatory effects in mouse models. Giving dextran-sulphate to induce colitis in mice we could show that lactoferrin and certain of its peptides given perorally significantly decreased the number of CD4 positive cells, F4/80 positive macrophages and TNF-α producing cells in the colon mucosa. The level of IL-1β in the blood was also reduced, as was the typical shortening of the colon.[17] In parallel there was a reduced number of cells producing the immunosuppressive cytokine IL-10 in the colonic submucosa. The anti-inflammatory effect of lactoferrin is presumably due both to its anti-infectious effect and its capacity to inhibit the transcription factor NFκB, which is instrumental in bringing about the production of the main pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in leucocytes.[18]

In a model of urinary tract infections caused by *Escherichia coli* in mice orally given human lactoferrin, it appeared in the urine 2 hours later and killed bacteria. The urinary tract infection was significantly prevented compared to animals just given the vehicle ($p< 0.009$ for kidneys, $p< 0.0001$ for bladder). The number of urinary leucocytes and IL-6 levels in blood and urine were also reduced. One of the tested lactoferrin peptides also provided perorally gave significant protection.[19] These experimental data agree well with clinical observations showing that breast-feeding protects against urinary tract infections.[20]

It is likely that the protection against urinary tract infections via lactoferrin is also supported by the milk secretory IgA antibodies since they coat the gut bacteria, which are the major cause of such infections.[21] Such coating would prevent the bacteria from passing through the gut mucosa infecting via the blood as may occur in early infancy. It would also prevent the gut bacteria from climbing the urinary tracts from below, which is the most common route of infections in the urinary tract.

Lactoferrin has been reported to have several enzyme activities, including that of ribonuclease.[22] We asked the question whether or not bacteria killed in the gut, for instance by lactoferrin, might leak nucleotides and that these may be degraded and taken up in the intestine. In preliminary experiments in germfree rats we could actually see that addition of nucleotides to the diet enhanced their weight gain significantly and increased mitosis in enterocytes.[23] Human milk in addition of course contains nucleotides.

In further ongoing studies we find evidence that lactoferrin shows some capacities in similarity to heat shock proteins (HSPs), which are very conserved structures appearing in cells under stress. In similarity to HSPs lactoferrin seems to bind to ATP and it also has ATP-ase activity, although weak.[24] It might be possible that lactoferrin can interact with ATP coming from bacteria killed by anti-microbial agents, possibly such as lactoferrin.

Many components of human milk influence the microbial gut flora of the breast-fed infant, contributing to the microflora, which is the main stimulus for the expansion of the immune system, which is tiny, but more or less complete at birth.[25] This flora also has a central role in making the expanding immune system able to develop immunological tolerance against foods, pollen, autoantigens etc. This is the background to the Hygiene hypothesis, claiming that today neonates are often not permitted to be colonised with the proper microflora, originating mainly from being delivered next to the mother´s anus, like all mammals.[25] In addition many milk components contribute to the development of this flora. Lactoferrin for instance seems to promote growth of Bifidobacteria and hinders adherence of enteropathogenic *E. coli* to the gut mucosa.[26] Through its serine protease activity lactoferrin cleaves two colonisation factors of *Haemophilus influenzae*,[27] presumably

decreasing their appearance on, and pathogenic attachment to respiratory epithelium.

5. HUMAN MILK ANTIBODIES

The only milk components proven in clinical studies to protect against infections are secretory IgA (SIgA) antibodies directed against various intestinal pathogens such as *Campylobacte* and *Vibrio cholerae*. [25] It is likely that there is similar protection against respiratory infections and, as already mentioned, urinary tract infections, due to the capacity of the milk SIgA antibodies in preventing microbes attaching to the various cellular structures on mucosal membranes, which they use to initiate the contact with the host. Many milk oligosaccharides and glycoconjugates act in a similar way by appearing as analogues to the structures on mucosal epithelium that microbes need to bind to for starting an infection.[28] In the next step the microbes would invade deeper tissues where they meet circulating antibodies like IgM and IgG, as well as T cell mediated immunity. These forms of defence always activate phagocytes and result in production of pro-inflammatory cytokines giving clinical symptoms such as local pain, fever, loss of appetite, inflammatory tissue damage and increased energy consumption.

Human milk contains numerous anti-inflammatory factors.[29] Defence against both infection and inflammation is provided via human milk. Host defence via serum antibodies, the complement system and phagocytes regularly induce inflammation when activated. In early infancy the complement system, as well as the phagocytes are not fully functional. [25] Thus the transplacental IgG antibodies may not provide efficient defence. This may be why neonatal septicaemia/meningitis is such a threat to non-breast-fed infants. [30]

Due to the entero-mammaric link of the lymphoid cells producing the milk SIgA antibodies, they are directed against the microbes passing through the mother's intestine.[31] These include microbes not only in the gut microflora, but also swallowed respiratory tract pathogens. As a result the maternal milk provides protection against a very broad spectrum of microbes present in the mother's milieu. It is remarkable how SIgA antibodies of a very broad spectrum of specificities remain present throughout lactation, for instance against numerous *E. coli* O and K antigens, regardless of bacteria with those antigens were present in the gut at the time of sampling the milk.[32-33] It is not quite clear whether this represents antibodies also produced by memory cells to maintain significant levels against very many antigens throughout lactation. Usually SIgA responses are expected to be of

a relatively short duration and be dependent on re-exposure to be kept up. Whereas Swedish infants usually showed a rather stable microflora in the gut with respect to *E. coli* strains, Pakistani children had a rapid turnover of various serotypes of *E. coli*.[34-36] It is probably of great help for the infant that the milk can contain SIgA antibodies of such a wide range of specificities and remain throughout lactation.

The milk SIgA antibodies promote growth of *E. coli* with type 1 adhesins, because they bind to carbohydrate side chains of such antibodies.[37-38] These *E. coli* are generally of low virulence. Recently it has also been suggested that SIgA antibodies favour the formation of a biofilm of microbes on an epithelial surface.[39] This may favour normal microbial colonisation of the breast-fed infant.

We found SIgA and IgM antibodies to *E. coli* and poliovirus in saliva and meconium samples obtained during the first day of life in Swedish newborns.[40] It should be added that poliovirus does not exist in Sweden because of general vaccination with killed poliovirus vaccines. These antibodies did presumably not originate from their mothers, since they were also present in the offspring of a mother with hypogammaglobulinemia, (lacking IgA and IgM) and of mothers with IgA deficiency.[41] Normally IgA and IgM do not pass across the placenta. The SIgA and IgM antibodies in the neonates may have been induced by anti-idiotypic antibodies specific for *E.coli* and poliovirus, because we found such antibodies in serum and milk from Swedish mothers.[42] In an experimental model we even found evidence that anti-idiotypic antibodies given in the neonatal period could enhance specific immune responses over two generations.[43] Such long-term enhancement of immune responses may take place via transfer of maternal anti-idiotypic antibodies via the placenta, as well as the milk.

6. α-LACTALBUMIN

Recent work by Svanborg *et al.* has shown that the major milk protein α–lactalbumin can take the shape of HAMLET, human α-lactalbumin made lethal to tumour cells. In small aggregates of α-lactalbumin exposed to the low pH in the stomach, the protein is unfolded and forms a binding site for oleic acid, which may originate from triglycerides degraded in the stomach. In this form HAMLET kills cancer cells. This activity has been demonstrated against some 40 malignant human cell lines.[44] *In vivo* it was found that HAMLET killed human glioblastoma cells in an animal model. Most recently a clinical effect was found against human papilloma.[45] Human glioblastoma cells in brain xenografts were also killed by HAMLET.[46]

Presently it is not clear whether the fact that breast-feeding provides significant protection against breast cancer is in any way is linked to the effects of HAMLET.[47] It is unknown if breast-feeding via HAMLET can protect against tumours in infants. There have been some suggestions that breast-feeding may decrease the risk of e.g. childhood leukaemia and some other tumours, but this is debated.[25, 48]

7. MATERNAL SIGNALS FROM THE MOTHER TO THE INFANT: CYTOKINES, HORMONES, GROWTH FACTORS ETC

Some studies suggest that breast-feeding may enhance vaccine responses in the offspring.[49-52] Other studies have not seen such effects.[53-57] It is not really surprising that different outcomes have been obtained. Different vaccines have been applied in different populations with varying pre-vaccination degrees of exposures and immune responses and with different length of breast-feeding. The transplacental IgG antibodies in the infant may inhibit vaccine responses relating to their levels.[58]

One explanation for a stimulatory effect of breast-feeding may be that the milk may contain anti-idiotypic antibodies against the vaccine agents as mentioned above.[42] The long-term enhancement of the IgG2 antibody response *to Haemophilus influenzae* type b (Hib) in breast-fed children may be due to the IFN-γ in the milk specifically enhancing this kind of protective antibody production. This is seen especially against bacterial polysaccharide capsules, which are important virulence factors on pneumococci, meningococci, certain *E. coli* and Hib.[59] This observation agrees with the finding that exclusive breast-feeding for >13 weeks compared with < 13 weeks resulted in enhanced protection against invasive infections with *H. influenzae* type b for up to 10 years.[60-61] In agreement with this higher levels of antibodies to Hib in preschool children related to the longer duration of breast-feeding.[62] The enhancement of the protective IgG2 anti-Hib responses correlated with the appearance of the idiotypes Id-1 and Id-2 identified for the anti-Hib, indicating a rather specific enhancing effect of breast-feeding.[63] Recent evidence suggests that breast-feeding for >90 days results in a higher proportion of children who up to 13 months of age have protective levels of antibodies to Hib and to pneumococcus 6B, compared to those breast-fed for a shorter period (Silfverdal and Ekholm, this volume abstract A62).

The higher levels of IFN-γ in breast-fed infants compared to non-breast-fed, who attract RSV infection may relate to one or some of the many signals in the milk.[64] That may also explain the higher levels of IgA and lactoferrin

in the urine of breast-fed infants and the elevated niveau of SIgA in the saliva during the first 6 months of life in breast-fed compared to non-breast-fed infants.[65] For instance the TGF-β in the milk may be responsible for this. High levels of TGF-β in the milk may also help prevent or delay allergic disease [66], possibly since this cytokine is anti-inflammatory and lower levels of TGF-β1 are related to higher IgE production against cow´s milk protein by B cells from cow's milk protein allergic children.[67] In contrast high levels of IL-4 in milk relates to higher IgE production. Furthermore TGF-β levels in the mothers´ milk were inversely related to the risk of the infant to develop wheezing.[68] This would agree with another recent observation: a significant positive relation was found in milk between TGF-β2 and the proportion of polyunsaturated fatty acids and a negative association between TGF-β2 and saturated fatty acids.[69] This again may be considered to agree with our results in the experimental model where a low ratio of *n*-6:*n*-3 fatty acids given to pregnant and lactating rats favoured the development of neonatal tolerance to a food protein in the offspring, as described earlier.[9]

Actually, many factors in milk may be linked to the risk of the offspring developing allergic disease. A recent finding suggested that the presence of subclinical mastitis relates to the risk of atopic disease and positive skin-prick-tests in babies.[70] Possibly this results from an increased exposure of the breast-fed offspring to allergens from the mother via the milk.

The fact that the thymus of fully breasted infants reaches twice the size of the thymus of non-breastfed infants illustrates another effect of signalling via the milk.[71] In fact the content of IL-7 in the milk relates to the size of the thymus.[72] This cytokine also enhances the development in the Tγδ cells, which are found as intraepithelial lymphocytes in the gut.[73-74] A larger thymus shows a higher output of T cells and a small thymus at birth predicts a higher infant mortality from infections in a study from Africa, independent of other factors known to reduce the size of the thymus, such as low birthweight and malnutrition.[75] A small thymus due to undernutrition expands on refeeding. It is not known whether IL-7 in milk is also instrumental in this effect on the thymus, but it is likely that leptin is, as detailed below.

There are numerous other cytokines in milk and also some soluble cytokine receptors, such as TNF-αR, which may modify the effects of the TNF-α in the milk.[76] The TNF-α enhances the production of the polyIgR, which transports IgA dimers into milk. The IL-6 in milk, like the TGF-β, enhances the development of B lymphocytes and also the production of α1-antitrypsin by phagocytes, possibly explaining why this protein is present in the stools of breast-fed infants.[77-78]

Human milk contains many hormones and growth factors. Some of these have effects on the immune system. A striking example of this is leptin,

which has the structure of a cytokine. Leptin is increased by the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, which is one explanation why infections usually lead to a loss of appetite.[2] Leptin stimulates the differentiation and proliferation of haematopoietic cells, increases the functions of monocytes/macrophages and influences T cell functions. Leptin also stimulates proliferation and survival of T cells in the thymus.[2] Insulin-like growth factor, epidermal growth factor and prolactin in milk seem to act as immuno modulatory components in the gut.[79] Such factors as well as the many anti-inflammatory factors demonstrated in human milk provide an interesting area where many more biologically interesting functions may be waiting to be defined.

8. MILK LYMPHOCYTES

Several experimental studies, recently summarised, suggest that there may in fact be a transfer of lymphocytes via the milk to the offspring.[80] It really seems as if there can be an uptake not only of the cells by the lymphoid system, but even a transfer of immune capacity to judge from experiments in sheep showing transfer via milk of priming of a vaccine response.[81] In humans the observation showing that the child develops tolerance to the mother's HLA, may help explain how the maternal lymphocytes may be accepted by the infant's immune system.[82]

9. LONG TERM EFFECTS OF BREAST-FEEDING ON PROTECTION AGAINST CERTAIN INFECTIONS, TUMOURS, OBESITY ETC

The previously mentioned possible effects on the immune system of the offspring via milk cytokines, growth factors, anti-antibodies, the possible transfer of immunological information via sub-cellular elements from milk lymphocytes and so on, may help explain the observations that the protection afforded by breastfeeding against certain infections may be long lasting. The first observation was made by Saarinen.[83] She noted an enhanced protection by breast-feeding against otitis media lasting for the 3 years of observation. Similar observations have since been reported for gastroenteritis, and respiratory infections for up to 7 years.[84] The improved protection by breast-feeding against invasive Hib infections was still present 10, but not 15 years later and paralleled increased levels of protective IgG2 antibodies up to school age.[61] This may be linked to the long term enhancement of the IgG2

antibody response against protective *H. influenzae* type b by breast-feeding previously mentioned.[59] One study supports the suggestion that breast-feeding resulted in enhanced protection against urinary tract infections for the first 2 years of life.[20] A protective effect against more severe manifestations of clinical measles by breast-feeding lasting up to the age of 10 years was recently proposed (Silfverdal and Montgomery, this volume, abstract A63).

Long-term protection against allergic disease has been much debated and questioned, but recent critical studies provide evidence that breast-feeding offers significant protection, although weak, against both wheezing, asthma and eczema.[85-87]

Similarly, long-term protection by breast-feeding has been claimed against obesity as recently summarised.[25] It is likely that further work on long-term effects of breast-feeding, especially on protection against infections and inflammatory and certain other diseases will provide additional interesting information.

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Chapter 9

FLAVOUR PROGRAMMING DURING BREAST-FEEDING

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1. INTRODUCTION

As omnivores, humans exploit a wide range of potential plant and animal foods. Thus, developmental processes must act to ensure that an individual is not restricted to a narrow range of foodstuffs by virtue of few preferences and strong aversions for novel foods.[1] Nevertheless, a cursory view of cultural differences in cuisine serves to show that not all of the potential foods available to individuals within a culture are actually consumed. Individual experiences, which can be conceived as the mechanism by which one absorbs culture, must serve to specify acceptable and preferred foods.[2]

After defining the concept of flavour, this essay summarises the insights gleaned from scientific research on the sensory capabilities of the human infant and describes one of the first ways that mothers pass on to daughters and sons their gastronomic culture. This body of research reveals that, like other mammals, the foods eaten by women during pregnancy and lactation flavour amniotic fluid and mothers' milk, respectively. Such experiences bias acceptance of particular flavours and may 'program' later food preferences.

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2. DEFINITION OF FLAVOUR

Flavour, a powerful determinant of human consummatory behaviour throughout the lifespan, is a product of several sensory systems, most notably those of taste and smell. The perceptions arising from these two senses are often confused and misappropriated,[3] with such sensations as vanilla, fish, chocolate and coffee being erroneously attributed to the taste system, *per se*. In fact, there are only a small number of primary taste qualities (e.g., sweet, salty, bitter, sour and savoury tastes) which can be perceived in all areas of the tongue. Smell sensations, on the other hand, encompass thousands of diverse qualities, some of which are noted above. The receptors for the olfactory system, located high in the nasal chambers, are stimulated not only during inhalation (orthonasal route), but during sucking in infants and deglutition in both children and adults, when molecules reach the receptors by passing from the oral cavity through the nasal pharynx (retronasal route). It is this retronasal stimulation arising from the molecules of foodstuffs that leads to the predominant flavour sensations.

3. ONTOGENY OF FLAVOUR PERCEPTION

Emerging scientific research revealed that the taste and olfactory systems are well developed before birth (see reference 4 for review). The apparatus needed to detect taste stimuli make its first appearance around the 7th or 8th week of gestation, and by 13 to 15 weeks the taste bud begins to morphologically resemble the adult bud, except for the cornification overlying the papilla.[5] Taste buds are capable of conveying gustatory information to the central nervous system by the last trimester of pregnancy, and this information is available to systems organising changes in sucking, facial expressions, and other affective behaviours.

Likewise, the olfactory bulbs and receptor cells needed to detect olfactory stimuli have attained adult-like morphology by the 11th week of gestation. Olfactory marker protein, a biochemical correlate of olfactory receptor functioning in fetal rats[6], has been identified in the olfactory epithelium of human fetuses at 28 weeks of gestation.[7] Because the epithelial plugs that obstruct the external nares resolve between gestational weeks 16-24, there is a continual turnover of amniotic fluid through the nasal passages such that by the last trimester of pregnancy, the fetus swallows significant amounts of amniotic fluid, inhaling more than twice the volume it swallows. Even in air-breathing organisms, volatile molecules must penetrate the aqueous mucus layer covering the olfactory epithelium to reach receptor

sites on the cilia. Thus, there is no fundamental distinction between olfactory detection of airborne versus water-borne stimuli.

One source of chemosensory stimulation during fetal life is the maternal diet. That is, the environment in which the fetus lives, the amniotic sac, changes as a function of the food choices of the mother since dietary flavours are transmitted and flavour amniotic fluid.[8-10] As will be discussed in the next section, some of these same flavours are also transmitted to breast-milk.[11-16]

4. BREAST-FEEDING: A FLAVOUR LINK TO AMNIOTIC FLUID

Research in a variety of non-human mammalian species revealed that young animals learn about the dietary choices of their mother via flavour cues in amniotic fluid and mothers' milk and, in turn, develop preferences for these flavours. This chemosensory information has been shown to facilitate the transition to solid foods. For example, the growth rate of weanling pigs improved when a flavour that had been incorporated into the sow's feed during lactation was added to the weanling's feed.[17] Moreover, weanling animals actively seek and prefer the flavours of the foods eaten by their mother during nursing[18-20] and are more likely to accept unfamiliar flavours if they experience a variety of different flavours during the nursing period.[21] This learning occurs when flavours are experienced during either gestation or lactation.[20] Such redundancy of dietary information may be important biologically because it provides complementary routes of transferring information on the types of foods available in the environment, should the mothers' diet change during the course of pregnancy and lactation. At weaning, the young animal is faced with learning what to eat and how to forage. Exposure to dietary flavours in amniotic fluid and mother's milk may be one of several ways that the mother teaches her young what foods are "safe".[22] Consequently, young animals tend to choose a diet similar to that of their mothers when faced with their first solid meal.

During the past 15 years, we and others have been asking similar questions of the human mother-infant dyad. This research has shown that experiences with flavours in amniotic fluid lead to increased enjoyment and preference for these flavours at birth [9-10] and during weaning.[16] Some of these same flavours will later be experienced by infants in their mother's milk, a predominantly sweet liquid. Like amniotic fluid, human milk is composed of flavours which directly reflect the foods, spices and beverages eaten by or inhaled by (e.g., tobacco) the mother.[11-16] In other words, the flavour principles of the child's culture are experienced prior to their first

taste of solid foods.[2] That amniotic fluid and breast-milk share a commonality in flavour profiles with the foods eaten by the mother suggests that breast-milk may 'bridge' the experiences with flavours *in utero* to those in solid foods. Moreover, the sweetness and textural properties of human milk, such as viscosity and mouth coating, vary from mother to mother.[23-24] This suggests that breast-feeding, unlike formula feeding, provides the infant with the potential for a rich source of varying chemosensory experiences. The types and intensity of flavours experienced in breast-milk may be unique for each infant and serve to identify the culture to which the child is born.

To test the hypothesis that pre- and post-natal flavour experiences enhance the acceptance and enjoyment of flavours during infancy, we conducted an experimental study in which pregnant women, who planned on breast-feeding their infants, were randomly assigned to one of three groups.[16] Women in one group drank carrot juice for several days per week during the last trimester of pregnancy; mothers in a second group drank the carrot juice for a similar time period during lactation, whereas those in the control group drank water during both pregnancy and lactation. All mothers refrained from eating carrots or drinking carrot juice during and between the two exposure periods. Approximately 4 weeks after the mothers began complementing their infants' diet with cereal, and before the infants had ever been fed foods or juices containing the flavour of carrots, the infants were videotaped as they fed, in counterbalanced order, cereal prepared with water during one test session and cereal prepared with carrot juice during another.

Like that observed in other mammals,[20] infants who had exposure to the flavour of carrots in either amniotic fluid or mothers' milk behaved differently in response to that flavour in a food base than did non-exposed control infants. Specifically, previously exposed infants displayed fewer negative facial expressions while feeding the carrot-flavoured cereal when compared to the plain cereal. Moreover, those infants who were exposed to carrots prenatally were perceived by their mothers as enjoying the carrot-flavoured cereal more when compared to the plain cereal. Postnatal exposure has similar consequences thus highlighting the importance of a varied diet for both pregnant and lactating women.

When an infant is exposed to a flavour in amniotic fluid or breast-milk, and is tested sometime later, the exposed infant accepts the flavour more than do infants without such experience. Presumably, learning (e.g. elimination of neophobia, conditioning, mere exposure) has occurred. Teleologically, one can argue that the infant has acquired information that the food associated with the flavour is not dangerous or is nutritionally valuable or both. It is important for the infant to accept and be particularly attracted to the flavours consumed by the mother. All else being equal, these

are the flavours that are associated with nutritious foods, or at least, foods she has access to, and hence the foods to which the infant will have the earliest exposure.

There is some evidence, however, that the exposure needed to enhance later acceptance may not require experience with the actual flavour. Rather, research in humans[25-27] and animal models[21] suggests that experience with flavour variety enhances the acceptance of novel foods during weaning. Of interest is the finding that breast-fed infants are more willing to accept a novel vegetable upon first presentation than are formula-fed infants.[27] One explanation for this finding is that, unlike the formula-fed infant who experiences a monotony of flavours in infant formula, the breast-fed infant is exposed to a variety of flavours in breast-milk, setting the pattern for a diversified diet.

5. LONG TERM BENEFITS OF BREAST-FEEDING

The long-term consequences of breast-feeding on the development of aspects of food and flavour preferences have been the subject of a few studies in recent years. In an 8-year longitudinal study conducted in the United States, fruit and vegetable consumption by school-aged children was predicted by either breast-feeding duration, food-related experiences during early life, or mothers' preferences.[29-30] Similar findings have been recently reported in the United Kingdom[31] and France.[32] Clearly, more research in this area is needed, but the finding that infants are learning via flavour cues in breast-milk suggests one (but not the only) mechanism underlying these associations.

6. CONCLUSION

Food traditions mirror a people's beliefs and are among the last characteristic of a culture that is lost during the immigration of an individual or group into a new culture. Many families around the world, including those who have immigrated to new countries, continue to eat 'traditional' family foods common to their homeland.[1] Deep-rooted traditions, passed from generation to generation, educate the child about its cuisine, the classification of foods as meals or snacks, and which foods are suitable for which meals. What identifies a cuisine is often the distinctive combinations of a small number of flavouring ingredients, which have been coined the term 'flavour principle'.[33] In other words, a culture's flavour principles not

only identify its cuisine for those outside the group but provide a sense of familiarity for those who share the tradition.

The research presented herein suggests that prenatal and early postnatal exposure, at the very least, predisposes the young infant to favourably respond to the now-familiar flavour, which, in turn, facilitates the transition from fetal life through the breast-feeding period to the initiation of a varied solid food diet. In this way, culture-specific flavour preferences are probably initiated early in life and early experiences in a sense, educate the young child to appreciate the flavours typical of the culture into which she or he was born. Significant traces of this may remain as children become adults and pass on their food habits to the next generation.

To be sure, many continue to learn and develop preferences for flavours and foods experienced later in life. However, the large olfactory component of flavours may shed light as to why flavours experienced early in life remain preferred, and to some extent, provide "comfort". That is, memories evoked by odours and flavours are more emotionally charged than those evoked by other sensory stimuli[34] because of the olfactory system's intense and immediate access to the neurological substrates underlying emotion.[35-36] The emotional potency of odour- and flavour-evoked memories, and the reward systems that encourage us to seek out pleasurable sensations together play a role in the strong emotional component of food habits – an integral part of all cultures that has its beginnings during gestation and breast-feeding.

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Chapter 10

METHODOLOGICAL CHALLENGES IN STUDYING LONG-TERM EFFECTS OF BREAST-FEEDING

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1. INTRODUCTION

Many short- and long-term child health benefits have been reported with breast-feeding. Many of these alleged health benefits exhibit dose-response relationships, with greater exclusivity and duration of breast-feeding associated with greater degrees of benefit. The short-term benefits have included reduced morbidity and even mortality from infectious diseases, particularly gastrointestinal and respiratory infection.[1-2] Atopic eczema and cow's milk and other food allergies have also been reported to be less frequent in breast-fed infants. The clearest short-term benefits have been shown to accrue to infants during the actual period of breast-feeding, but a number of studies have suggested that breast-feeding, particularly exclusive and prolonged breast-feeding, may confer protection against such long-term health outcomes as asthma, other allergic diseases, type 1 diabetes, inflammatory bowel disease, lymphoma, leukemia, obesity, hypertension, and hypercholesterolemia, as well as lead to taller stature and improved neurocognitive development.[1]

It is unfeasible and probably unethical to randomise newborn human infants to breast-feeding vs formula-feeding, or even to more vs less

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exclusive, or to longer vs shorter durations of breast-feeding. Thus most of the evidence bearing on the child health benefits of breast-feeding is based on observational (nonexperimental) studies, with numerous potential sources of bias.[3-4] This is true even for short-term benefits, but relating long-term health outcomes to feeding in infancy and early childhood presents even greater methodological challenges for epidemiological studies. Given the recent interest in "programming" and the early origins of adult chronic diseases,[5,6] it is timely to reflect on these challenges and how they can be addressed in future studies.

The major methodological issues can be subdivided into those related to random vs systematic error. Systematic error, or bias, can be in turn subdivided into considerations of bias due to measurement, selection, confounding, and reverse causality. These issues will be discussed in turn in the remainder of this chapter.

2. RANDOM ERROR

Random error is due to sampling variation, i.e., chance variation in the observed association between "exposure" (breast-feeding vs formula-feeding, exclusive vs partial breast-feeding, prolonged vs short duration of breast-feeding) and the health outcome under study. No matter how representative the sample of the source population of interest, the observed magnitude of the association between feeding and health outcome may differ from the "true" magnitude merely by chance. The size of the error (i.e., the difference between the observed and true magnitude) reflects the degree of sampling variation and depends primarily on the sample size, with small samples being much more prone to random errors. This is a particularly important issue when using cohort (forward-directional) designs to study rare outcomes, such as type 1 diabetes, cancer, inflammatory bowel disease, and other autoimmune diseases, since very large samples are required to ensure an adequate number of cases of the outcome to detect differences in incidence due to feeding mode.

Random error is easily quantified by standard statistical theory based on known sampling distributions such as the normal, binomial, and Poisson distributions. It is these known distributions that enable quantitative estimates of sampling error by such well-known techniques as confidence intervals, hypothesis tests, and P values.

Table 10-1 provides an illustrative example of random error in a hypothetical cohort (follow-up) study of 500 breast-fed (BF) and 500 formula-fed (FF) infants for the development of a health outcome (O+) of interest. In the upper 2 x 2 table, the absolute risks in the BF and FF groups

are 1% and 2%, respectively, suggesting a 50% reduction in risk associated with breast-feeding. The wide 95% confidence interval, however, indicates that the result is far from statistically significant and is compatible (at the 95% level) with both a 6-fold risk reduction and a 45% increase in risk. Most investigators would recognise the insufficient statistical power here, and few reviewers or editors would be enthusiastic about publishing such a result. If the study were 10 times larger (i.e., a total sample size of 10,000 instead of 1,000) but the absolute risks remained at 1 and 2%, the relative risk (RR) would remain 0.50, but the 95% confidence interval would narrow considerably, providing a more meaningful (and publishable) result. The lower 2 x 2 table shows a second example: a null result, i.e., no protective effect of breast-feeding. But here, too, the 95% confidence interval is statistically compatible with both a halving of risk, as well as a 2.5-fold higher risk! This example is more insidious than the first, because many investigators, reviewers, and editors would probably accept the result as convincing evidence against an association with breast-feeding.

Table 10-1.

Random Error: An Illustrative Example

	O+	O-	
BF	5	495	RR=0.50 (0.17-1.45)
FF	10	490	If n=10,000: CI=0.36-0.70

	O+	O-	
BF	11	489	RR=1.10 (0.47-2.57)
FF	10	490	If n=10,000: CI=0.84-1.44

One important, but frequently overlooked, problem in studies of breast-feeding and health outcomes is the underestimation of random error due to failure to consider clustering of the outcome within study groups. Thus studies comparing community A vs community B, participants vs non-participants in a breast-feeding promotion program, or changes over time before and after introduction of a breast-feeding promotion program usually ignore the fact that the health outcomes under study are more likely to be similar in members of the same community than in members of different communities, among participants or nonparticipants in a program, and within different specific time periods. Failure to consider the intraclass correlation, or clustering, of outcomes within these geographical or temporal groups

leads to underestimation of true sampling variation, leading to overly narrow confidence intervals and falsely low P values.[7]

3. SYSTEMATIC ERROR

3.1 Measurement bias

Imprecise ("sloppy") measurement of either infant feeding or the health outcome under study will lead to a bias toward the null, i.e., toward no association between breast-feeding and the outcome. This is illustrated by the results of a hypothetical case-control study of type 1 diabetes (T1D) and infant feeding shown in Table 10-2. The true 2-fold protective effect of BF is reduced and rendered statistically nonsignificant by nondifferential (i.e., similar in cases and controls) misclassification of the infant feeding history. The degree of misclassification shown in the table is not large: the sensitivity (Se) is 70% (classifying 30% of truly breast-fed subjects as having been formula-fed), and the specificity (Sp) is 90% (classifying 10% of truly formula-fed subjects as having been breast-fed).

Table 10-2.

Measurement Bias: Nondifferential Misclassification of Infant Feeding

		T1D	CTL	
"Truth"	BF	100	130	OR = 0.54 (0.35-0.82)
	FF	100	70	
		T1D	CTL	
Se=70% Sp=90%	BF	80	98	OR = 0.69 (0.40-1.05)
	FF	120	102	

One of the frequent causes of measurement error in studies of health effects of breast-feeding relates to the inadequate distinction among feeding groups. Defining infants as either having been breast-fed or formula-fed will result in diminished associations with outcomes whenever those outcomes

depend on the exclusivity or duration of breast-feeding. This is particularly true whenever the relation exhibits a threshold effect, i.e., when a minimum degree (proportion of total intake) or duration is required to produce the effect. In that case, the absence of effect among infants who received only small amounts or short durations of breast-feeding will "dilute" the effects observed among infants with greater degrees or longer durations. This is dramatically illustrated by Table 10-3, which summarises results from another hypothetical case-control study of type 1 diabetes (T1D) and infant feeding. In this example, the observed results (upper 2 x 2 table) show no association with infant feeding, but the "true" effect of BF requires a threshold (minimum) of 3 months of exclusive BF. When the infant feeding is reanalysed by combining with infants who do not meet this threshold classified with the formula-fed infants (lower 2 x 2 table), the results show a significant 3-fold reduction in risk.

Table 10-3.

Measurement Bias: Consequences of Ignoring Threshold Effect of Breastfeeding

	T1D	CTL	
BF	130	130	Observed
FF	70	70	OR = 1.00 (0.65-1.54)

	T1D	CTL	
BF	50	100	"Truth": Effect requires EBF ≥3 mo
FF	150	100	OR = 0.33 (0.21-0.52)

Another, entirely different, source of measurement error can occur with non-blinded ascertainment of the outcome. This type of systematic error often leads to bias away from the null, i.e. towards a stronger protective effect of breast-feeding.[4] It is likely to occur whenever observers who assess the health outcome under study are aware of the feeding status of the subjects whose outcomes they are assessing. Although blinding of outcome assessment is often incorporated into randomised trials, it is often feasible to implement in observational (nonexperimental) cohort studies as well. In case-control studies, where subjects are selected based on the presence or absence of the outcome at the time the study groups are assembled, those who obtain infant

feeding histories (whether by interview, chart extraction, or other means) should be blinded to the case vs control status of the subjects and, preferably, to the investigators' research hypothesis.

3.2 Selection bias

Selection bias is of particular concern in studies of breast-feeding effects, because infants who receive exclusive and prolonged breastfeeding are those who grow well and remain free of major illness in infancy. Breast-feeding has been described as a "one-way street".[8-9] In other words, infants who are breast-fed, particularly those who are exclusively breastfed, can be supplemented or even weaned if they fail to grow adequately or become ill. Formula-fed infants, on the other hand, can rarely if ever be switched to breast-feeding when they fail to thrive or develop illness. Thus, inevitably, infants with prolonged and exclusive breast-feeding are those who have grown well, thrived, and remained healthy, i.e., those whose health problems have not resulted in the mother's or health care provider's decision to add complementary feeding, supplement with formula, or completely wean from the breast. Because infants with prolonged and exclusive breast-feeding are selected to be those who are healthiest in infancy, it would not be terribly surprising if prolonged and exclusive breast-feeding were also associated with longer-term outcomes that were themselves associated with (and perhaps caused by) the excellent growth and good health in infancy, even if breast-feeding had nothing to do with it.

Particularly when using case-control designs to investigate rare study outcomes, it is essential to ensure that cases and controls are selected in such a way as to avoid bias in infant feeding histories. A good rule-of-thumb is to select controls from the same population base as the cases and thus to ensure that had the control subjects developed the case condition, they would have been selected as cases for the study. Table 10-4 illustrates what can happen when this rule-of-thumb is ignored in yet another hypothetical study of type 1 diabetes (T1D) and infant feeding. The upper 2 x 2 table shows the (correct) results when both the cases and controls are selected using a population-based strategy, e.g., a registry of T1D cases and birth registry-based controls covering the same population. The lower 2 x 2 table shows what can happen when the cases are population-based (the same T1D registry) but the controls are selected from births at a single maternity hospital that has achieved "baby-friendly" status with a successful program of breast-feeding promotion.

Any study of long-term health outcomes requires prolonged follow-up, with inevitable risk of losses to follow-up. Whenever the losses are substantial, and particularly if they are asymmetrical (unequal) among the

infant feeding groups, concern will increase that the observed association among those who remain in follow-up will be systematically different from (i.e., biased) *vis à vis* the association that would have been observed in the entire cohort in the absence of losses to follow-up. Losses to follow-up should always be minimised, and efforts to retain subjects should be vigorous and equal in all infant feeding groups. Investigators often compare subjects who remain vs those who are lost to follow-up according to measured baseline characteristics. Similarity in these characteristics says little, however, about outcomes in those lost vs those retained and thus do not provide convincing reassurance about selection bias.

Table 10-4.

Selection Bias Due to Source of Controls

	T1D	CTL	
BF	130	130	Population-based cases and controls
FF	70	70	OR = 1.00 (0.65-1.54)

	T1D	CTL	
BF	130	160	Population-based cases, but controls selected from baby-friendly hospital
FF	70	40	OR = 0.46 (0.29-0.78)

3.3 Confounding

Confounding is a challenge for all observational studies but is even more so when the confounding factor is behavioural or interactional, as in studies of breast-feeding and neurocognitive development or behaviour. Confounding occurs whenever a third factor (i.e., a factor other than infant feeding or the health outcome under study) is associated with the infant feeding received, is an independent cause of the health outcome under study, does not lie on the causal path between infant feeding and the outcome, i.e., is not a ***mediator*** of the effect of infant feeding on the outcome, and is not affected by either infant feeding or the outcome.

Table 10-5 shows a hypothetical example of confounding by socioeconomic status (SES) in a study of infant feeding and child obesity. In

this example, SES is strongly associated with both infant feeding and obesity; low-SES mothers are far less likely to breast-feed (20 vs 80%), and their children are far more likely to become obese (40 vs 20%). Since SES is not on the causal path between infant feeding and child obesity and is not affected by either (at least not in children), it meets the criteria for a confounder. The observed ("crude") results shown in the upper panel of the table are confounded, whereas the stratum-specific results shown in the middle and lower panels show no association whatsoever between breast-feeding and subsequent obesity.

Table 10-5.

Confounding of BF-Obesity Association by Socioeconomic Status (SES)

		Obese	Non	
Observed ("Crude")	BF	240	760	RR = 0.67 (0.58-0.77)
	FF	360	640	
		Obese	Non	
Low SES	BF	80	120	RR = 1.00 (0.83-1.21)
	FF	320	480	
		Obese	Non	
High SES	BF	160	640	RR = 1.00 (0.73-1.36)
	FF	40	160	

Figure 10-1 illustrates what can happen when causal pathways are ignored in a hypothetical study of infant feeding and subsequent asthma and atopy. Breast-feeding is known to protect against a variety of infections in infancy.

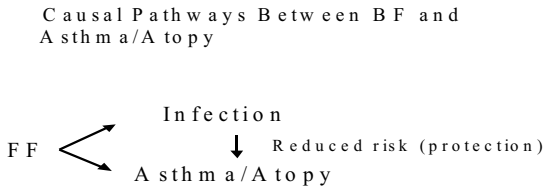

Figure 10-1.

One of the leading theories about the development of atopic disease is the so-called "hygiene hypothesis," which posits that some early infections (depending, perhaps, on the age of the infant/child and the type of infecting microorganism) may "program" T-helper cells in the immune system toward a type-1 (infectious) response and away from a type-2 (atopic) response to foreign antigens. If this hypothesis is correct, then the increased risk of infection associated with formula feeding (FF) could theoretically reduce the FF-associated risk of asthma or atopy (as has been reported in some recent studies), rather than increase it. It is important to emphasise that infection is ***not*** a confounder of the feeding-atopy association, since it lies on the causal path between infant feeding and the development of atopy and therefore should not be "controlled for" in analysing data bearing on that association.

When confounding does occur, a variety of methods have been developed to minimise and control for the bias it induces. The best of these methods is randomisation. Despite comments made earlier about the feasibility and ethical issues involved in randomising humans to different feeding modalities, several trials have succeeded in randomly allocating interventions in ways that affect the type of feeding received. These include Lucas *et al's* trial of preterm infants randomised to pooled human milk vs preterm vs term formula[10], Dewey *et al's* trials of 4 vs 6 months' introduction of complementary foods in breast-fed infants in Honduras[11-12], and Morrow *et al's*[13] and our[14] trials of randomisation to breast-feeding promotion interventions.

In the absence of randomisation, control for confounding requires valid and precise measurement of the confounding variables. The confounding can then be reduced through stratification (i.e., comparisons within strata of the confounder, as illustrated in Table 10-5), matching on the confounders, or (most commonly) multivariate statistical models. The latter approach, of course, requires that an appropriate model be chosen.

A major difficulty arises, however, when behavioural or interactional factors confound the relationship between infant feeding and outcome. This is particularly a problem in studying neurocognitive, developmental, and behavioural outcomes in the offspring. It is difficult to measure such potential confounders as maternal motivation, parenting style, stimulation, bonding, love, warmth, patience, and similar factors that may differ systematically in mothers who choose to breast-feed (particularly those who breast-feed exclusively and for a prolonged duration) and those who do not.

3.4 Reverse causality

Reverse causality is a known potential source of bias in observational studies, particularly in cross-sectional studies, where the exposure and outcome are measured at the same point (cross-section) in time. For example, in some cross-sectional studies of breast-feeding and infection, it may not be clear whether weaning led to infection or whether early signs and symptoms of infection made the infant or mother unable to continue breast-feeding and themselves led to weaning.[3-4] Reverse causality can also occur with studies of long-term outcomes such as growth and behaviour, however, because growth and temperament in infancy can influence the mother's feeding decisions and are themselves predictive of, and possibly causally related to, long-term growth and behavioural outcomes.

4. PROBIT: ATTEMPTING TO OVERCOME THE METHODOLOGIC OBSTACLES

I will conclude with a very brief overview of the methods and early results of PROBIT (Promotion of Breastfeeding Intervention Trial), a cluster-randomised trial of a breast-feeding promotion intervention in the Republic of Belarus.[14] The study is a collaborative effort among the Maternal and Child Health Department of the Belarussian Ministry of Health, the Belarussian Maternal and Child Health Research Institute, McGill University, and the University of Toronto, as well as the European Regional Office of the World Health Organization. The experimental intervention was based on the WHO/UNICEF Baby-Friendly Hospital Initiative, and the clusters were maternity hospitals and their affiliated polyclinics (outpatient pediatric clinics). Those hospitals/clinics randomised to the experimental intervention underwent a period of extensive training based on a standard WHO/UNICEF course for the medical, midwifery, and nursing staff of the maternity hospitals and the pediatrician and nursing staff

at the polyclinics. The course was designed to implement the 10 steps of the Baby-Friendly Hospital Initiative and to promote the degree and duration of breast-feeding in the weeks and months following discharge from the maternity hospital. Those maternity hospitals and polyclinics randomised to the control intervention were asked to maintain the practices and policies in place at the time of randomisation. Thirty-one hospital/polyclinic pairs were randomised, with a total sample size of 17,046 mother-infant pairs. This large sample size was necessary because of the anticipated overlap in breast-feeding exclusivity and duration between the experimental and the control groups and the need for adequate statistical power, given this overlap, to detect differences in important health outcomes.

Several papers have been published based on outcomes during the first year of life, with the primary focus on gastrointestinal infection, respiratory infection, atopic eczema, and growth.

Our results showed a significant increase in the proportion of infants breast-fed to any degree, beginning the first month postpartum and continuing throughout the first 12 months of life. Very large differences were seen between the experimental and control groups in exclusive and predominant breast-feeding, particularly at 3 months, but even at 6 months. These differences in exclusivity and duration of breast-feeding were reflected in statistically significant reductions in gastrointestinal infection and atopic eczema, although no significant differences were observed in respiratory infectious outcomes.[14] The experimental group also exhibited somewhat increased growth in weight and length in the first 3 months, but this difference diminished and was no longer present by the age of 12 months.[15]

Thus PROBIT provides two cohorts with substantially different degrees and durations of breast-feeding, where the differences were created by randomisation, rather than by the choice of the mother or health care provider. PROBIT's large sample size and (at least up to the present time) high rates of follow-up create a unique scientific opportunity for studying the long-term effects of breast-feeding exclusivity and duration. This resource should help overcome many, albeit not all, of the methodological difficulties and challenges discussed in this paper.

The Canadian Institutes of Health Research is currently funding a follow-up of PROBIT children at the age of 6½ years. The major outcomes of interest are those potentially related to "programming," including stature, obesity and adiposity, blood pressure, asthma, hay fever, skin test hypersensitivity, IQ, and behaviour as independently rated by parents and teachers. The 6½ year visits are now nearly completed, and we are hoping for a follow-up rate over 80%. Funding has also been requested for follow-up at 11 years of age, when we hope to obtain fasting blood samples for studying

glucose tolerance and lipoproteins, as well as repeat anthropometric and blood pressure measurements.

Many excellent observational studies have related infant feeding to important child health outcomes. For most of the health outcomes of interest, careful attention to the inherent methodological problems of observational studies can often result in large studies with low potential for random error and excellent control for most sources of systematic error (bias). The quality of these studies has improved substantially over the last 10 or 15 years, and through continued improvements in observational designs and the more frequent use of randomised trials, we should continue to learn much about the potential long-term effects of breast-feeding for infant, child, and adult health.

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Chapter 11

THE LONG TERM EFFECTS OF EARLY POSTNATAL DIET ON ADULT HEALTH

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1. INTRODUCTION

It has been recognised for over seventy years that the early environment in which a child grows could have long-term effects on its health.[1] This was suggested based on observations in England, Scotland and Sweden that death rates in specific age groups at any time depended more upon the date of birth of individuals than upon the year under consideration.[1] Further support came from a study by Forsdahl *et al* looking at geographical variations in current death rates from arteriosclerotic heart disease in Norway.[2] This showed that there was a significant positive correlation between these current death rates and geographical variations in past infant mortality rates. No such relationship was observed with current infant mortality rates[2]. It was subsequently shown that the geographical pattern of mortality from cardiovascular disease in England and Wales was related to maternal and neonatal mortality earlier in the century.[3] This was suggested to reflect a relationship between poor nutrition in fetal and early life, and cardiovascular disease.

A large number of epidemiological studies have subsequently demonstrated that there is a relationship between poor fetal and early growth and the development of adult diseases such as type 2 diabetes and the metabolic syndrome.[4] This relationship has been observed in a wide range of populations worldwide in different ethnic groups; thus the existence of the

G. R. Goldberg et al. (eds.), Breast-Feeding: Early Influences on Later Health,

relationship is widely accepted.[5] However the mechanistic basis of this relationship and the relative role of genes and the environment and the interaction between the two remains the subject of much debate. Rare mutations have been identified in the glucokinase gene which have been associated with a low birthweight and the development of a rare monogenic form of diabetes, maturity onset diabetes of the young.[6] However extensive genome scans have failed to identify common diabetes susceptibility genes/polymorphisms. Recent studies have identified interesting gene-birth size interactions in the case of peroxisome proliferator receptor (PPAR)-gamma 2 gene polymorphisms.[7]

Some of the strongest evidence for the importance of the early environment in mediating the relationship between poor early growth and adult disease has come from the study of twins. A study of middle-aged twins in Denmark revealed that, in both monozygotic and dizygotic twin pairs who were discordant for type 2 diabetes, the diabetic twin had a significantly lower birthweight than the normoglycaemic co-twin.[8] Monozygotic twins are genetically identical, and therefore the difference in birthweight must be related to the fetal environment. A second study of twins in Italy who were significantly younger (mean age 32) than the cohort in Demark revealed similar findings.[9] These studies thus provide strong evidence for the importance of a non-genetic intrauterine factor in the development of type 2 diabetes in later life.

In light of the epidemiological observations it has been proposed that poor fetal nutrition leads to programming of metabolism in a manner beneficial to survival under conditions of poor postnatal nutrition.[10] This would give rise to a "Thrifty Phenotype" in which the organism was adapted to store carbohydrate. This metabolic programming was proposed to become detrimental if the fetus was born into conditions of either adequate or over nutrition, and obesity occurred. It may also increase the risk of the development of obesity. This has therefore drawn attention to the possibility that postnatal nutrition may be as important as antenatal nutrition in determining long-term health. More specifically the focus has been directed towards the role played by nutrition during the neonatal period.

2. EVIDENCE

2.1 Interactions between poor fetal growth and adult obesity

The apparent conflict between fetal nutritional experiences and adult obesity has been demonstrated by a number of human epidemiological studies. A study of 64-year old men in Hertfordshire U.K. demonstrated that the individuals with the worst glucose tolerance were those who were born small and who were currently obese.[11] Individuals with a high birthweight were relatively protected from the detrimental effects of obesity. Individuals who were born small but remained thin were also protected from diabetes. This would explain why in countries where there is chronic malnourishment, the prevalence of the metabolic syndrome is very low. Studies of individuals who were *in utero* during the famine known as the Dutch Hunger Winter have also shown that for any current body mass index, glucose tolerance was worse in those individuals exposed to the famine *in utero* compared to those born the year before the famine.[12] This meant that frank diabetes was generally only present at age 50 in those individuals who were malnourished *in utero* and were currently obese. The detrimental effects of poor early nutrition and adult obesity have also been shown in animal models. For example early protein restriction and adult obesity (induced by the feeding of a cafeteria diet) have been shown to independently and additively cause an increase in systolic blood pressure in rats.[13]

2.2 Catch-up growth

The importance of rates of growth during early postnatal life has become apparent from a number of epidemiological studies. A study by Crowther *et al* on 7 year old South Africans revealed that those children born with low birthweights but who underweight rapid childhood weight gain had the worst glucose tolerance and were thus proposed to be most susceptible to development of type 2 diabetes in adulthood.[14] Further studies by the same group have related these differences to alterations in ß cell secretory activity.[15] Children who had low birthweights and low weights at age seven had the lowest ß cell secretory response. However the children who were born small but had high weights at age 7 years had the greatest ß cell response and were most insulin resistant (as assessed by HOMA) but had the poorest glucose tolerance. They also had the lowest percentage of mature insulin and the highest percentage of pro-insulin and partially processed

intermediates. It was concluded that poor fetal growth followed by higher postnatal growth results in low ß cell numbers and reduced whole body glucose uptake which leads to reduced efficiency in the processing of pro-insulin. Similar deleterious effects of poor early growth followed by rapid catch up growth have been observed in a cohort of Finnish men and women.[16] The incidence of type 2 diabetes was shown to increase with decreasing birthweight. It was also observed that individuals who developed diabetes had above average heights at age 7 and age 15. This again suggests that the increased risk of diabetes that is associated with small size at birth is further increased by high growth rates in childhood. A study of pre-term children who were aged 9-12 years has shown that glucose concentrations 30 minutes after a glucose load were negatively related to birthweight.[17] Fasting split pro-insulin concentrations were highest in children who showed the greatest increase in weight centile between birth and time of study. Rapid growth during early life has also been associated with increased risk of cardiovascular disease. A study in Finland showed that the highest death rate from coronary heart disease occurred in men who were thin at birth but whose weight caught up postnatally such that they had an average or above average body mass from the age of seven years.[18] It was concluded that death from coronary heart disease may be a consequence of poor prenatal nutrition followed by improved postnatal nutrition. Accelerated neonatal growth has also been associated with lower flow-mediated endothelium-dependent dilation in young adolescents.[19]

Studies in rodents have also demonstrated that differences in early growth rates can ultimately affect longevity. The offspring of rat dams fed a low (8 %) protein diet during pregnancy have around a 15 % reduction in birthweight compared to control offspring of dams fed a 20 % protein diet. If these animals are cross-fostered to normally fed dams at birth they undergo rapid catch-up growth during the lactation period. This catch-up growth observed in these recuperated animals has a detrimental effect on longevity, resulting in approximately a 25 % reduction in lifespan in male rats.[20] The major cause of death in male rats is reported to be renal failure. Consistent with this is the observation that the reduced longevity in the recuperated animals is associated with accelerated loss of kidney telomeric DNA.[21] In contrast control offspring suckled by low protein fed dams and which therefore grew slowly during the lactation period have a substantial increase in longevity. Similar findings have recently been observed in mice.[22] These studies in mice revealed an interesting interaction with the post-weaning diet. An obesity-inducing cafeteria-style diet reduced the longevity of both control and recuperated animals. In contrast the animals that were suckled by low protein fed dams and thus grew slowly during the lactation period were completely protected from the detrimental effect of the obesity-inducing diet

to reduce longevity. The mechanisms underlying these effects are not known however there is an urgent need to see if similar mechanisms operate in humans.

There is some recent evidence from a study of young teenage children who were born pre-term to suggest that relative under-nutrition early in life may have beneficial effects on insulin resistance.[23] It was shown that those children who had been randomised to receive a nutrient-enriched diet neonatally had higher fasting 32-33 split pro-insulin concentrations than those given a lower nutrient diet. Breast-milk feeding, which is associated with slower growth than formula-feeding has been shown to be associated with reduced C-reactive protein and LDL to HDL cholesterol ratio in 13-16 year old children who were born pre term and breast-milk fed compared to those who were formula fed.[24] This suggests that breast-feeding is protective against the risk of atherosclerosis. Weight gain from birth to two years has also been shown to be a predictor of overweight at age six.[25]

2.3 Programming of appetite

In addition to being key to the full expression of the Thrifty Phenotype, it is also possible that obesity itself is a manifestation of the Thrifty Phenotype. Several studies have linked low birthweight to changes in body composition in adulthood including increased central fat[26] and reduced lean mass.[27] There is some evidence that the timing of the nutritional insult may also have an impact on the future susceptibility to obesity. Studies of men who were exposed to the Dutch Hunger Winter in early life have revealed that those who were exposed to the famine during the first half of pregnancy were more obese at age 19. In contrast those who were exposed to the famine during the last trimester of pregnancy and in early postnatal life had reduced obesity.[28] This provides some of the earliest evidence from human studies that nutrition during the neonatal period can have long terms effects on future susceptibility to obesity. It pointed towards slow growth during the lactation period actually having a beneficial outcome.

The detrimental effects of poor antenatal growth are exaggerated by rapid postnatal weight gain. Rapid weight gain in infancy has been shown to be a risk factor for future obesity. Infants who were growth restricted *in utero* and underwent postnatal catch-up growth between birth and 2 years of age have been shown to be fatter and to have more central fat than other children.[29] The mechanisms underlying postnatal catch-up growth are not well defined. However such rapid growth may be the consequence of programmed changes in gene expression that were established *in utero*. This therefore makes the interpretation of the role of postnatal growth patterns and scope for intervention complicated. It has been shown in a rodent model

that maternal protein restriction is associated with increased expression of insulin receptors which could drive post natal weight gain.[30] It may also be a consequence of programmed changes in appetite. Thus epidemiological studies showing relationships between post-natal growth and obesity with the long-term risk of disease might simply reflect patterns of gene expression already established during fetal life.

Increased rates of postnatal weight gain have been associated with reduced satiety in small for gestational age (SGA) infants (as assessed by volume of milk consumed by bottle-fed infants).[31] Leptin is one of many potential candidates that could mediate these changes in appetite. It has been shown that cord blood leptin is inversely related to rates of growth during infancy and that SGA infants have lower leptin concentrations.[32] One possibility therefore is that low leptin levels in SGA infants lead to reduced satiety and rapid postnatal weight gain. There are also a number of studies that have also suggested that breast-feeding is protective against obesity risk in later childhood. [33] These effects are not confounded by socioeconomic status. [34] It is thought that the reduction in risk of obesity is related to properties of human milk than to factors associated with breast-feeding.[35] Bottle-fed infants are known to have higher total energy and protein intakes than breast-fed infants [36] so one possible explanation is that early breast-feeding may affect subsequent appetite regulation.

Studies in rodents have also revealed that early nutrition can have long-term consequences on appetite. Early studies with rats where nutrition during lactation was manipulated by altering litter size revealed that reduced nutrition during lactation resulted in a permanent reduction in appetite.[37] More recent studies of litter size manipulation have revealed that rats in small litters have increased plasma insulin levels increased weight gain.[38] The authors hypothesised that hyperinsulinism during brain differentiation (as caused by overfeeding in a small litter) was a pre-disposing factor for the development of obesity, diabetes and cardiovascular disease. Further studies have shown that rats from small litters have reduced responsiveness of their paraventricular neurons in the brain.[39] Severe energy restriction during pregnancy has also been shown to lead to hyperphagia in adult life.[40] These findings suggest that appetite can be programmed upwards or downwards depending on the timing of the insult and suggest that the early postnatal period may be a time window for targeted intervention. Recent studies in mice have also pointed towards both the fetal and postnatal period being critical time windows for appetite regulation. Mice who were growth restricted *in utero* by maternal protein restriction but were cross-fostered to normally fed dams at birth and underwent rapid postnatal catch-up growth were permanently heavier than control offspring.[41] This effect was exaggerated when the animals were weaned onto an obesity-inducing

cafeteria-style diet. In contrast control offspring who were suckled by low protein fed dams and therefore grew slowly during the lactation period were permanently smaller than controls despite being weaned onto standard laboratory chow fed *ad-libitum*. They also gained less weight when weaned onto a cafeteria-style diet suggesting that they were relatively protected from its normal obesity-inducing effects.

Further evidence for the importance of the lactation period has come from cross-fostering experiments with diabetic rats. Control offspring that were cross-fostered and suckled by rats that had diabetes developed changes in the orexigenic and anorexigenic circuits in the brain.[42] If these changes were permanent they could have lifelong changes in regulation of food intake. There is evidence to suggest that these findings may be relevant to humans. A study of two year-old children showed that those who had breast-milk from diabetic mothers had an increased risk of becoming overweight and developing impaired glucose tolerance.[43]

The identification of the lactation period as a critical period for determination of appetite has been further emphasised by recent studies of transgenic animals. These demonstrated that neural connection pathways from the arcuate nucleus of the hypothalamus were permanently disrupted in leptin deficient mice.[44] Leptin treatment in adulthood, although normalising body weight was unable to reverse the neuro-anatomical defect. However treatment during the perinatal period completely restored the density of innervation to that observed in wild-type mice.[44] It is known that the timing of opportunity for rescue coincides with surge of leptin in control animals. This therefore provides a potential mechanism by which manipulation of food intake during a critical time period can have effects on adult appetite.

3. CONCLUSIONS

There is now overwhelming evidence to suggest that experiences during both the fetal and neonatal period can have long terms effects on health. Poor fetal growth followed by rapid postnatal catch-up growth appears to be particularly detrimental. The interpretation of the relationship between the two is complex. Therefore the designation of suitable intervention strategies is not straightforward and requires further research effort.

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Chapter 12

PROTECTIVE EFFECT OF BREAST-FEEDING AGAINST OBESITY IN CHILDHOOD: CAN A META-ANALYSIS OF PUBLISHED OBSERVATIONAL STUDIES HELP TO VALIDATE THE HYPOTHESIS?

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1. INTRODUCTION

In recent years the relationship between breast-feeding and childhood obesity has been a focus of interest. Since 2000 16 studies regarding this issue have been published - some have found a protective effect, while others have not.

The conclusions drawn from these data regarding the potential protective effect of breast-feeding on childhood obesity diverge. While a narrative review of Dewey suggests an effect of breast-feeding[1], an editorial in the British Medical Journal cites two recent studies without such an effect and cites inconclusive evidence.[2] A recent meta-analysis suggested a small but significant protective effect of breast-feeding.[3] The objective of this paper is to discuss the strengths and limitations of the meta-analysis approach and to summarise the results of our meta-analysis.

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1.1 Why does it make sense to conduct a meta-analysis?

The assumption in a meta-analysis is that all studies measure the same exposure and effect. In this case different odds ratios are explained by chance and are related to differences in the size of the study. The meta-analysis summarises the effects of the included studies. Statistical power is increased to allow for more precise estimates.

1.2 What are the limitations of a meta-analysis on breast-feeding and childhood obesity?

The reported studies do not only differ with respect to sample size but also with regard to a number of other study characteristics. The studies included used different approaches to measure the exposure "breast-feeding".[4-12] Most of the studies compare children in the broad categories "never breastfed" with children "ever breastfed" while some studies use other more elaborate definitions of breast-feeding taking account of the exclusiveness and duration of breast-feeding. Also the assessment of potential confounders and the definition of the outcome are not consistent over the different studies. Overweight or obesity was defined by BMI percentiles $\geq$ 90, 95 or 97 with varying reference populations. Testing for heterogeneity can be conducted to rule out bias generated by these different approaches.

Publication bias; (small) studies which do not show a significant effect are less likely to be published.[13] Publication bias can be detected by a funnel plot. For example a measure of the effect of breast-feeding on childhood obesity (for example the log of the odds ratios) can be plotted against a measure of precision reflecting the study size (for example the inverse of the standard error of the log odds ratio). It is assumed that the point estimates for more powerful studies will be closer to the pooled estimate. The plot therefore constitutes a funnel, the tip being formed by the more powerful studies (Figure 12-1). The objective is to assess symmetry as an indicator of the absence of publication bias. A funnel plot regression analysis can also be conducted. This is likely to have sufficient power if the number of studies included is 20 or more.[13] In this approach the degree of funnel plot asymmetry can be measured as the intercept from a linear regression of the standardised effect sizes against precision. In the absence of publication bias this intercept will be zero.

Inclusion criteria for a meta-analysis may account for selection bias if knowledge of results of eligible studies leads to the exclusion of studies with negative findings. To minimise selection bias inclusion criteria should be

defined *a priori* in a study protocol and eligibility should be assessed by at least two independent observers not familiar with the study results.

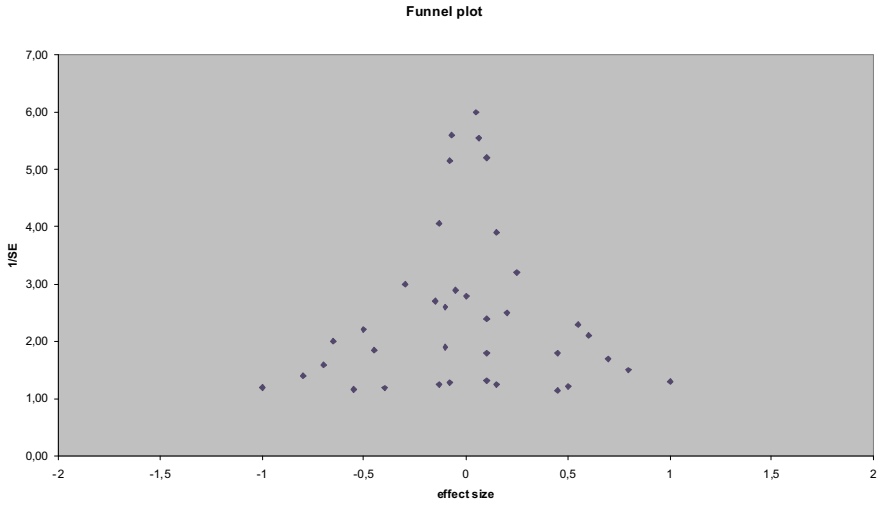

Figure 12-1. Typical funnel plot generated from simulated studies.

1.3 Does a meta-analysis take us further towards causality?

Meta-analyses can never be better than the primary studies included. If residual confounding is a problem of the observational studies there will also be potential residual confounding in the meta-analysis. For example, breast-feeding might be a surrogate for other exposures that could not be assessed or adjusted for. Parental overweight, smoking and socio-economic status are related to both breast-feeding and to childhood obesity and may account for confounding. Estimates of these factors are used in the analysis in order to adjust for confounding. However the estimates of these potential confounders may not be precise enough for full adjustment (residual confounding). Residual confounding may also arise from unknown factors - associated both with the exposure and the outcome - for which data have not been collected.

1.4 Methods and main results of a recent meta-analysis on breast-feeding and childhood obesity

Cohort, cross-sectional or case-control studies were included in this meta-analysis[3]. Only studies with adjustment for at least three potential confounding factors such as birthweight, dietary factors, physical activity, parental overweight, parental smoking and socio-economic status were included. Other inclusion criteria were: comparable risk estimates such as odds ratio or relative risk had to be reported; age at the last follow-up had to be between 5 and 18 years; feeding-mode had to have been assessed and reported; and obesity as the outcome had to be defined by BMI percentiles ≥ 90, 95 or 97 to allow for comparison of studies. Inclusion criteria were defined *a priori* by a person initially not familiar with the study results.

A systematic computerised literature search of published studies for breast-feeding, obesity and children was conducted. Identification of further studies was carried out through handsearches in the references of original articles and reviews.

The pooled odds ratios of the eligible studies were calculated, heterogeneity was tested to determine whether the studies were measuring the same effects and stratified analyses were carried out to detect potential sources of heterogeneity by testing the stability of the findings across different approaches in study design, exposure ascertainment and selection of study participants. The potential impact of inclusion of other studies - not matching the inclusion criteria for the meta-analysis - on the pooled estimates was also assessed.

Nine studies with more than 69000 participants met the inclusion criteria. The adjusted odds ratio for breast-feeding on childhood obesity was 0.78, 95% CI (0.71, 0.85) in the fixed-effects model. The results of the included studies were homogeneous (Q-test for heterogeneity, $p>0.3$). Stratified analyses showed no significant differences regarding different study types, age groups, definition of breast-feeding or obesity and number of confounding factors adjusted for (Table 12-1).

Table 12-1. Stratified analyses of studies that met the inclusion criteria for the meta-analysis *

Component		Pooled odds ratio and 95%CI (fixed effects)
Study type	Cohort study	0.73 (0.64, 0.85)
	Cross-sectional study	0.76 (0.67, 0.86)
Age group	Up to 6 y	0.75 (0.63, 0.90)
	Older than 6 y	0.76 (0.68, 0.85)
Definition of breast-feeding	Never - ever	0.76 (0.67, 0.86)
	Other definition	0.74 (0.64, 0.85)
No. of confounding factors adjusted for	<7	0.69 (0.59, 0.81)
	≥7	0.78 (0.70, 0.87)
Definition of obesity	≥95th Percentile	0.79 (0.68, 0.91)
	≥97th Percentile	0.76 (0.65, 0.89)

*From: Arenz *et al* [3]

The funnel plot was asymmetrical due to one particular study. Funnel plot regression gave no indication of publication bias, however the statistical power might have been insufficient due to the small number of included studies.

It is difficult to definitely rule out publication bias. Some studies which found no significant effect in a crude analysis, did not report adjusted estimates and therefore had to be excluded from the meta-analysis. Including these studies might reduce the protective effect of breast-feeding. However, most of the recently published studies with weak or absent effects in the crude analysis presented estimates with adjustment for confounding.

To assess potential selection bias a pooled estimate of all eligible studies which reported adjusted odds ratios with confidence intervals was calculated (including those studies excluded from the original meta-analysis because individuals were either too young or too old to meet the original inclusion criteria.[14,15]). In this analysis the AOR of 0.77 (95%CI: 0.72, 0.82) was similar to the base case.

In conclusion our meta-analysis indicates that breast-feeding has a small but consistent protective effect on obesity risk in childhood. Since it is difficult to rule out residual confounding and publication bias there remains some uncertainty. Regarding publication bias we felt reassured when we looked at unpublished data from candidates for the Bavarian school entry examinations in 1999 and 2002 and compared the observed effect estimates for breast-feeding on childhood obesity to the original publication based on data from 1997.[4] Whilst in 1999 no significant protective effect could be seen, in 2002 the effect became significant again (Table 12-2).

Table 12-2. Adjusted odds ratios for breast-feeding and overweight or obesity in candidates for school entrance examinations in Bavaria.

Year	Adjusted odds ratio and 95%CI for breast-feeding	
	Overweight	Obesity
1997	0.79 (0.68, 0.93)	0.75 (0.57, 0.98)
1999	0.84 (0.66, 1.06)	0.91 (0.60, 1.38)
2002	0.79 (0.63, 0.99)	0.70 (0.47, 1.04)

1.5 Why is it still tempting to assume that breast-feeding prevents childhood obesity?

There are some hints for biological plausibility of a protective effect of breast-feeding including behavioural and hormonal mechanisms and differences in macronutrient intake. Formula-fed infants have higher plasma-insulin concentrations compared to breast-fed infants. This could stimulate fat deposition and lead to early development of adipocytes.[16] Bioactive factors in breast-milk might modulate growth factors which inhibit adipocyte differentiation *in vitro*. [17-18] Protein intake and energy metabolism is lower in breast-fed than in formula-fed infants.[19] A longitudinal study showed a significant positive association between early protein intake and later BMI, suggesting that a higher amount of protein intake early in life might increase the risk of obesity in later life.[20] In animal studies the availability of protein during fetal and early postnatal development was found to have a long term effect on glucose metabolism and body composition.[21-22]

If true, a causal relationship between breast-feeding and childhood obesity might be relevant at the population level. Even a small protective effect with an odds ratio near one would have a large public health impact which is reflected in the population attributable risk (PAR: reduction in the prevalence of childhood obesity by breast-feeding of all children) and the population attributable risk fraction (PARF: fraction of formula-fed children with obesity where obesity could have been prevented by breast-feeding of all children). Data from the Bavarian school entry examinations in 4916 children with an overweight prevalence of 10.4% showed a breast-feeding prevalence of 76.3% and an adjusted odds ratio for breast-feeding of 0,75 resulting in a potential reduction in the prevalence of overweight from 10.4 to 9.6% if all instead of 76% of the children would have been breastfed (population attributable risk). In this setting 7.3% of the risk for childhood overweight could be explained by not breast-feeding (population attributable risk fraction).

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Chapter 13

BREAST-FEEDING INFLUENCES ON LATER LIFE - CARDIOVASCULAR DISEASE

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1. INTRODUCTION

Cardiovascular disease represents a major challenge to public health globally. While there is now a clear downward trend in mortality from coronary heart disease (CHD) and stroke in many Western high income countries,[1-2] it is projected that in many other parts of the world mortality will increase steeply in the next 20 years. In an authoritative review, Yusuf *et al* have estimated that by 2020 the annual number of deaths worldwide from CHD will be double the level seen in 1990.[3] These increases will affect low as well as middle income countries. In part the trend is a consequence of population aging. However, it is believed that, in many areas of the world, the increase in cardiovascular disease is also being driven by profound shifts in behaviour and diet. These changes are occurring as part of a process that includes the wide-spread migration of people from rural areas to cities, which is happening in almost all low and middle income countries.

In some areas of the world communicable diseases remain the top priority for public health. However, even in sub-Saharan Africa, circulatory diseases are rapidly becoming major contributors to mortality: here, in 1990 it was estimated that the number of deaths from cardiovascular disease was only slightly lower than those from infectious and parasitic diseases.[3] In all other global regions the numbers of CHD deaths appreciably outnumbered those from infectious and parasitic diseases.

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The increasing global importance of cardiovascular disease justifies a continual review and appraisal of our current understanding of the aetiology of these conditions and the prospects for both primary and secondary prevention. The 52 country INTERHEART study,[4] which includes many low and middle-income countries, has recently made an assessment of risk factors. It concluded that abnormal lipids, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, consumption of fruits, vegetables, and alcohol, and regular physical activity account for most of the risk of coronary heart disease events (myocardial infarctions) worldwide in both sexes and at all ages in all regions.

Interventions in adult life have been shown to be effective in primary and secondary prevention of cardiovascular disease. Smoking, diet and exercise are clear priority areas. Where available and affordable, pharmaceutical treatments for hypertension and adverse lipid profiles have been shown to be highly effective in reducing morbidity and mortality. However, in addition to these later-life risk factors, there is intriguing evidence that cardiovascular risk may also be related to circumstances in early life.

The possibility that experiences in infancy and childhood could contribute to cardiovascular disease in adult life has been discussed for decades.[5] However, in recent years this debate has focussed on the possible links between impaired fetal growth and later disease risk, encapsulated in the so-called "fetal origins hypothesis".[6] As reviewed elsewhere,[7] historical prospective cohort studies from a number of countries (including Britain,[8-9] Sweden,[10] Finland,[11] USA[12] and India[13]) have found that the risk of coronary heart disease events declines as size at birth increases. This inverse association has been found in all published studies with the exception of one small investigation from Gothenburg, Sweden.[14] Most of these studies are restricted to mortality as an endpoint, although a number also include non-fatal myocardial infarction and other indicators of CHD such as angina. This inverse association is little affected by adjustment for socio-economic factors and is evident without adjustment for adult body size. A smaller number of studies have also been able to look at the association of size at birth with risk of stroke.[15-18] These have also found the existence of an inverse association.

One interpretation of the link between size at birth and later cardiovascular disease is that it reflects the long-term consequences of impaired nutrition *in utero*. These could include effects on final cell number or organ structure and function that in turn could result in differences in risk in later life. Size at birth is thus simply a proxy measure of *in utero* nutrition. It has been argued, however, that it may be difficult to differentiate between the effects of impaired fetal growth and accelerated (or catch-up) growth in the post-natal period.[19] Indeed, it has recently been argued that it is nutrition in infancy rather than *in utero* that is the important early-life influence on

later cardiovascular risk.[20] Clearly one crucial aspect of nutrition in infancy is breast-feeding. Over the past few years there has been increasing interest in whether breast-feeding is indeed associated with cardiovascular disease risk in later life.

This chapter will review the current evidence for an association between breast-feeding and cardiovascular disease risk in later life. The evidence relating breast-feeding with cardiovascular events will be examined first. Following this, the association of breast-feeding with cardiovascular risk factors such as blood pressure and serum lipids will be reviewed. As detailed below, systematic reviews and meta-analyses of each of these areas have been published. We have not attempted to replicate this very detailed work. Instead we aim to bring all this evidence together in one place. This will involve summarising the key findings of these meta-analyses and any additional later studies that have been published. However, we also aim to identify some of the broader issues in the interpretation of the evidence and to assess its significance. Finally we consider important evidence from one of the few randomised trials that has contributed to this topic.

2. METHODOLOGICAL ISSUES

Before preceding to outline the substantive results found in the literature it is necessary to reiterate some methodological cautions made elsewhere, which should be borne in mind when assessing the evidence.[21-22]

2.1 Defining the exposure "breast-feeding"

It is important to make one obvious point at the outset. Investigations of the effect of breast-feeding on outcomes in later life are complicated by the difficultly of being precise and consistent in defining what constitutes the exposure "breast-feeding". First, do we define a breast-fed infant as one who is exclusively breast-fed, or simply as an infant who is predominantly breast-fed before weaning? Moreover, is there a minimum qualifying period of breast-feeding required? Second, the nature of the 'unexposed' group is unusual in the case of infant feeding. In contrast to an exposure such as tobacco smoking, where we can compare smokers with never smokers, in the case of breast-feeding those not breast-fed actually receive some other food in infancy. Thus the comparison is implicitly between breast-feeding and an alternative food. This creates a central difficulty in interpretation: is any difference in later disease outcome due to the biological effect of breast-feeding or to the effect of the alternative food ? Third, the alternative food can vary: the composition of artificial bottle feeds has changed dramatically

over time. This is of particular importance when considering the evidence linking breast-feeding with cardiovascular events, such as myocardial infarctions or deaths from coronary heart disease. The study populations required to investigate this will generally have been born in the first 50 years of the 20th century if they are to be old enough to have developed cardiovascular disease. The composition of infant feeds in, for example, Caerphilly, Wales in the 1930s were very different to feeds given to neonates in hospital settings in the 1990s. [23]

2.2 Confounding and reverse causality

Finally, although common to all observational studies and in no way unique to the long-term effects of breast-feeding, there is concern about whether all potential alternative explanations for an association (confounders) have been adequately accounted for. Moreover, there is the question of whether or not breast-feeding in infancy may have itself been influenced by illness or behavioural characteristics that are predictive of, for example, cardiovascular disease. This is the problem of reverse causality (see also Chapter 11 by Kramer).

The potential problems of confounding and reverse causality mean that there is often uncertainty about whether any statistical association between breast-feeding and later outcomes really represents an effect of breast-feeding *per se*. For this reason, properly conducted randomised trials of breast-feeding should provide a much more persuasive level of evidence. However, as we discuss at the end of this chapter, the main randomised trial evidence available about the association of breast-feeding with later cardiovascular risk factors is not as persuasive as it first appears.

3. BREAST-FEEDING AND CARDIOVASCULAR EVENTS

A meta-analysis of 4 studies that presented data on breast-feeding in relation to subsequent cardiovascular mortality was published by Martin *et al* in 2004.[24] It included a total of 3093 cardiovascular deaths among subjects born between 1904 and 1939. Three of the studies were from the UK and one from California, USA.[25] Of the UK studies, the results of one (from the Boyd-Orr cohort) were published as part of the meta-analysis for the first time, a second was in press although subsequently published [23], while the third (the Hertfordshire cohort) contributed data that had been updated from the original publication.[26] The information on breast-feeding in infancy was

collected prospectively in one study, was from maternal recall during the subject's childhood in two studies and was based on subject's self-report in middle age in the fourth study.

The meta-analysis compared infants who were exclusively or partially breast-fed with those who were never breast-fed, i.e. were bottle-fed. With adjustments for a range of factors, which varied across studies (including age, birthweight, socio-economic position in childhood and adult life), the meta-analysis produced a pooled rate ratio for all causes of death of 1.01 (95% CI 0.91 – 1.13) among those who were ever breast-fed relative to those who were never breast-fed. For cardiovascular disease deaths (including coronary heart disease and stroke) the equivalent rate ratio was 1.06 (0.94 – 1.20). Thus, from these four studies there was no evidence that people who were ever breast-fed had a different risk of death from all causes or cardiovascular disease than those who were never breast-fed in infancy. An analysis looking at the effect of prolonged breast-feeding on cardiovascular mortality in three of the studies also failed to find any statistically significant association.

Subsequent to this meta-analysis an additional study looking at breast-feeding in relation to cardiovascular events was published from the US Nurses Health Study.[27] This was based on 2039 incident fatal and non-fatal myocardial infarctions, strokes and deaths from coronary heart disease occurring between 1992-2000 among 87252 US nurses born between 1921-46. Whether subjects were breast-fed in infancy was based on self-report in 1992. A validation of these data was made by asking the surviving mothers of 3515 nurses about breast-feeding. This found self-report to have a specificity of 71% and a sensitivity of 90%. The study found that the rate ratio for any cardiovascular event was 0.91 (95% CI 0.83 – 1.01) among those who were ever breast-fed compared to those never breast-fed. This rate ratio was adjusted for age, subject's cigarette smoking in adult life and birthweight. The direction of the effect seen in this study is opposite to that produced by Martin *et al's* meta-analysis.

Without conducting a further formal meta-analysis it is thus clear that the evidence published to date suggests that there is no association between risk of cardiovascular events and ever having been breast-fed in infancy. However, data are limited, most notably by the absence of prospectively gathered information on breast-feeding of a consistent type. It is therefore possible that a small real association between breast-feeding and cardiovascular events does exist.

4. BREAST-FEEDING AND CARDIOVASCULAR RISK FACTORS

The number of studies able to investigate the link between breast-feeding and cardiovascular disease is inevitably limited, as this requires information on breast-feeding in very large cohorts of people who are already well into middle age. Nevertheless, we can gain further insights by examining whether breast-feeding is linked to cardiovascular disease risk factors such as blood pressure and serum lipid profiles. These can be examined in much younger cohorts than are required for looking at cardiovascular events *per se*, opening up many more possibilities for *ad hoc* studies of contemporary populations of children and adolescents as well as adults.

In the following two sub-sections we review the evidence for an association between breast-feeding and both blood pressure and serum lipids. Equally relevant in this context would be to examine the link between breast-feeding and insulin-mediated glucose uptake and type 2 diabetes, and obesity. These outcomes are dealt with in Chapters 11 and 12.

4.1 Blood pressure

There have been two meta-analyses of the association between breast-feeding in infancy and blood pressure in later life, one published in 2003 by Owen *et al*[28], the second in 2005 by Martin *et al*. [21] Both found that, compared to people who were exclusively or predominantly bottle-fed, those who were breast-fed had a slightly lower systolic blood pressure (SBP). They both also found some evidence of publication bias, with the size of the effect declining as study size increased. Neither study found any evidence of a different effect of infant feeding by the age at which blood pressure was measured. The later meta-analysis, unlike the earlier one, also found a small statistically significant reduction in diastolic blood pressure (DBP) associated with breast-feeding.

The meta-analysis by Owen *et al*[28] included 24 studies. These covered 8 analyses of systolic blood pressure as an outcome in infancy, 12 in childhood and in 6 in adult life. Half the studies included subjects born before 1980, the other half being born later. The total number of subjects who were classified as breast-fed was slightly less than 8,500 while those who were bottle-fed numbered just under 11,300. One of the studies included was a randomised controlled trial, the others being observational studies.

Overall, this meta-analysis[28] found that the difference in SBP between breast-fed and bottle-fed infants was -1.10 mmHg (95% CI -1.79, -0.42) i.e. the breast-fed had lower SBP. The equivalent estimate for DBP was -0.36

mm Hg (-0.79, +0.08). Formal tests showed that the largest effects were observed for the 13 estimates based on less than 300 subjects while the smallest in the 4 studies with more than 1000 subjects (p=0.046).

The most recent meta-analysis by Martin *et al* [21] only included information from 14 studies, in contrast to the 24 included by Owen *et al*. How is this discrepancy to be explained? Firstly, Martin *et al* excluded the studies of blood pressure in infancy. However, it appears that a number of the other studies not included by Martin *et al*, but included in the earlier meta-analysis, were those where Owen *et al* had admirably obtained results directly from researchers that went beyond those available in published papers – a procedure that would be intended to minimise publication bias.

On the plus side Martin *et al* included three studies [23, 29-30] on this issue that had not been published at the time Owen *et al* had produced their meta-analysis, two of which were very large.[29-30] Overall, Martin *et al* included just over 17,500 subjects, although the breakdown by category of infant feeding was not given.

Despite the fact that the two meta-analyses only shared 11 studies in common, the summary result produced by Martin *et al* is very similar to that produced by Owen *et al*. Their pooled difference in SBP among the breast-fed compared to bottle-fed was -1.4 mmHg (95% CI -2.2, -0.6) i.e. the breast-fed had lower SBP. The equivalent estimate for DBP was -0.5 mm Hg (-0.9, -0.04). As found in the earlier meta-analysis there was also statistically significant evidence of publication bias, with the size of effect being inversely proportional to study size.

In summary there is evidence that individuals who were classified as having been breast-fed in infancy had lower blood pressure in childhood and adult life. The difference in blood pressure is relatively small, however, at just over 1 mmHg SBP. Interestingly, Owen *et al* describe this as being of "limited clinical or public health importance." In contrast, Martin *et al* suggest that, if causal, this small difference "could confer important benefits on cardiovascular risk at the population level."

This takes us to the key issue of whether this association is indeed a real effect: the evidence for publication bias is strong, with the smaller effects seen in the larger studies being of marginal statistical significance. The second important caveat concerns the great heterogeneity in definitions of infant feeding seen across the different studies. One could argue that a more consistent definition that encapsulated the key aspects of infant feeding might yield larger effects than observed, although this is pure speculation. Finally, it is notable that neither of the meta-analyses were able to consistently include estimates adjusted for a broad range of confounders. The potential importance of this omission is illustrated by the ALSPAC study,[29] in which adjustment for social, economic, maternal and

anthropometric variables reduced the effect of breast- vs. bottle-feeding by a third from -1.2 mmHg to -0.8 mmHg SBP.

4.2 Lipid profiles

The first systematic review and meta-analysis of infant feeding in relation to cardiovascular risk factors looked at blood cholesterol in infancy, childhood and adult life. This was published in 2002 by Owen *et al*[31], the group that subsequently undertook the meta-analysis of blood pressure discussed in the previous section. It included 37 studies with 52 observations on total serum cholesterol : 26 in infancy, 17 in childhood or adolescence, and 9 in adulthood. With serum low-density lipoprotein (LDL) as the outcome there were 7 observations in infancy, 4 in childhood or adolescence and 6 in adulthood. Most of the studies were of subjects born in the late 1960s or later, with the exception of the 3 oldest cohorts of people born between 1920 and 1946. All the studies were observational, the majority being cohort studies.

As in the case of the other meta-analyses reviewed above, there was considerable variation across studies in the criteria used to categorise individuals as having been breast-fed or bottle-fed. Studies were included in the meta-analysis even if it was not possible to identify groups that had either been exclusively breast or bottle-fed before they were weaned. The vast majority of studies obtained information on breast-feeding during infancy either directly from clinical records or from a parental questionnaire.

The authors of the meta-analysis decided *a priori* to look at the association of infant feeding with serum lipids in three pre-defined age groups (<1 year, 1-16 years, 17+ years). The results confirmed their view that the strength and direction of the association did indeed vary across the three age groups ($p<0.001$). In infancy, breast-fed babies had higher total cholesterol concentrations than bottle-fed (+0.64 mmol/L; 95% CI +0.49, +0.79). In children and adolescents there was no difference in total cholesterol between those who were breast and those who were bottle-fed (0.00 mml/L; 95% CI -0.07, +0.07). In adults, those who were breast-fed had lower total cholesterol than those who were bottle-fed (-0.18 mmol/L; -0.06, -0.30). The patterns observed for LDL cholesterol were similar those seen for total cholesterol throughout.

Since this meta-analysis was undertaken several new studies have been published that have also looked at the association of infant feeding with serum lipids. The Caerphilly cohort study [23] reported on total cholesterol and LDL cholesterol levels in middle age (45-59 years), in relation to infant feeding. It found no evidence of a statistically significant difference. However, it is striking that contrary to the results of the meta-analysis, this

study suggested, if anything, that in adults total cholesterol was higher in breast compared to bottle-fed (+0.05 mmol/L; 95% CI -0.09, +0.18). The Caerphilly study is in fact relatively large compared to the other adult studies in the meta-analysis. It included 1159 breast and 421 bottle-fed infants, while the meta-analysis as a whole included 1519 breast and 495 bottle-fed infants. Given this, it is plausible that if formally combined into a new single meta-analysis, there would no longer be a statistically significant difference in total cholesterol in adult life by whether people were breast or bottle-fed in infancy.

The second paper of relevance that was published after Owen's 2002 meta-analysis was based on the follow-up of a randomised controlled trial of infant feeding in pre-term babies.[32] Out of a total of 926 infants recruited into two parallel infant feeding trials, this paper reported the results concerning serum lipoprotein profile in 216 participants followed up to age 13-16 years. The authors found that the LDL/HDL ratio was lower (a good thing for cardiovascular risk) among people who were assigned to banked breast-milk compared to those who were not (p=0.04). However, they found no statistically significant differences in total cholesterol or LDL cholesterol per se. They concluded that their data provided evidence "for the long-term benefits of breast-feeding". There are, however, a number of issues concerning the interpretation of results from this trial (which also reported on blood pressure in relation to infant feeding) that are best dealt with separately in the next section.

5. RANDOMISED TRIALS OF BREAST-FEEDING

Many of the limitations of the observational data that have been summarised above would be overcome if it were possible to follow-up people who had been randomised in infancy to well-characterised feeding regimes. However, the view that breast-milk provides, in most situations, optimal infant nutrition creates an almost insuperable ethical barrier to randomisation of infant feeding. However, in special situations trials are justifiable. An excellent example is the trail of pre-term feeding established by Lucas et al in 1982.[33] This was established to look at the effects of different feeds on post-natal growth in pre-term infants weighing less than 1850g at birth.

Members of this trial were followed-up in childhood, at ages 7.5-8 years, and in adolescence, at ages 13-16 years. A series of papers have emerged looking at infant feeding in relation to a range of outcomes, including blood pressure[34-35], lipid profiles[32] and insulin resistance.[36] Two parallel randomised trials were conducted. The first trial allocated 502 babies to

either banked breast (N=253) or to pre-term formula (N=249). The second trial allocated babies to either normal term formula or pre-term formula feed. As breast-milk was not a component of the randomisation further details of this second trial are not discussed here.

Within the first trial the feed received by each infant was not solely determined by the randomisation. Some infants received the allocated feed as a supplement. The numbers of subjects in each arm of the trial and the actual feed they received is shown in Table 13-1 together with the numbers followed up into childhood and adolescence.

What is immediately apparent is that the follow-up to adolescence was very far from complete. However, on the reasonable assumption that equal efforts were made to trace all subjects in the trial regardless of feeding regime, *a priori* there is little reason to think that the groups should be biased other than by chance due to the relatively small numbers included. This is confirmed by analyses presented in one of the papers, [35] which suggests that there were minimal differences between those who were followed up and those who were not. Table 13-2 shows mean blood pressures at first follow-up, aged 7.5-8 years of age, according to trial arm.[34]

The differences in SBP and DBP are very small between the various groups, although no formal statistical test between the two arms of the trial was conducted in the paper.[34] Table 13-3 shows mean blood pressures, this time by allocated arm of the trial for the even smaller number of subjects followed-up to age 13-16 years.[35]

Thus it appears that while in childhood there are minimal differences in blood pressure according to infant feeding, by adolescence there is evidence of a difference. With respect to serum lipids, Table 13-4 shows mean levels at age 13-16 years.[32]

Table 13-1. Numbers of subjects at recruitment and follow-up of a randomised trial of pre-term feeding by assignment (see reference 33)

	Actual feeding regime		Follow-up at ages	
			7.5 – 8 years	13 – 16 years
Arm 1	Banked breast-milk	N=83	N=66	N=13
	Banked breast-milk + mother's milk	N=170	N=133	N=53
Arm 2	Pre-term formula	N=76	N=60	N=17
	Pre-term formula + mother's milk	N=173	N=146	N=47

Table 13-2. Mean blood pressures at age 7.5 – 8 years of age by trial arm (see reference 34) and feeding received (mmHg)

Initial randomisation			SBP	DBP
Arm 1	Banked breast-milk	N=66	98.7	61.0
	Banked breast-milk + mother's milk	N=133	99.3	61.4
Arm 2	Pre-term formula	N=60	98.6	61.7
	Pre-term formula + mother's milk	N=146	99.2	61.8

Table 13-3. Mean blood pressures at age 13 – 16 years of age by trial arm (mmHg) (see reference 35)

	Banked breast-milk (n=66)	Pre-term formula (n=64)	p-value for difference
Diastolic BP	61.9	65.0	0.016
Systolic BP	113.6	116.3	0.075
Mean arterial pressure	81.9	86.1	0.001

Table 13-4. Mean serum lipid levels at age 13 – 16 years of age by trial arm (see reference 32)

	Banked breast-milk (n=66)	Pre-term formula (n=64)	p-value for difference
LDL/HDL ratio	2.2	2.5	0.04
Mean total cholesterol (mmol/L)	3.8	4.1	0.06

These results, plus others not shown in this chapter, led the investigators to conclude that "Together with other epidemiological data and our experimental observations … our findings suggest that breast-milk has a major beneficial effect on cardiovascular health".[32] The strength of this conclusion – at least in terms of results from this particular trial – may be questioned. As can be seen from Table 13-1, two thirds of infants randomised to receive pre-term formula actually received this as a supplement to breast-milk. In total, only 76 (15%) out of 502 infants randomised in this trial received no breast-milk at all (either from the mother or a milk bank). Of the 130 subjects followed-up at 13-16 years, only 17 (13%) had not been fed breast-milk of one sort or another. It is therefore difficult to interpret the differences shown in Tables 13-3 and 13-4.

The trial was essentially a pragmatic one, not designed to look at the effects of breast-feeding *per se*. The fact that most of the subjects also received breast-milk was simply how it turned out. The real distinction between the two arms of the trial is that no one in arm 1 received pre-term formula, while everyone in arm 2 did (see Table 13-1). So, the key conclusion one might draw here is that at age 13-16 years, those who were given pre-term formula are more likely to display markers of cardiovascular

disease risk. One could not really claim that the trial provides strong evidence for the beneficial effects of breast-milk *per se*. Thus, despite the advantage of experimental design, unfortunately this trial at least has relatively little to contribute to the evidence base concerning breast-milk or breast-feeding and their relationship to cardiovascular risk factors or events.

6. SUMMARY AND CONCLUSIONS

Current evidence, almost exclusively from observational studies, provides a rather mixed picture. From the few studies that have been able to look at fatal or non-fatal cardiovascular events, there is little indication that breast-feeding is associated with either an increased or decreased risk. With respect to blood pressure, the meta-analyses suggest a small but statistically significant lowering of around 1mmHg SBP associated with having been breast-fed in infancy. However, there is a strong indication from the meta-analyses that even this small effect may partly be accounted for by publication bias. The strongest evidence for an effect of breast-feeding reviewed in this chapter is for serum lipids, where there is good evidence that being breast-fed is associated with an increase in serum total cholesterol in infancy. In childhood there appears to be no association, while in adults there is some indication of breast-feeding being associated with a small decline in total cholesterol levels.

As already outlined at the start of the chapter, this whole area of research is made particularly difficult by the fact that breast-feeding can be defined in many different ways. Some studies use definitions that are equivalent to exclusive breast-feeding prior to weaning, while others define it as having ever been breast-fed. This problem of classification is likely to dilute any real associations that may exist. The other major problem is one of interpretation. A result implying that breast-feeding is a "good thing" for cardiovascular health could equally be construed as evidence for a "bad" effect of bottle-feeding. From these data alone, we cannot convincingly determine which conclusion is correct. This is not simply a philosophical debating point. As discussed above in relation to the interpretation of results from the randomised trial of infant feeding, the issue has implications for all research on this topic.

Some progress in this area will be made if studies are conducted which define breast-feeding in a more precise and comparable way, and take account of the composition of alternative infant feeds. This will be most easily done by following up more recent study populations that were originally recruited to look at shorter-term effects of infant feeding on outcomes such as growth. With respect to randomised trial evidence, looking

at the cardiovascular disease risk profiles of children (and later adults) who were part of the PROBIT trial in Belarus (see Chapters 5 and 10) is likely to prove fruitful.

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Chapter 14

DO INFANTS WHO ARE BREAST-FED HAVE AN ALTERED RISK OF DEVELOPING CANCER?

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1. OUTLINE

The idea that breast-fed infants may be at an increased risk of cancer has been around for at least 60 years. In the 1930s, John Bittner hypothesised on the basis of mouse studies that a factor, perhaps an oncogenic virus, transmitted through the mother's milk was a cause of breast cancer in the offspring.[1] Indeed, in the late 1960s mothers with a family history of breast cancer were advised not to breast-feed their daughters for fear of increasing their child's risk of breast cancer.[2] This historical context highlights the potentially important health policy and public health implications of understanding the long-term health consequences of being breast-fed. Whilst associations with cardiovascular disease have been the subject of a series of recent studies [3-4], there has been less focus on cancer. As well as breast cancer[5], other cancers have been reported in the epidemiological literature to be associated with having been breast-fed in infancy, including childhood haematological and solid tumours[6], and testicular cancer in young men[7] (Box 1).

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Box 1: Cancers associated with having been breast-fed in infancy
Childhood leukaemia
Childhood lymphoma
Childhood CNS cancers & neuroblastoma
Malignant germ call tumours
Breast cancer
Testicular cancer

Epidemiology is notorious for unearthing data dredged, confounded and biased associations.[8] Nevertheless, the possibility that breast-feeding may influence later cancer risk is not without biological plausibility and a number of mechanisms have been proposed (Box 2). In this chapter we appraise the direct and indirect epidemiological evidence that having been breast-fed as an infant influences the development of cancer in later life and we speculate on possible biological mechanisms that might underlie any observed associations. We do not address whether a mother who has breast-fed has a reduced risk of cancer herself, although there is a growing literature on this topic, especially in relation to breast cancer.[9]

Box 2: Suggested biological mechanisms linking breastfeeding with cancer in the offspring
Transmission of a tumour agent through the mother's milk
Immune modulating properties of breast-milk (Greaves hypothesis)
Presence of persistent organic compounds (e.g. dioxins, polychlorinated biphenyls) in breast-milk
Influence of breastfeeding on later growth and insulin-like growth factors (IGFs)
Influence of breastfeeding on insulin resistance, indirectly via an effect of insulin on IGFs or a direct mitogenic effect of insulin resistance

2. EARLY NUTRITION AND THE LATER DEVELOPMENT OF CANCER

The idea that postnatal nutrition might permanently influence health beyond infancy and into adulthood has received much attention in animal studies. Robert McCance manipulated litter sizes in rats, so that rats from large litters received less breast-milk than those from small litters[10]. Rats who were food restricted before weaning were permanently lighter, regardless of the amount of food available after weaning. In contrast, undernutrition between 9-12 weeks of age resulted in reversible weight loss. These observations suggest that under or overfeeding in infancy may have irreversible ("programming") effects on later growth and development, but only during critical periods. More specifically, energy restriction limited to

very early life has been associated with a reduced risk of cancer in animal models.[11]

2.1 Breast-feeding, height and leg-length

The opportunities to directly examine the relationship between early nutrition and cancer many years later are limited, because few cohort studies have information from birth until old age. To date, therefore, most epidemiological studies investigating early life origins of cancer have used indirect markers of childhood nutritional exposures (for example, birthweight, height and leg length), and their relation with cancer.[12] Such markers are used chiefly because they are often readily available from existing records or personal recall in retrospective cohort studies.[13] In contrast, finding records of breast-feeding history, or relying on data from the long-term recall of infant feeding, that can then be related to cancer risk many years later is more problematic. Birthweight is often used as a marker for *in utero* nutrition, but is also influenced by maternal size, smoking, ill health, gestational age and birth order.[13] Adult height is a marker for diet and health during childhood but is influenced by birthweight and the genetic determinants of growth. [13] As long ago as 1893 it was noted that potential army recruits in France were more likely to be rejected on grounds of poor physique if they had been artificially fed.[14] Since breast-feeding in the 19th century was more common amongst the least affluent, this observation is unlikely to be explained by socio-economic confounding.

Epidemiological evidence on associations between height and cancer risk have been systematically reviewed by Gunnell *et al*.[12] Overall, these studies indicate that taller individuals are at a 20-60% increased risk of a range of cancers (comparing the tallest groups versus the shortest). This evidence indicates the possible importance of factors that promote growth in childhood and adolescence, such as early nutrition, in the development of cancer in later life. The most consistent associations have been found in relation to breast cancer, although associations have also been found for other cancer sites, in particular colorectal cancer, prostate cancer, endometrial cancer and haematopoietic cancers.

In those studies that investigated associations of the components of stature and cancer, leg-length (a suggested marker of exposures influencing pre-pubertal growth, such as early socio-economic circumstances, infection load and nutrition[13-15]) was the component of height most often associated with increased cancer risk. For example, in the Boyd Orr cohort, a long-term follow-up of children surveyed between 1937-1939 and followed up in adulthood using the NHS central register, there was a positive association between childhood leg-length and mortality from cancers unrelated to

smoking[16] (Figure 14-1). The observed association was most obvious for mortality from sex-hormone dependent cancers (breast, uterus, ovary, prostate, other genital organs): the risk of death increased by 126% for every unit increase in z-score for leg length (approximately 3-4 mm). There was no evidence that trunk length was associated with cancer.

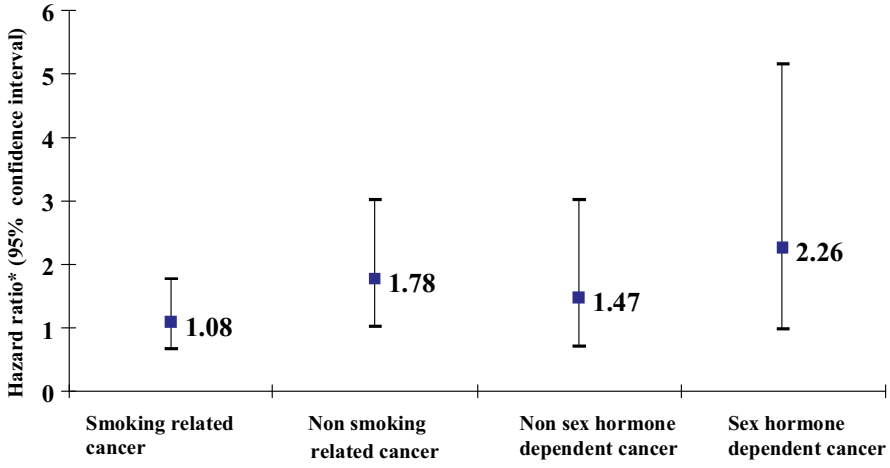

Figure 14-1. Hazard ratios for cancer mortality in relation to one z-score increase in leg length, Boyd Orr cohort. *Adjusted for age, sex, trunk length, childhood and adult SES, survey district. Source: Data obtained from Table 1 in Gunnell *et al*[16]

Leg-length is a suggested marker for growth before puberty since pre-pubertal increases in stature arise more from increases in leg-length than trunk-length.[13] This is demonstrated by changes in the trunk-length:height ratio during growth. At birth the ratio of trunk-length to total height is approx 0.66, but by puberty it has declined to 0.52. From puberty, linear growth occurs equally in the trunk and legs. This suggests that the biological mechanism underlying the relation between height and cancer may have its origins in exposures which influence pre-pubertal long-bone growth.

The positive leg-length – cancer association is unlikely to reflect the long-term effects of prenatal exposures on cancer risk, since associations of birthweight (a marker for *in-utero* exposures) with leg-length and trunk-length are similar.[17] Leg-length is, however, a sensitive marker for childhood diet and socio-economic circumstances.[15] More specifically, leg-length (but not trunk-length) in both childhood and adulthood has been associated with breast-feeding in infancy in analyses of the Boyd Orr cohort[18] (Figure 14-2) and in the 43 year follow-up of the 1946 UK national birth cohort.[19] The specific association between breast-feeding and leg-length, which persists

after controlling for socio-economic circumstances[18-19], suggests that breast-feeding may be a biologically relevant early-life exposure, rather than a marker for a broader social class effect, underlying the observed associations of leg length with cancer. In the Boyd Orr cohort, a mother's leg-length but not trunk-length as a child was associated with her offspring's birthweight, suggesting the intriguing possibility that early nutrition and growth may have trans-generational effects on later health, including cancer risk.[20]

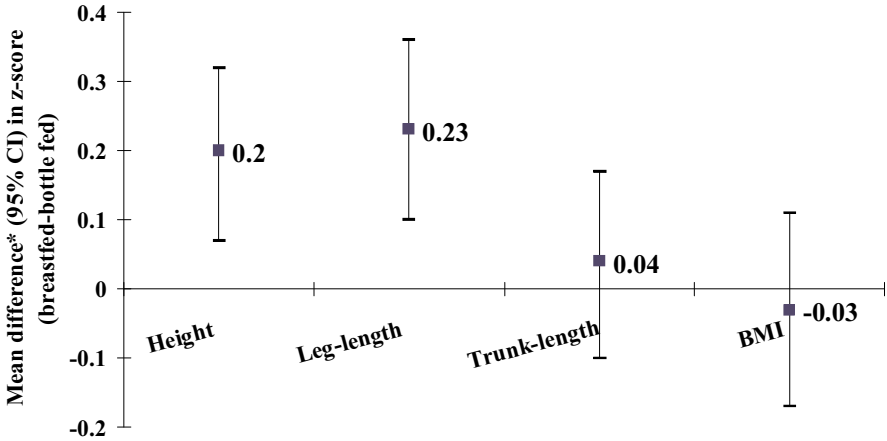

Figure 14-2. Breastfeeding and stature in childhood, boys (n = 1059), Boyd Orr cohort. CI: confidence interval. *Controlling for childhood social factors, family-level clustering and survey district. Source: data obtained from Table 3, Martin *et al.*[18].

There was little social patterning of breast-feeding in 1920-1940, providing some reassurance against residual socio-economic confounding in the Boyd Orr and 1946 birth cohorts. Nevertheless, while breast-feeding may have been associated with leg-length in these early British cohorts, nowadays differences in growth may have decreased because of improvements in the nutritional adequacy of infant formulas. Evidence for an attenuation in the effect of breast-feeding on growth in more recent times comes from the 1958 British birth cohort (n = 10953).[21] Those who were breast-fed were taller at age 7 versus those not breast-fed by a standard deviation (SD) score of 0.17 (1.2 cm) for boys and 0.12 (0.9 cm) for girls, and they remained taller in young adulthood. The effect of breast-feeding was substantially weakened, however, after controlling for parental height, birthweight, family size and social class (adjusted SD score at age 7: 0.08 for boys and 0.03 for girls).

A protective effect of breast-feeding on childhood infections in the setting of high infectious morbidity [22-23] may also underlie associations of

breast-feeding with childhood leg-length in cohorts breast-fed before the second world war. In a recent study from Brazil (n = 2250), height at age 18 increased steadily with increasing duration of breast-feeding (difference in height between longest and shortest durations of breast-feeding: 1.2 cm; p for trend: 0.01).[24] After controlling for birth, socio-economic, maternal, and current lifestyle factors, the difference in height between longest and shortest durations of breast-feeding was 0.9 cm (p for trend: 0.06).

2.2 Insulin-like growth factors, nutrition and cancer

Insulin-like growth factors (IGFs) are multifunctional peptides that regulate cell proliferation, differentiation and apoptosis (cell suicide) and play a fundamental role in somatic growth. Circulating IGF-binding proteins (IGFBPs) modulate the availability of IGF-I to circulating tissue. Over 90% of circulating IGF-I is bound to either a binary or ternary binding complex involving IGFBP-3. Circulating IGF-I is positively associated with height in childhood[25], suggesting that height and leg-length may be acting as anthropometric markers for levels of IGFs.[12]

Recent prospective studies using stored blood up to 14 years before the onset of the disease have linked raised levels of IGF-I or low levels of IGFBP-3 with the later development of pre-menopausal breast cancer, prostate cancer, and colorectal cancer; these are the same sites for which height-cancer associations have been most frequently shown.[26]-[27] Weaker evidence links the IGF axis with childhood leukaemia, but studies of height-childhood leukaemia associations are inconclusive.[12]

Since childhood stature is a marker of the activity of IGF-I, the IGF axis could provide a mechanism explaining associations of height and leg-length with cancer.[12] While adult height is not strongly associated with IGF-I in cross-sectional studies, it may be a marker for this growth factor in childhood and this may be the period during which it acts to increase cancer risk in later life. IGF-I is mainly secreted from the liver in response to growth hormone (GH) but dietary intake also influences IGF-I levels with energy and protein deficient diets resulting in marked reductions in IGF. [28] Furthermore, studies have related diet to IGF-I levels in well-nourished humans.[29-31] For example, in cross-sectional analyses based on 1037 healthy women participants in the Nurses Health Study, total energy and protein intake were positively associated with IGF-I levels when adjusted for covariates.[29] The association with protein intake was largely attributable to higher IGF-I levels among women who consumed higher amounts of milk.

An analysis based on the Boyd Orr cohort [32] found that a lower energy intake in childhood was associated with lower cancer risk in later life (relative hazards for all cancer mortality and cancers not related to smoking

= 1.15 and 1.20 per MJ, respectively, in fully adjusted models; p = 0.001). In animals energy restriction reduces risk of cancer primarily by reducing circulating concentrations of IGF-I.[33] It is therefore plausible that height-cancer associations may reflect an association between early diet and cancer risk that is mediated via the IGF system.

If height and leg-length are markers for IGF-I activity in childhood [26], could the positive association of breast-feeding with stature [18,19] be mediated by an association between breast-feeding and IGF-I? In a study based on 33 preterm infants (gestational age: 28-37 wks) who were appropriate size for their gestational age, levels of IGF-I and IGFBP-3 were measured at one and three weeks after birth.[34] There were strong positive correlations between neonatal protein ($r = 0.40$; $p < 0.1$) and energy intakes ($r = 0.45$; $p < 0.001$) and IGF-I and IGFBP-3, suggesting that very early nutritional intake influences levels of these growth factors.

Breast-fed infants may be at reduced risk of early overnutrition and accelerated growth [35] compared with those who are formula-fed since formula-fed infants consume greater volumes of milk than breast-fed infants and appear to have higher protein and energy intakes in infancy.[36-39] The lower energy and protein intakes of breast-fed infants compared with those who are formula-fed, may lower hepatic IGF-I production at the time of breast-feeding, which would be compatible with the slower growth rate of breast-fed compared with formula-fed infants.[35]

Could programming of IGF-I levels by infant nutrition underlie associations between childhood stature and cancer? Indirect evidence for a programming effect of early nutrition on long-term levels of IGF is provided by one study based on 87 postmenopausal women living in Utrecht.[40] In this study, the degree of childhood exposure to the 1944-1945 Dutch famine (when daily rations dropped from 1500 kcals in Sept 1944 to below 700 kcal in Jan 1945 until liberation in May 1945) was associated in a dose response manner with increased plasma levels of IGF-I and IGFBP3 at age 50-69. These data suggest that a relatively short period of energy restriction was associated with increased long-term levels of IGF-I. No differences were found for c-peptide levels, a marker of insulin resistance. The results are opposite to immediate responses seen under starvation, which would have caused a lowering of IGF-I, and could indicate a permanent resetting up of the IGF-axis upon improvement of diet after the famine. We have recently observed that IGF-I levels were lower at age 25 years in individuals who received milk-supplementation to their diets as children up to the age of 5 years.[107] In the Avon Longitudinal Study of Parents and Children, having been breastfed was positively associated with IGF-I levels at age 7-8 years[108]. These two studies would be compatible with increased nutritional intake in infancy, primarily protein intake, causing a direct increase in

hepatic IGF-I production which then feeds back to suppress pituitary growth hormone output with a long-term resetting of the pituitary resulting in lower IGF-I levels if the nutritional intake reverts. Thus, a relatively low IGF-I level at the time of breast-feeding would be consistent with the slower infant growth rate at that time and a consequent resetting up of the pituitary, due to less feedback, could then result in a relatively high IGF-I level subsequently in later life. This would be compatible with our findings of greater height in later childhood and adulthood associated with breast-feeding.

Given that leg-length is sensitive to early diet, including breast-feeding [18-19], and leg-length may be a marker for childhood levels of IGF-I [25] which are influenced by dietary factors [29-30], including nutrition in the neonatal period [34], breast-feeding may be positively associated with future levels of IGF-I. Since IGF-I is associated with sex-hormone dependent cancers [26-27], we also hypothesise that breast-feeding may be positively associated with breast, prostate and colorectal cancers (Figure 14-3).

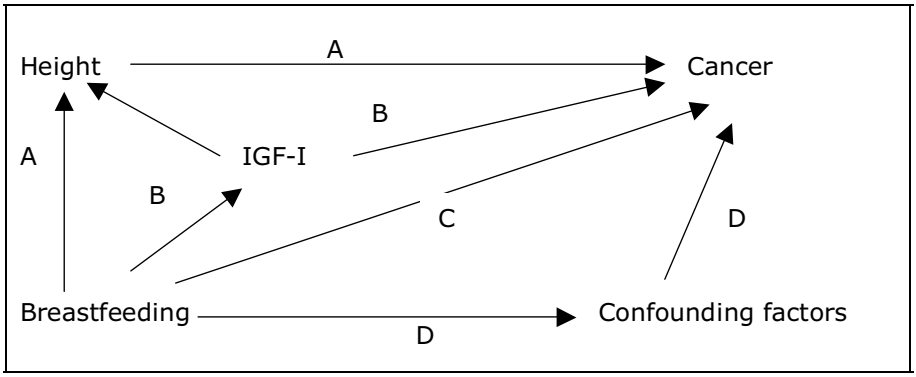

Figure 14-3. Possible pathways linking breast-feeding with cancer

In pathway A, breast-feeding influences childhood height and leg–length. In turn, taller individuals are at increased risk of cancer by mechanisms linking total body size with cancer.
In B, breast-feeding is positively associated with later levels of IGF-I, which increases cancer risk via the biological properties of IGF-I.
In C, breast-feeding more directly influences cancer risk (e.g. via maternal transmission of a virus[1] or organochlorines[41]).
In D, breast-feeding is a marker for a confounding factor.

Insulin is closely related to the IGF system. Hyperinsulinaemia has been associated with cancers, such as breast [42] and prostate cancers [43], and an influence of breast-feeding on insulin resistance [44] is another possible mechanism that could link breast-feeding with cancer, perhaps via an effect

of insulin resistance on insulin-like growth factors (hyperinsulinaemia increases circulating levels of IGF-I by suppressing the hepatic production of IGFBP-1) or by a direct mitogenic effect.

3. OTHER HYPOTHESES RELATING BREAST-FEEDING WITH THE LATER DEVELOPMENT OF CANCERS

As indicated in Box 2, several other hypotheses have been developed which link breast-feeding in infancy with later cancer risk. In John Bittner's experiments, descendants from a line of albino mice with a high incidence of breast tumours (80%) were either breast-fed by their own mother or immediately fostered by mothers with incidence rates of breast tumour of 14% (intermediate tumour stock females) or 1.0 % (low tumour stock females). Mice nursed by low or intermediate tumour stock females had significantly lower incidence rates of breast cancer compared with mice nursed by their own mothers of high tumour stock. Bittner hypothesised that *"…some influence is transmitted through the mother's milk which is of prime importance in determining the incidence of breast tumours"*.[1] This hypothesis would speculate that breast cancer risk would be increased among women who were breast-fed by a mother who subsequently developed breast cancer. Epidemiology studies that have tested this hypothesis are reviewed in section 5.

Greaves has proposed that breast-milk may play an important role in the prevention of childhood leukaemias by actively stimulating or modulating the immune system and promoting its development in early life.[45] A childhood peak of common acute lymphoblastic leukaemia at age 2-5 emerged in the early 20th century in developed countries. To explain both these temporal trends [46] and observed clusters of childhood leukaemia cases [47], and drawing on animal models and molecular evidence, Greaves hypothesised that childhood common acute lymphoblastic leukaemia may arise as a result of delayed exposure to common infections until the ages represented by the childhood peak (when lymphocytes may be more vulnerable to spontaneous mutations) or because of a failure (in more developed countries) of appropriate immune-modulating infectious exposures in infancy.[45] Greaves proposed that delayed exposure or a poorly developed immune system results in an abnormal immunological response which in susceptible individuals could increase the proliferation of premalignant clones and enhance the risk of further mutations and of childhood leukaemia. In contrast, early stimulation of the immune system is

suggested to promote adequate immune modulation, increasing the appropriateness of response to later infection.

Greave's hypothesis is supported by studies showing that the risk of childhood leukaemia is inversely associated with early day-care attendance [48-50], a history of early common infections [48-49, 51-52], more crowded households [52-53], higher rates of immunization [54], and population mixing, which tends to increase contacts between susceptible individuals in rural areas and infected individuals from urban areas.[47] Although breast-feeding has anti-infective properties, it also has immune stimulatory effects, and breast-fed infants appear to develop aspects of their own immune systems more rapidly than artificially fed infants.[55] Greaves specifically hypothesised that breast-feeding protects against childhood common acute lymphoblastic leukaemia because of these early immune modulating effects. This hypothesis has stimulated several reports of the association between breast-feeding and childhood acute lymphoblastic leukaemia, but has also been used as the basis for investigating a wide range of childhood cancers, such as malignant germ cell tumours[56], central nervous system tumours[57] and neuroblastoma.[58] Whether these associations arose as a result of *a priori* hypotheses or are reports of significant findings following multiple *post-hoc* analyses is unclear.

Others have noted that mutagenic and carcinogenic organic compounds, such as dioxins and polychlorinated biphenyls, can be transferred to the offspring via breast-milk.[41] Greater plasma concentrations of these organochlorines have been observed in breast-fed compared with formula-fed infants and are related to the duration of breast-feeding. It has been proposed that if mammary epithelial cells are exposed to lipid-soluble carcinogens sequestered by the adipose tissue, carcinogenesis can result. There is some evidence to support this idea. In adult women, plasma organochlorine levels have been associated with breast cancer[59], suggesting the hypothesis that they may increase breast cancer risk in the offspring of mothers who breast-feed. However, prospectively measured plasma levels of polychlorinated biphenyls were not associated with the subsequent development of breast cancer in a recent case control study (n = 230 case: control pairs)[60], and in the absence of more direct evidence this hypothesis remains speculative.

4. BREAST-FEEDING AND CANCER IN CHILDHOOD

The cause of most childhood leukaemia and lymphomas is unknown. Few childhood cases can be explained by exposures to agents known to be

leukaemogenic in adults, such as ionising radiation, cancer chemotherapy or Down's syndrome. There is increasing evidence that very early exposures are associated with childhood cancers.[47-54] Knowledge of the relationship between breast-feeding and childhood leukaemias and lymphomas is important. Based on a review of six published studies suggesting reductions in risk of childhood leukaemia or lymphoma of between 30%-50%, Parker calculated that 25% of the 500 annual UK cases of childhood acute leukaemia or lymphoma may be prevented if the prevalence of breast-feeding at six months was increased from current levels of around 20% to 100%. [61] Parker's review suggests that if effect estimates from studies on childhood leukaemia and lymphoma are true, there could be important public health consequences of increasing efforts to promote breast-feeding. However, our own review of the literature suggests that the relationship of breast-feeding with childhood cancers is not clear-cut (see below).

4.1 Results of systematic literature search

We undertook a systematic search of the literature up to September 2004 and identified 5 review articles [6, 62-65] and 29 individual studies which documented the association between breastfeeding and at least one childhood cancer (age < 20 years) (Table 14-1). A comprehensive update of this review and detailed meta-analyses, including new publications up to April 2005, has subsequently been published.[109]

The largest studies were: i) the Children's Cancer Group (>2500 cancers since 1988)[75]; ii) UK Childhood Cancer Study (>3000 cancers since 1992)[6] and iii) the Oxford Survey of Childhood Cancers (>3000 cancers since 1955)[66]. The Children's Cancer Group is a cooperative clinical trials group with around 118 affiliated institutions in the USA, Canada and Australia. The USA participating institutions treat around 50% of all paediatric cancer cases throughout the USA. The UK Childhood Cancer Study and the Oxford Survey of Childhood Cancers are national population-based case control studies. Different cases were included in these two studies. Table 14-2 summarises the retrieved studies by cancer site and study design. Only one cohort study was retrieved. [53] This was based on breast-feeding on discharge from place of confinement collected by the Northern Ireland Child Health System which was linked with the subsequent development of cancer identified in the Northern Ireland Cancer Registry.

Table 14-1. Studies documenting the association between breast-feeding and a childhood cancer. (ALL: acute lymphoblastic leukaemia; AML: acute myeloid leukaemia, ANLL: acute non-lymphoblastic leukaemia. HD: Hodgkin's disease; NHL: Non-Hodgkin's lymphoma.)

1st author, study, country (year), reference (ordered by year of publication)	No. of cancers
Jourdan-Da Silva, France (2004)[48]	ALL: 393; AML: 59
Lancashire, Oxford Survey of Childhood Cancers, UK (2003)[66]	All childhood cancers: 3376; leukaemia: 1342 (ALL: 948; ANLL: 394); all other non-haematological cancers: 1708
Perrillat, France (2002)[67]	Leukaemia: 246 (ALL: 218; ANLL: 28)
Daniels, USA (2002)[68]	Neuroblastoma: 393
Murray, Northern Ireland (2002)[53]	ALL: 178
Beral, UK Childhood Cancer Study, (2001)[6]	All childhood cancers: 3500; leukaemia: 1637 (ALL: 1401; AML: 214); HD: 114; NHL: 228; all other cancers: 1521
Schuz, Childhood Cancer Registry, Germany (2001)[57]	All CNS cancers: 465
Hardell, Sweden (2001)[69]	All childhood cancers: 835; leukaemia: 235 (ALL: 204; AML: 26); HD: 28; NHL: 52; CNS: 264; neuroblastoma: 34
Benner, United Arab Emirates (2001)[70]	Leukaemia: 117 (ALL: 69); HD: 22; NHL: 27
Infante-Rivard, Canada (2000)[50]	ALL: 491
Rosenbaum, USA (2000)[71]	ALL: 255
Dockerty, New Zealand (1999)[72]	ALL: 97
McKinney, Scottish case-control study (1999)[51]	Leukaemia: 144 (ALL: 124); lymphoma: 45; CNS: 75; other solid tumours: 126
Smulevich, Russia (1999)[73]	All childhood cancers: 593; leukaemia: 199 (ALL: 109; AML: 25); HD: 47; NHL: 70; CNS: 57; neuroblastoma: 42
Schuz, Childhood Cancer Registry, Germany (1999)[74]	Leukaemia: 1001 (ALL: 682)
Shu, Children's Cancer Group, USA / Canada / Australia (1999)[75]	Leukaemia: 2200 (ALL: 1744; AML: 456)
Grufferman, Pediatric Oncology & Children's Cancer Groups, USA/Canada (1998)[76]	HD: 444
Petridou, Greece (1997)[77]	153 (89% ALL)
Shu, Shanghai (1995)[75]	Leukaemia: 159 (ALL: 108; ANLL: 51); HD: 14; NHL: 68
Shu, Children's Cancer Group, USA / Canada (1995)[56]	Childhood malignant germ cell tumours*: 105
Mathur, India (1993)[78]	Leukaemia: 65 (ALL: 48; ANLL: 14); HD: 8; NHL: 11
Schwartzbaum, USA (1991)[79]	ALL: 522; ANLL: 107; HD: 133; NHL: 104; neuroblastoma: 104
Golding, 1970 birth cohort, UK (1990)[80]	All cancers: 33
Birch, Inter-regional epidemiological study of childhood cancer, UK (1990)[81]	CNS: 78
Magnani, Italy (1988)[82]	Leukaemia: 163 (ALL: 141; ANLL: 22);

1st author, study, country (year), reference (ordered by year of publication)	No. of cancers
	NHL: 19
Davis, Colorado childhood cancer study, USA (1988)[83]	All childhood cancers: 201; ALL: 52; ANLL: 11; HD: 13; NHL: 13; CNS: 38; other: 59
van Duijn, Childhood Leukaemia Study, Netherlands (1988)[84]	ALL: 492
Hartley, Inter-regional epidemiological study of childhood cancer, UK (1988)[54]	All childhood cancers: 520
McKinney, Inter-regional epidemiological study of childhood cancer, UK (1987)[85]	Leukaemia: 171; lymphoma: 63

Table 14-2. Published studies giving an estimate of the effect of breast-feeding on childhood cancer by cancer site and type

Cancer type	No. of studies	Case control / cross-sectional studies	Cohort /nested studies
All childhood cancers	7	6	1
Leukaemia	13	13	0
Acute lymphoblastic leukaemia	17	16	1
Acute non-lymphoblastic leukaemia	9	9	0
Lymphoma	2	2	0
Hodgkin's disease	8	8	0
Non-Hodgkin's lymphoma	7	7	0
CNS	6	6	0
Neuroblastoma	3	3	0
Other solid cancers	4	4	0
Malignant germ cell tumours	1	1	0

Table 14-3. Characteristics of 17 studies investigating the association between breast-feeding and acute lymphoblastic leukaemia

Characteristic	No (%) unless stated
Year published (range)	1988-2004
Year of birth (range)	1956-1999
Exclusive breastfeeding compared with exclusive bottle feeding	1 (6%)
Median (IQR) % ever breastfed	59% (49%-77%))
Median (IQR) % breastfed > 6 mo	21% (9%-35%)
Influence of duration of breastfeeding investigated	14 (82%)
Measurement of infant feeding exposure based on maternal recall	14 (82%)
Response rate in cases < 80%	5 (31%)
Response rate in controls < 80%	10 (63%)
Population-based studies	14 (82%)
Controlled for at least one socioeconomic factor	12 (71%)

IQR: interquartile range.

4.2 Generalisability and quality of retrieved studies

To illustrate the generalisability and quality of these studies, the characteristics of the 17 reports investigating the association between breast-feeding and acute lymphoblastic leukaemia are summarised in Table 14-3. The year of birth of the participants in these studies varied between 1956-1999, during which time the composition of artificial feeds changed from unmodified diluted cow milk to formulae modified to more closely resemble breast-milk. Thus different studies could suggest different estimates of the association between breast-feeding and childhood leukaemia because the exposure of the reference group in different studies is heterogeneous. This will affect the generalisability of earlier studies to modern cohorts.

In general, the quality of the studies was poor for a number of reasons. Firstly, measurement of breast-feeding exposure was limited. Only one study compared a group defined as having been exclusively breast-fed with an exclusively bottle-fed group.[71] However, the meaning of exclusive breast-feeding was undefined. More studies investigated the influence of duration of any breast-feeding. The proportion of infants ever breast-fed and the proportion breast-fed for at least 6 months varied widely in different studies. As all studies effectively compared ever versus never breast-fed, without taking into account the duration or exclusivity of breast-feeding in each of the populations studied, effect estimates are likely to vary between studies, if duration or exclusivity of breast-feeding are important determinants of cancer.

Secondly, most studies relied on long-term maternal recall of breast-feeding after the diagnosis of cancer had been made; only three studies used data from existing child health or maternity records [53,69,51]. There is thus potential for misclassification of breast-feeding status. Retrospective recall may lead to recall-bias in case-control studies if the diagnosis affects responses or if misclassification of breast-feeding is socio-economically patterned, as suggested in at least one validation study.[86] Thirdly, although the majority of studies were population rather than hospital based (a potential advantage), the response rate in controls was less than the response rate in cases in most studies. This may have introduced a selection bias if the exposure prevalence in controls is not representative of the source population of cases. Since the second world war, breast-feeding has been increasingly more common among women from higher socio-economic backgrounds and in some studies participating controls were more advantaged than non-participating controls.[72] This selection bias could generate inverse associations of breast-feeding with leukaemia.

Fourth, publication bias is an issue in epidemiological studies, such as these case control studies, where vast amounts of exposure data are

collected, encouraging 'fishing expeditions' and multiple hypothesis testing. Thus although there is a high probability of finding a 'significant' result in such data-driven analyses, the tendency of both authors and editors is to only publish 'significant' results. Null results are often not published (publication bias) or the results are hidden in the text or tables so that literature searches do not retrieve them (citation bias). Whether observed associations arose as a result of *a priori* hypotheses or are reports of significant findings following multiple *post-hoc* analyses is unclear. Fifth, as can be seen from Table 14-1, the numbers of specific cancers in each study were generally small, and confidence limits in most of the studies were wide. Finally, although some studies attempted to control for socio-economic factors, the likelihood of residual confounding and confounding by unknown factors is a very real and often under-appreciated possibility in observational studies such as these.[8] Generally only around one third of studies presented both crude and adjusted effect estimates; these suggested that study specific crude and adjusted odds ratios were similar.

4.3 Results of studies relating breast-feeding with childhood cancers

The results of the retrieved studies are summarised in Table 14-4. For acute lymphoblastic leukaemia only 3 of 17 studies provided meaningful evidence ($p < 0.05$) of any association with breast-feeding. Although the report by Murray *et al* included only 178 cases, it was the only cohort study. The results were based on routinely recorded method of infant feeding on discharge from place of confinement, so recall bias is not a problem. This study is in line with the majority in suggesting no association of breast-feeding with acute lymphoblastic leukaemia (rate ratio = 0.98). The studies by Hardell [69] and McKinnney [51], which also used routinely recorded method of infant feeding as the source of exposure data, similarly found no evidence of any association. Furthermore, the two large population-based UK studies failed to find any evidence of a strong association (odds ratios = 0.99 and 0.91, respectively) [66,6] The large USA study, however, suggested a 20% reduction (95% CI: 7%-31%) in risk of acute lymphoblastic leukaemia in childhood [75].

For Hodgkin's Disease, 2 of 8 studies provided meaningful evidence ($p < 0.05$) of any association with breast-feeding. One of these was published as an abstract.[76] Three studies suggested an intriguing inverse association between breast-feeding and risk of neuroblastoma [68,69,73], although only one was significant at the 5% level (suggesting a 40% reduction; 95% CI: 10-50%) [68] and the biological mechanism explaining this association is unclear. Malignant Germ Cell tumours (including yolk sac tumour, germinoma,

dysgerminoma, seminoma, choriocarcinoma, gonadoblastoma, and malignant teratoma) were not related to breast-feeding in one exploratory study.[56]

Few studies provided strong evidence using appropriate statistical analyses that a duration-response effect of breast-feeding exists. In one study by Bener *et al*, however, any breast-feeding ≥ 6 months was associated with a 60% reduction (15%-81%) in risk of acute lymphoblastic leukaemia compared with any breast-feeding for 0-6 months.

5. BREASTFEEDING AND CANCER IN ADULTHOOD

The mouse experiments of John Bittner (see section 3.0**)** stimulated early epidemiological studies to explore the hypothesis that a transmissible cancer-causing agent was present in breast-milk. [87-90] For example in a paper published in 1948, Penrose *et al* compared the breast-feeding histories of 79 familial breast cancer cases (who had one or more relatives with breast cancer) with 360 women with sporadic breast cancer. The authors hypothesised that if a breast cancer-causing agent was transmitted in breast-milk then breast-feeding should be more common amongst women with familial compared with sporadic breast cancer. However, the proportion breast-fed was similar whether or not the case had a family history of breast cancer (Table 14-5). Penrose concluded that *"a search for evidence that mammary cancer can be inherited through maternal milk gave negative results".*[87] In 1957, Bucalossi and Veronesi compared the breast-feeding history of breast cancer cases whose mother had breast cancer with two control groups – sporadic breast cancer cases and hospital controls without cancer.[88] The proportions breast-fed were similar in all three groups (Table 14-5), leading the authors to conclude that: *"This result is not favourable to the hypothesis of transmission of a supposed 'tumoral agent' through mother's milk".* Although other early investigators reached similar conclusions [89-90], the possibility of a breast cancer virus attracted much interest in the 1970s and 1980s when viral RNA was found in breast cancer cells and in human breast-milk. However, in a large population-based case-control study by Titus Ernstoff *et al* published in 1998 [91], maternal history of breast cancer was found to confer the same increase in risk of breast cancer among women who had been breast-fed (OR: 1.88) and those who had not been breast-fed (OR 1.93). The p-value for interaction between having been breast-fed and family history of breast cancer in relation to the woman's breast cancer risk was 0.94. These results, argue strongly against a transmissible agent in breast-milk that increases breast cancer risk.

Table 14-4. Results of studies of breast-feeding and childhood cancer (ordered by year of publication).

Direction of association for ever breast-fed *vs* never (formula) breast-fed*

Author	Leukaemia	ALL	ANLL	HD	NHL	CNS tumours	Other	All childhood cancers combined
Jourdan-Da Silva[48]	-	Null	Null	-	-	-	-	-
Lancashire[66]	Null	Null	Null	-	-	-	Null	Null
Perrillat[67]	Null	Null	Null	-	-	-	-	-
Daniels[68]	-	-	-	-	-	Inverse	-	-
Murray[53]	-	Null	-	-	-	-	-	-
Beral[6]	Inverse	Null	Null	Null	Null	-	Null	Inverse
Schuz[57]						Null		
Hardell[69]	Null	Null	Inverse	Null	Positive	Null	-	Null
Benner[70]	Inverse	Inverse	-	Null	Null	-	-	
Infante-Rivard[50]	-	Inverse	-	-	-	-	-	-
Rosenbaum[71]	-	Null	-	-	-	-	-	-
Dockerty[72]	-	Null	-	-	-	-	-	-
McKinney[51]	Null	Null	-	Null†		Null	Null	-
Smulevich[73]	Inverse	-	-	Null	Inverse	Null	-	Inverse
Schuz[74]	Null	Null	-	-	-	-	-	-
Shu[75]	Inverse	Inverse	Null	-	-	-	-	-
Grufferman[76]	-	-	-	Inverse	-	-	-	-
Petridou[77]	Null	-	-	-	-	-	-	-
Shu[75]	Null	Null	Null	Null	Null	-	-	-
Shu[56]	-	-	-	-	-	-	Null	-
Mathur[78]	-	-	-	Inverse†		-	-	-
Schwartzbaum[79]	-	-	-	Inverse	-	-	-	-
Golding[80]	-	-	-	-	-	-	-	Null
Birch[81]	-	-	-	-	-	Null	-	-
Magnani[82]	Null	Null	Null	-	Null	-	-	-
Davis[83]	-	Null	Null	Null	Null	Null	Null	Null
van Duijn[84]	-	Null	-	-	-	-	-	-
Hartley[54]	-	-	-	-	-	-	-	Null
McKinney[85]	Null	-	-	Null†		-	-	-

ALL: acute lymphoblastic leukaemia, ANLL: acute non-lymphoblastic leukaemia. HD: Hodgkin's disease; NHL: Non-Hodgkin's lymphoma. Other: non-haematological (solid) cancers. *Null result implies no statistical evidence of an association at the 5% level. †Result for all lymphoma combined.

Table 14-5. .Breast cancer in mothers and daughters by breast-feeding history

Family history of breast cancer cases	No.	% breastfed
Penrose, UK (1948)[87]		
"Familial" breast cancer	79	93%
Sporadic breast cancer	360	87%
Bucalossi, Italy, (1957)[88]		
"Familial" breast cancer	81	82%
Sporadic breast cancer	3886	85%
Hospital controls without cancer	1177	84%

In section 2.2 we hypothesised that breast-feeding may be positively related to future levels of IGF-I. Since IGF-I is associated with sex-hormone dependent cancers, we also hypothesised that breast-feeding is positively associated with cancers of the breast and prostate. The following sections review published studies reporting on associations between having been breast-fed and future risk of cancer.

5.1 Results of systematic literature search

We conducted a MEDLINE search of studies between 1966 and September 2004. This search retrieved 11 studies relating having been breastfed with breast cancer, three which examined the risk of testicular cancer in men and two papers with follow-up into adulthood relating breastfeeding with all cancers (Table 14-6). A comprehensive update of this review and meta-analysis, including new publications up to July 2005 and novel data from the Boyd Orr cohort, has subsequently been published.[110]

The largest studies were by Titus Ernstoff *et al* (a population based case control study based on three registries in USA; n=4008 cases)[91], Michels *et al* (based on the Nurses Health Studies; n = 1073)[5] and Brinton *et al* (a case-control study nested in screening programme).[97]

5.2 Generalisability and quality of retrieved studies

The generalisability and quality of retrieved studies relating breast-feeding with breast cancer is summarised in Table 14-7. The participants in these reports were born between 1874-1972 during which time alternatives to breast-feeding changed, but before the development of modern modified formulae. Thus the relevance of these analyses to babies born now is unclear.

The quality of the studies was generally poor, for a number of reasons. Firstly, there were only 2 cohort studies, by Michels *et al* (Nurses Health Study)[5] and Ekbom *et al* (a record-linkage study based on the Swedish cancer registry).[96] Such studies are less likely than case-control studies to be

affected by recall bias and selection bias. Secondly, only one report compared exclusive breast-feeding compared with exclusive bottle-feeding[96], but the exposure data were limited to feeding method recorded at hospital discharge (classified as breast-fed only, partly breast-fed, and not breast-fed) so the effect of prolonged exclusive breast-feeding could not be ascertained. Thirdly, only two studies examined the influence of breast-feeding duration [5,93], the remaining simply comparing ever versus never breast-fed. The proportion of infants who were breast-fed for at least 6 months varied widely in the in different studies. This may be a source of between-study variation in estimates comparing ever versus never breast-fed.

Fourth, in 91% of studies breast-feeding history was obtained on interview of the participant's mother (n = 2) or the study participants themselves. This is a potential source of recall bias in the case-control studies, or misclassification bias in the cohort study reported by Michels *et al*.[5] In a retrospective cohort study, Ekbom *et al* abstracted data from hospital records completed by midwives or nurses[96], but these records were filled between 1874-1954 for non-research purposes and non-differential misclassification could lead to regression-dilution bias and attenuation of effect estimates. Fifth, in most of the case-control studies response rates were less than 80% in both cases and controls. This is a potential source of selection bias in these studies. Finally, control for potential confounding factors was patchy in these reports.

5.3 Results of studies relating breast-feeding with cancer in adulthood

The results of studies relating breast-feeding with all cancers, breast cancer and testicular cancer are summarised in Table 14-8. In general there was little evidence that having been breast-fed was associated with all cancers, breast cancers at all ages, or breast cancers stratified into pre- and post-menopausal age at diagnosis. At most, three studies reported borderline inverse associations between having been breast-fed and future breast cancer risk [94,95,97]. However, most of these studies had few cases and effect estimates were often imprecise, making type 2 errors possible. The two cohort studies[5,96] and the two studies that examined duration of breast-feeding [5,93] showed null results for breast cancer risk in relation to breast-feeding.

Three studies have examined the relationship between breast-feeding and testicular cancer[7,98,99]. The most recent was a population-based case-control study involving 446 men aged 15-49 years with testicular germ cell tumour diagnosed between 1984-1987 and identified at major cancer treatment centres and regional cancer registries.[7] The mothers of the participants

completed a questionnaire, including duration of any breast-feeding. The results provided some evidence of a duration-response relationship between any breast-feeding and testicular cancer in multiply adjusted models (p for trend = 0.05) (Figure 14.4). The study was established to investigate factors that might explain the 88% increase in age-standardised incidence rates of testicular cancer in the UK between 1971-1997. Therefore a large number of exposures were investigated and the possibility that this is a chance finding in the context of multiple hypothesis testing is a possibility. The two other studies provide contradictory evidence. One report, involving 78 cases diagnosed between 1972-4 in Los Angeles, showed a non-significant 11% reduction in risk of testicular cancer in men 15-40 yrs in models controlling for age, sex and neighbourhood (p = 0.4).[99] However, a Japanese study based on 37 cases showed a 5% increased risk of testicular cancer per month of breastfeeding in multiply adjusted models (p=0.1).[98]

Table 14-6. Studies documenting the association between breast-feeding and cancers in adulthood.

1st author, study, country (year), reference (ordered by year of publication)	No of cancers
ALL CANCERS	
Wingard, Terman Life cycle study, USA (1994)[92]	140
Tokuhata, USA (1969)[89]	75
BREAST CANCER	
Michels, Nurses' Health Studies, USA (2001)[5]	Pre-menopausal: 413; post-menopausal: 660
Titus Ernstoff, USA (1998)[91]	Pre-menopausal: 205; post-menopausal: 3803
Sanderson, USA (1998)[93]	< 45 years: 510
Weiss, Women's Interview Study of Health, USA (1996)[94]	20-45 years: 508
Freudenheim, USA (1994)[95]	Pre-menopausal: 229; post-menopausal: 299; all ages: 528
Ekbom, Sweden (1993)[96]	458 (< 50 yrs: n = 212; ≥ 50 yrs: n = 246).
Brinton, Breast Cancer Detection Demonstration Project, USA (1983)[97]	1192
Henderson, USA, (1974)[90]	235
Tokuhata, USA (1969)[89]	13
Bucalossi, Italy, (1957)[88]	81 familial (*vs* 3886 sporadic) breast cancers
Penrose, UK (1948)[87]	79 familial (*vs* 360 sporadic) breast cancers
TESTICULAR CANCER	
Coupland, UK, (2004)[7]	446
Mori, Japan (1990)[98]	37
Henderson, USA, (1979)[99]	78

Table 14-7. Characteristics of 11 studies investigating the association between having been breast-fed and breast cancer

Characteristic	No (%) unless stated
Year published (range)	1957-2004
Year of birth (range)	1874-1972
Cohort (or nested case-control) studies	2 (18%)
Exclusive breastfeeding compared with exclusive bottle-feeding	1 (9%)
Median (IQR) % ever breastfed	79% (49%-86%)
Median (IQR) % breastfed > 6 mo	39% (13%-65%)
Influence of duration of breastfeeding investigated	2 (18%)
Measurement of infant feeding exposure based on maternal recall or self report	10 (91%)
Response rate in cases < 80%[†]	6 (86%)[†]
Response rate in controls < 80%[†]	5 (83%)[†]
Controlled for at least one socioeconomic factor	5 (45%)
Controlled for at least one reproductive factor	6 (55%)
Controlled for family history of breast cancer	5 (45%)
Population-based studies	8 (73%)

[†]For case-control studies

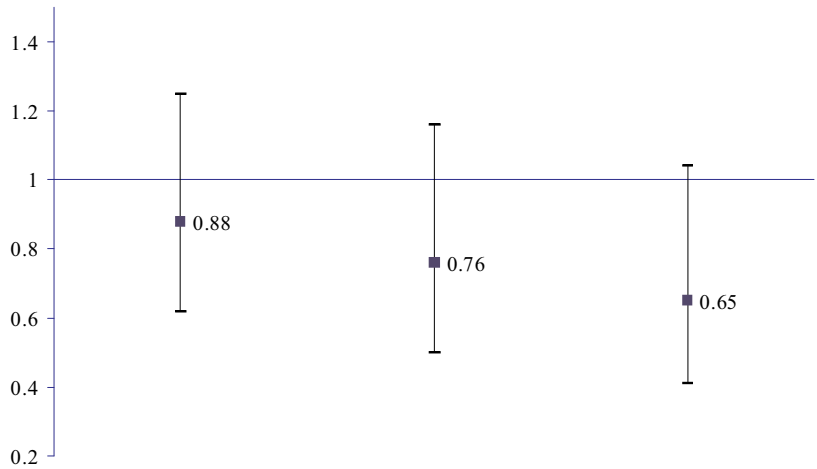

Figure 14-4. Testicular cancer and duration of any breast-feeding. *Adjusted for age, region, son's social class, undescended testis, inguinal hernia, maternal age at pregnancy. Source: Figure derived from data presented in Coupland *et al*. [7]

Table 14-8. Results of studies of breast-feeding and all cancers, breast cancer and testicular cancer (ordered by year of publication).

Author	Direction of association for ever breast-fed vs never (formula) breast-fed*	Variables controlled for
ALL CANCERS		
Wingard[92]	Null	Age, birthweight, infant health, childhood SES
Tokuhata[89]	Null	None
BREAST CANCER		
Michels[5]	Pre-menopausal breast cancer: Null Post-menopausal breast cancer: Null All ages: Null	Age, year of birth, premature birth, family history of breast cancer, height, BMI at 18 yrs, weight change since 18 yrs, history of benign breast disease, age at 1st child's birth, calorie intake, alcohol.
Titus Ernstoff[91]	Pre-menopausal breast cancer: Null Post-menopausal breast cancer: Null All ages: Null. (p for interaction by menopausal status =0.23)	Age, state, education, religion, family history of breast cancer, BMI, reproductive factors*.
Sanderson[93]	< 45 years: Null	Age, birth year, BMI, family history breast cancer, reproductive factors*, infertility, use of OCPs, birthweight, maternal age, birth order, maternal smoking.
Weiss[94]	< 45 years: Borderline inverse	Age, BMI, family history breast cancer, reproductive factors*, previous breast biopsy, alcohol, no. of mammograms.
Freudenheim[95]	Pre-menopausal: Null Post-menopausal: Null All ages: Borderline inverse	Age, education, BMI, family history breast cancer, reproductive factors*, history benign breast disease, duration breastfed own infant, fat & carotenoid intake, height.
Ekbom[96]	< 50 yrs: Null ≥ 50 yrs: Null All ages: Null	Maternal age, childhood SES, hospital stay, & reproductive factors*.
Brinton[97]	All ages: Null (borderline inverse)	Age at diagnosis
Henderson[90]	< 50 yrs: Null All ages: Null	'Race', date of birth, SES.
Tokuhata[89]	All ages: Null	None
Bucalossi[88]	All ages: Null	None

Author	Direction of association for ever breast-fed vs never (formula) breast-fed*	Variables controlled for
Penrose[87]	All ages: Null	None
TESTICULAR CANCER		
Coupland[7]	Borderline inverse	Age, region, social class, un-descended testis, inguinal hernia < 15 yrs of age, maternal age at pregnancy.
Mori[98]	Null	Year of birth, sex, age of mother at birth, birth order, no. of live births, number of induced abortions in mother.
Henderson[99]	Null	Sex, age & neighbourhood

*Null result implies no statistical evidence of an association at the 5% level. †Includes age at menarche, age at first full-term pregnancy, parity, age at menopause. BMI: body mass index. OCP: oral contraceptive pills. SES: socioeconomic status.

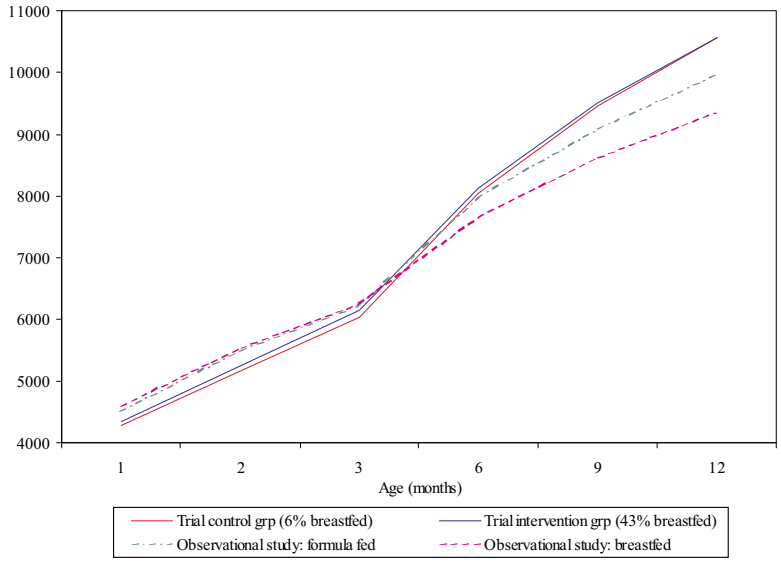

Figure 14-5. Growth of breast-fed and formula-fed infants from 0 to 12 months: Comparison of data from a randomised trial and from a prospective cohort study. Data for randomised trial, taken from Table 2, Kramer *et al* [101] and for the cohort study, taken from Table 2, Dewey *et al* [102].

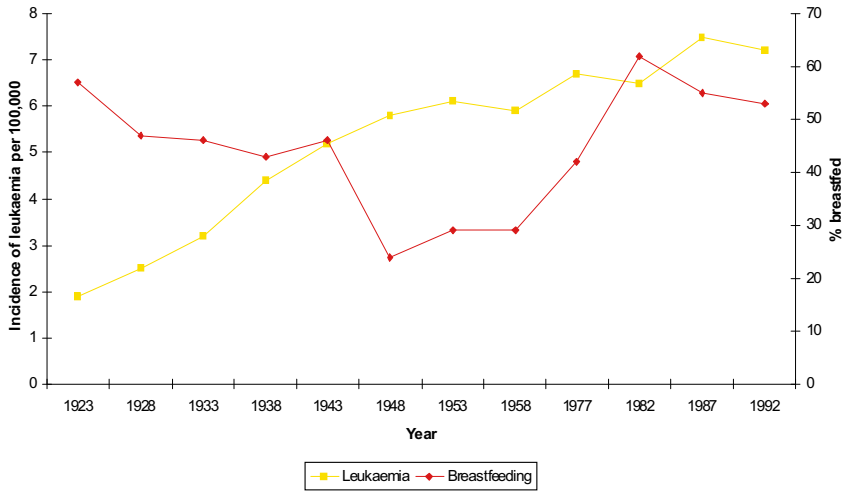

Figure 14-6. Temporal trends in percent breast-feeding and incidence of acute leukaemia among children aged 1-4 years, USA 1923-1992. Sources: Breast-feeding data: references 103, 104; Leukaemia data reference 105.

6. CONCLUSIONS

There has been a longstanding interest in the possibility of a relationship of breast-feeding with childhood acute lymphoblastic leukaemia[45] and sex-hormone dependent cancers in adulthood. [71] Indirect evidence supporting the potential importance of infant nutrition comes from a large body of evidence linking height and leg-length (a suggested marker of exposures influencing pre-pubertal growth, including diet [13,15] and breast-feeding history[18,19]) with later cancer risk.[12] There are also a number of plausible biological mechanisms (summarised in Box 2). We have systematically retrieved the available published evidence linking breast-feeding with cancers in childhood and adulthood. In general, the evidence that breast-feeding has a role in cancer development is weak. For acute lymphoblastic leukaemia, Hodgkin's Disease and breast cancer only 3 of 17 studies, 2 of 8 studies and 3 of 11 studies, respectively, provided strong evidence ($p < 0.05$) of any association with breast-feeding. Many of these studies were small and provided very imprecise effect estimates, so type 1 error is a potential problem with interpreting the evidence. The quality of the studies was

frequently poor (Box 3), and the generalisability of the results to modern cohorts is unclear. Perhaps the strongest conclusion from the literature is that a transmissible agent in breast-milk that increases future breast cancer risk in those who are breast-fed is unlikely.

The problem of confounding in observational epidemiological studies of the long-term effects of breast-feeding is illustrated by comparing their results with those from randomised controlled trials, where the effect of randomisation is to eliminate confounding. For example, in Belarus a cluster randomised controlled trial looked at growth amongst 17,000 infants randomised in 1996 to a breast-feeding health promotion intervention or usual practice (PROBIT trial).[100] The trial found that the intervention was associated with rates of exclusive breast-feeding at 3 months of 43% compared with 6% in controls. The results of the trial, shown in the solid lines in Figure 14-5, suggest little difference in weight gain between breast-fed and bottle-fed subjects. This is in marked contrast to results typically found observational studies which suggest that breast-fed infants grow more slowly than bottle-fed infants (shown by dotted lines in Figure 14-5). The difference could be due to confounding in the observational studies or a form of selection bias in observational studies whereby faster growing, hungrier infants are more likely to be bottle-fed.

Box 3: Limitations in the current epidemiological evidence

Inadequate measurement of infant feeding history (e.g. exclusivity and duration of breast-feeding; age at introduction of solids; weaning diet)
Retrospective assessment of infant feeding history (measurement error and recall bias)
Limited control for important confounding factors
Poor response rates
Selection bias in the recruitment of controls in case-control studies
Small number of cancers leading to imprecise effect estimates (wide confidence intervals)
Multiple hypothesis testing in single cohorts leading to chance findings
Lack of *a priori* hypothesis in relation to several cancers (e.g. childhood neuroblastoma; testicular cancer)
Possibility of publication and citation bias

An important link between infant nutrition and leukaemia is not supported by temporal trends in breast-feeding and these cancers (Figure 14-6). In the USA, over two-thirds of mothers breast-fed in the early 1900s but the rate and duration of breast-feeding began to decline slowly in the first decades of the twentieth century, with a large decrease after world war 2. The breast-feeding rate then reached its nadir in 1972 when only 22% of women breast-fed in the USA. By 1975, the breast-feeding rate began to increase with a small decrease in the late 1980s. A protective role for breast-feeding is not supported by the rapid rise in USA leukaemia incidence rates

among children aged 1-4 years during 1920-1950 (when breast-feeding was declining slowly) and the subsequent slow down in the rise in leukaemia, when breast-feeding was declining most rapidly. If breast-feeding was important, the upsurge in breast-feeding rates after the mid 1970s would be expected to be associated (with time lags of 1-4 years) with a large decline in childhood leukaemia rates. Similar analysis of temporal trends for the other cancers investigated here, in particular Hodgkins Disease, neuroblastoma, breast cancer and testicular cancer needs to be performed, with account taken of appropriate time lags and other intervening factors (e.g. the introduction of breast screening).

This review has identified several areas where the evidence base could be improved. Firstly, the relationship of breast-feeding with IGF, a risk marker for the future development of cancers of the prostate and breast, could be investigated in large modern birth cohorts with prospective and detailed data on infant feeding, growth, and potential confounding factors. The findings from the Avon Longitudinal Study of Parents and Childhood that having been breastfed was positively associated with IGF-I levels at age 7-8 years[108] need to be replicated. Secondly, investigators should attempt to publish associations that have been looked at in their studies, even when the results are negative or are qualitatively in unexpected directions. This, combined with the adequate reporting of the study[106] or provision of relevant data to a central resource, would allow meaningful pooling of the available evidence, along with meta-analyses to explore sources of heterogeneity.

Thirdly, if possible the relationship should be explored in populations where breast-feeding is not socially patterned.[8] This strategy would help reduce unease about residual socio-economic confounding, a major concern in infant feeding research because of the strong relationship of breast-feeding with education, income and other social factors.

Fourth, the relationship between breast-feeding and markers of later cancer risk could be investigated in large randomised controlled trials of measures to promote breast-feeding. For example, long-term follow-up of the 17,000 children in the PROBIT trial [100] and analysis by intention to treat, would provide a rigorous test of the hypothesis that breast-feeding is associated with markers of cancer risk, such as the IGF axis.

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Chapter 15

EFFECTS OF BREAST-FEEDING ON COGNITIVE FUNCTION

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1. INTRODUCTION

The effect of breast-feeding on cognitive function has been examined in many studies, and the results show a consistent pattern with a small but significant positive effect in most studies. The effect gets smaller when controlling for relevant confounders and it has been argued that the remaining effect could be due to residual confounding. The aim of this short review is to summarise the main results from these studies and to discuss the possible causes of such an effect, possible confounders and the potential impact of residual confounding. Furthermore, data on the effects of breast-feeding on other aspects of brain development (i.e. visual acuity and head circumference) and the potential negative effects of environmental contaminants in human milk on cognitive function will be covered briefly. The most important meta-analysis and critical reviews will be discussed together with a few recent important papers.

G. R. Goldberg et al. (eds.), Breast-Feeding: Early Influences on Later Health,

2. METHODOLOGICAL ISSUES

Studies that compare breast-fed infants with infants who were not breast-fed are most often observational since it is unethical to randomise infants to breast-feeding or formula. The methodological challenges of interpreting observational studies of the effects of breast-feeding are discussed in detail in chapter 10 by Michael Kramer. In most studies from industrialised countries breast-feeding is more prevalent among mothers with high education and high social status, and most studies find a positive association between intelligence and education as well as socio-economic status. Consequently, it is essential that studies comparing indicators of brain development in breast-fed and non breast-fed infants control for potential confounders such as maternal education, family socio-economic status and, if possible, maternal intelligence. Even after such adjustments it is difficult to exclude that residual confounding and reverse causation could explain some of the association, as discussed in the chapter by Kramer. Another problem when comparing studies is that the definition of breast-feeding and the way information about duration of breast-feeding is handled, is often very different.

Cognitive performance can be examined in many different ways, but quality measures of general intelligence usually show substantial intercorrelations, and for IQ or other summary scores derived from a battery of subtests measuring different abilities the observed effects of breast-feeding should not depend much on the choice of test. However, results for individual tests will of course depend on the specific cognitive functions assessed and cannot necessarily be generalised to other types of tests or other cognitive functions. Many studies of cognition and breast-feeding have assessed intellectual development in children. Intelligence assessed in middle childhood is a very strong predictor of adult intelligence [1] while assessment at younger ages will show lower correlations with adult intelligence and assessment at age three or younger is too early for long-term prediction.[2] In general, tests of specific cognitive functions can be expected to show a similar pattern, but lower correlations with assessment in adulthood. Although correlations for assessment in middle childhood and adulthood are high, intellectual development is influenced by many other factors than breast-feeding, and the effects of breast-feeding on cognition may be progressively diluted through the life span. Even if this should prove to be the case, effects of breast-feeding on child and young adult intelligence may be associated with educational attainment and thereby affect the individual through most of the lifespan.[3]

3. BREAST-FEEDING AND COGNITIVE DEVELOPMENT

There has been an increasing interest in the possible effect of breast-feeding on cognitive function since the study by Hoefer and Hardy in JAMA in 1929.[4] Since then more than 50 studies have been published many of which have been included in the three reviews published between 1999-2001 which will be discussed here.

Anderson *et al.* [5] performed the only meta-analysis. They identified 20 studies which met three inclusion criteria: 1) comparing predominantly breast-fed with predominantly formula-fed subjects; 2) using a widely applied test of cognitive development yielding a single score; and 3) testing subjects between infancy and adolescence. The included studies assessed cognitive development at ages between six months and 15 years. Eleven of the studies controlled for ≥ 5 covariates and presented both un-adjusted and adjusted results. Six of the studies included a measure of maternal intelligence in the analysis. The unadjusted benefit of breast-feeding was 5.3 IQ-points, and after adjustment for appropriate covariates the increment was still 3.16 IQ-points and highly significant (Table 15-1). In addition, the effect was supported by a significant dose-response effect with better cognitive development with increased duration of breast-feeding. For infants only breast-fed for 4-7 weeks there was no effect, whereas for children breast-fed for more than 28 weeks the effect was 2.9 IQ-points (Table 15-1). All the estimated effects, except the 4-7 week category, were significant ($p<0.001$), when compared to infants not breast-fed. In an analysis by category of birthweight Anderson *et al* found that the effect of breast-feeding was significantly larger in low birthweight children (5.2 IQ-points) compared to normal birthweight children (2.7 IQ-points). It has been hypothesised that the positive effect of breast-feeding on mental development could be explained by long-chain polyunsaturated fatty acids (LCPUFA) in breast-milk, which are not present in infant formulas. This dependence of the effect on birthweight supports the LCPUFA hypothesis, since preterm infants are born with a low LCPUFA status and therefore more dependent on LCPUFA-supply.[6] The LCPUFA hypothesis will be explained later in this chapter. Another interesting finding from the analysis was that the effect seemed to be independent of the age at which mental performance was examined. The magnitude of the effect in subjects measured between 6-23 months was not different from that seen in subjects examined at the age of 2-5 years, 6-9 years, or 10-15 years. Thus, the effect seems to be stable from early in life throughout childhood. This is further supported by the two large studies mentioned below in which an effect of the same magnitude was seen in adults.

Twenty four studies about breast-feeding and mental development were included in a "critical evaluation" by Drane and Logmann.[7] There is considerable overlap between the studies included in this review and the studies included in the meta-analysis of Anderson *et al.*[5] but several studies were only included in one of these. Drane and Logman did not perform a meta-analysis but concluded that four out of six studies fulfilling strict inclusion criteria found a 2 to 5 IQ-point advantage of breast-feeding for term infants and an 8 IQ-point advantage for low birthweight infants. They concluded that "the question of whether breast-feeding and formula-feeding have differential effects on cognitive development has not yet been comprehensively answered".

A more recent "critical review" by Jain *et al.* included 40 studies.[8] Twenty-seven (68%) of these studies concluded that breast-feeding promoted intelligence. Each study was evaluated according to 8 principles of clinical epidemiology. Jain *et al* only found two studies that included full-term infants and fulfilled all their quality criteria. One of these showed an effect of 3.8 IQ-points that was decreased to a non-significant 0.8 IQ-point after adjusting for relevant confounders, and the other showed an effect of 5 IQ-points that was reduced to 4.6 IQ-points after adjustment for confounders. They concluded that although the majority of studies concluded that breast-feeding promotes intelligence, the evidence from higher quality studies is less persuasive.

Since the publication of these three reviews a few other studies have examined the effect of breast-feeding on cognitive development. In a large study from Australia 1450 children were tested at the age of 6 years with a verbal cognitive IQ test and 1375 from the same cohort were tested at the age of 8 years with a non-verbal subtest.[9] There was a highly significant association between breast-feeding and verbal cognitive ability in an adjusted model, while there was no association with the non verbal subtest. Interestingly they found a significant interaction with the strongest effect of breast-feeding among the mothers with the highest education.

Table 15-1. Weighted mean difference in cognitive development score between breast-fed and formula-fed children from the meta-analysis by Anderson *et al*[5]

		Mean difference (breast-fed – formula fed)	95 % CI
Overall effect	Unadjusted	5.3	4.5 – 6.1
	Adjusted	3.2	2.4 – 4.0
Duration of breast-feeding	4-7 weeks	0.0	-0.7 – 0.7
	8-11 weeks	1.7	1.1 – 2.3
	12-19 weeks	2.2	1.4 – 2.9
	20-27 weeks	2.8	1.9 – 3.6
	≥ 28 weeks	2.9	1.7 – 4.1
Birthweight	Normal or mixed	2.7	2.2 – 3.2
	Low	5.2	3.6 – 6.8
Age at assessment	6-23 months	3.1	1.8 – 4.4
	2-5 years	2.5	1.9 – 3.2
	6-9 years	3.0	2.0 – 4.0
	10-15 years	3.2	1.9 – 4.5

4. BREAST-FEEDING AND IQ IN ADULTS

In the three reviews only one study was included which assessed cognitive function in adults.[10] In this study around 1000 individuals were examined using a computerised test of logical, verbal and arithmetic reasoning. There was a significant positive association with breast-feeding, but this disappeared when family and perinatal factors were included in the analysis. The assessment of feeding was approximate, dividing the children into breast-fed, bottle-fed and mixed feeding, and only 53 were bottle-fed. Furthermore, cognition was assessed when the participants were 60-70 years old. At this age, test scores may be affected by individual differences in age-related decline in cognitive function, which could weaken the association between breast-feeding and cognition. Since then three other studies have assessed the effect of breast-feeding on IQ in adults. They are based on large birth cohorts, one from the Copenhagen Perinatal Cohort born 1959-1961, one from the British 1946 Birth Cohort and a cohort of men born in South Wales 1920-35.

We have examined the effects of breast-feeding on IQ in the Copenhagen Perinatal Cohort Study.[11] The cohort comprises 9125 infants born between 1959-1961. Information about duration of breast-feeding was collected at an interview with the mother when the child was aged 12 months. Intelligence was assessed in adulthood in two non-overlapping sub-groups. A group of 973 men and women were examined with the Wechsler Adult Intelligence Scale (WAIS) at a mean age of 27.2 years. For 2280 male participants in the cohort, intelligence scores at a mean age of 18.7 years were obtained from

draft records from the military authorities. These IQ-scores were measured by a 45 minute intelligence test (Børge Priens Prøve (BPP)), which had a score range from 0 to 78. Thirteen potential confounders were included as covariates in the analysis: parental social status and education; single mother status; mother's age, height, weight gain during pregnancy and cigarette consumption during the third trimester; number of pregnancies; estimated gestational age; birthweight; birth length; and indexes of pregnancy and delivery complications. In both samples we found a highly significant dose response relationship, indicating higher IQ for longer duration of breast-feeding (Figure 15-1). In both samples there were no significant differences between the 7-9 month and the > 9 months' breast-feeding categories, indicating that breast-feeding beyond nine months was not associated with additional IQ benefits. The WAIS full scale IQ increase corresponds to nearly half a SD. For the conscript sample the increase corresponds to about one fifth of a SD.

In a study based on the British 1946 Birth Cohort three cognitive tests were administered at the age of 53 years in 1739 cohort members, and data collected on potential confounding variables.[12] The three cognitive functions assessed were reading ability (National Adult Reading Test), verbal memory and mental speed and concentration (visual search speed). The likelihood of obtaining advanced educational qualifications at the age of 26 years was also assessed. Breast-feeding was positively associated with educational attainment at 26 years of age. Those breast-fed for > 7 months had an odds ratio of obtaining an advanced education of 1.58 compared to those not breast-fed. This effect was independent of early social background, but largely accounted for by cognitive ability at 15 years. Among the cognitive scores obtained at the age of 53 years breast-feeding was only associated with reading ability. The association was still significant ($p=0.02$) after adjustment for early social background, education and social class, but if cognitive ability at 15 years was included the association was non-significant ($p=0.12$). This is in agreement with the results from the meta-analysis by Anderson *et al*. in which there was no effect of age at assessment on the effect on cognitive function.[5] According to Richards and co-workers reading ability is a function that is stable over time, whereas the other cognitive functions that were tested in the British 1946 Birth Cohort may be more vulnerable to age related decline, which is very variable among individuals.

In a more recent study also from the UK, 779 men were tested when they were 60-74 years old.[13] In those with birthweight at or above the median the adjusted mean cognitive function was only slightly and non-significantly lower in those that had not been breast-fed, while not being breast-fed was significantly associated with poorer cognitive outcome in those with a low birthweight. Again, differences in age related decline in cognitive function

may weaken the association so that the effect was only significant among those with low birthweight, in accordance with the meta-analysis showing that the effect is stronger among preterm infants.[5]

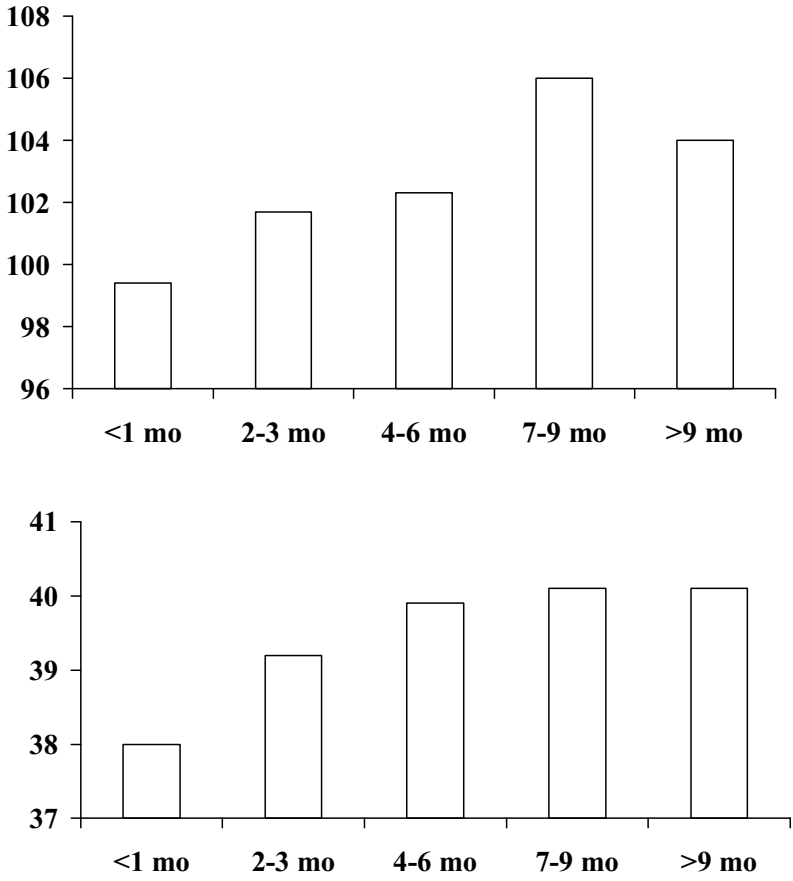

Figure 15-1. Adjusted means for intelligence test scores in relation to duration of breast-feeding from the Copenhagen Perinatal Cohort. The upper figure represents the WAIS full scale IQ for 973 men and women with a mean age of 27.2 years. The lower figure shows the data from 2,280 male conscripts tested at a mean age of 18.7 years with the BPP test. The differences between the feeding groups were significant in both samples (upper: $p<0.001$, lower: $p=0.01$).

5. THE LCPUFA HYPOTHESIS

There are several potential mechanisms that could explain the effects of breast-feeding on cognitive function. It could be unidentified factors that correlate with both breast-feeding and cognitive development, so called residual confounding or it could be factors associated with the feeding situation, i.e. physical and psychological contact between the mother and infant. It could also be components in human milk stimulating cognitive development. One of the most plausible explanations is that the effect is caused by the breast-milk content of long-chain polyunsaturated fatty acids (LCPUFA), especially the *n*-3 LCPUFA docosahexaenoic acid (DHA).[6] Breast-milk contains LCPUFA whereas most infant formulas, especially those produced before 1990, do not. The cellular membranes of the central nervous system (CNS) contain uniquely high levels of LCPUFA, especially DHA, and in particular the retina, in which DHA contributes up to half of the fatty acids in the photoreceptor disk membranes. During the CNS growth spurt, rapid accretion of DHA takes place.[14] Formula-fed infants, who do not receive exogenously preformed DHA, have lower levels of DHA in their CNS membranes.[15,16] It is plausible that the differences in DHA accretion could contribute to the observed difference in mental abilities between breast-fed and formula-fed children.

Small cross sectional studies have also observed relationships between the DHA content of human milk, or infant or maternal plasma, and measures that reflect CNS maturation, such as sleep pattern, cognitive abilities, speech perception and language development of breast-fed infants.[17-21] Some randomised LCPUFA supplementation studies have shown beneficial effects on cognitive development [22-24], but other studies have not.[18,25-27] A few studies supplementing lactating mothers with LCPUFA from marine oils have investigated whether the DHA-intake of breast-fed infants may have beneficial effects on mental development.[28-31] One such maternal *n*-3 LCPUFA-supplementation trial found that an increase in breast-milk DHA was associated with a positive effect on the Bayley Mental Development Index score at one year of age, which was however no longer evident at two years.[28] A recent Norwegian study has shown that *n*-3 LCPUFA supplementation during pregnancy and lactation resulted in an increased mental processing score at four years of age.[30]

5.1 Visual development

Examination of visual acuity is also a way to examine early brain development. During infancy visual acuity gradually increases until it reaches a plateau around the age of 12 months.[31] Visual acuity is determined

by the rod and cone photoreceptors in the retina. Visual acuity has been assessed mainly by behaviourally and electrophysiologically based methods. The main behavioural method, the Teller acuity card procedure or "forced choice preferential looking", evaluates an infant's tendency to gaze at a pattern, rather than a blank field.[32] The procedure is somewhat subjective and requires an integrated behavioural response that includes factors other than strictly visual ones (e.g. alertness, attention, motor control). In the electrophysiologically based methods one measures visual evoked potentials (VEP) in the visual cortex. These methods are far less subjective and based on specific visual factors, including the process of signal conduction from the eye to the visual cortex.

Studies examining the effects of diet on visual acuity in infants have been evaluated in a systematic review by SanGiovanni *et al.*[33] Among the non-randomised studies he found that breast-fed infants had significantly better visual acuity than formula-fed infants at the age of two months (0.49 octaves, $p<0.000001$) and four months (0.18 octaves, $p=0.04$) when assessed by behavioural methods and at four months (0.37 octaves, $p=0.02$) when assessed by electrophysiological methods. The review also covered studies in which formula-fed infants were randomised to *n*-3 LCPUFA supplementation or no supplementation. Based on this evaluation the authors conclude that *n*-3 LCPUFA intake is associated with performance on visual acuity tasks at two, and possibly four, months of age in healthy term infants, but that it has not been shown whether n-3 LCPUFA intake confers a lasting advantage in visually based processes.

Since then we have examined visual acuity by visual evoked potentials (SWEEP-VEP) in a group of 39 term breast-fed four month old infants and related it to the DHA content of their mothers' milk.[34] In this cross-sectional study we found a significant positive association ($p=0.02$) with higher visual acuity the more DHA the mother had in her milk. Forty seven percent of the variation in milk DHA content could be explained by the mothers' fish intake. A similar Canadian cross-sectional study found a positive correlation between the levels of DHA in erythrocyte phospholipids, which are closely associated with DHA-intake, of breast-fed infants at 2 months of age and visual acuity at 12 months of age.[35] We saw a similar significant trend in a study in which we randomly assigned lactating mothers to supplementation with *n*-3 LCPUFA from fish oil.[36]

5.2 Brain growth

Head size in early life has been reported to be associated with developmental scores in later life, especially in infants with low birthweight and in disadvantaged children.[37] Head size tracks throughout life, but studies

on the influence of head size at birth on later mental performance have been conflicting (as discussed by Gale *et al.* [38]). In a very interesting recent study Gale *et al.* investigated whether brain growth during different periods of pre- and postnatal development influence later cognitive function in a follow-up study of 221 children, whose mothers had participated in a study of nutrition in pregnancy.[38] They found that the mental performance of 9 year old children, after adjustment for confounding factors, including maternal IQ, was related to current head size and head size at 9 months of age, but not to head size at birth or at 18 weeks of gestation. These findings are interpreted as evidence that postnatal brain growth is important in determining higher mental function.

Brain growth (measured as head circumference and estimated brain weight) has also been shown to be associated with the level of DHA in infant erythrocytes, which is a well accepted biomarker for *n*-3 LCPUFA intake.[39] In a randomised trial we found that maternal *n*-3 LCPUFA-supplementation during the first four months of lactation resulted in a 5 mm increase in head circumference at 2½ years of age, also after adjustment for head circumference at 2 month of age.[40] A small Swedish study has shown that brain growth in the first months of lactation was positively associated with the arachidonic acid (AA) to DHA ratio in breast-milk.[41] This seems contradictory to our observation, as we found that the ratio of AA to DHA in breast-milk decreased with maternal *n*-3 LCPUFA-supplementation.[36] This may however be caused by the specific supplementation conditions and not be applicable to habitual dietary intake, as *n*-6 and *n*-3 PUFA breast-milk content has been shown to be positively correlated in observational studies.[42] A meta-analysis of all randomised controlled LCPUFA-treatment trials in term formula-fed infants with growth outcome did not show an effect of LCPUFA on growth within the first year of life measured as weight, length and head circumference.[43]

Head circumference is known to correlate closely with brain volume.[44,45] Lipids account for approximately 50-60 % of the brain dry weight in adults, and approximately 35% is PUFA.[6] Myelination and synaptogenesis take place during the post-natal brain growth spurt and continues into late childhood, but are dependent on neurogenesis, which is complete by 16 weeks. Neuronal cells have been shown to be dependent on DHA *in vitro*. It is therefore plausible that these processes may be affected by the *n*-3 LCPUFA supply, which may explain the effect of *n*-3 LCPUFA intake on brain growth, which in turn could explain the effect on mental development and later IQ. However, it has also been hypothesised that the DHA-content of neuronal membranes could influence nerve cell function and information processing via interactions with neurotransmission and ion transport.

5.3 Environmental contaminants

Human milk contains environmental contaminants such as methylmercury and PCBs at considerably higher levels than infant formula. Prenatal exposures to high levels of these substances have been shown to have negative effects on brain development,[46-47] and theoretically the additional exposure to these contaminants through breast-feeding could have a negative effect on development. However, intrauterine exposure is larger than the exposure through breast-milk, which happens at a later stage of development. The prenatal brain seems much more vulnerable to adverse influences of contaminants.[48] One study suggested that PCBs transmitted through breast-milk could have a negative effect on neurodevelopment.[49] However, the data are not conclusive, since it is difficult to tease out pre- and postnatal exposure.[48] This is even more difficult because breast-feeding mothers with a high fish intake will have both high levels of DHA, which is likely to promote cognitive development and high intakes of contaminants which could have negative effects. There is, however, general agreement that the positive effects of breast-feeding on cognitive function and the other benefits of breast-feeding on the health of the child outweigh the potential negative effects of pollutants, but also that efforts to reduce the level of these contaminants in the environment, which would reduce both the pre- and postnatal exposure should be given high priority.

6. MATERNAL BEHAVIOUR

The behaviour of the mother can influence the cognitive development of the infant. In studies of the association between breast-feeding and cognitive performance in the offspring behaviour can be either a confounder or a mediator. If the behaviour of the mother leads to both an increased duration of breast-feeding and a better cognitive development through support and stimulation of the child it is a confounder of the association between breast-feeding and cognitive development. However, breast-feeding may affect the behaviour of the mother through a hormonal influence (e.g. oxytocin and prolactin) and through bonding due to breast-feeding, and if this leads to maternal behaviour that stimulates the cognitive behaviour of the infant, such an effect is mediated by breast-feeding. In an interesting study of preterm infants Feldman and co-workers found that the mothers of infants receiving the most breast-milk during their admission in neonatal departments were also more affectionate and had lower maternal depression scores at 37 weeks gestational age.[50] At 6 months corrected age those receiving more breast-milk also showed better behavioural scores. In their paper Feldman *et al* also review the literature that shows how breast-feeding,

via hormonal responses (prolactin and oxytocin), can influence maternal behaviour. Other studies have shown a temporal impairment in memory and attention during lactation.[51] It is likely that this effect is caused by oxytocin because oxytocin delivered by nasal spray to adult men caused a short-term selective amnesic effect.[52] Heinrichs *et al*. refer to other studies showing impaired cognitive performance in the presence of improved social memory or social behaviour. Thus it is suggested that this effect may isolate the mother from distracting stimuli during lactation and focus maternal attention on interaction with the infant. Such a change in behaviour may stimulate cognitive behaviour in the infants. However, the available studies do not allow an estimation of how important this effect is on the cognitive development of the child.

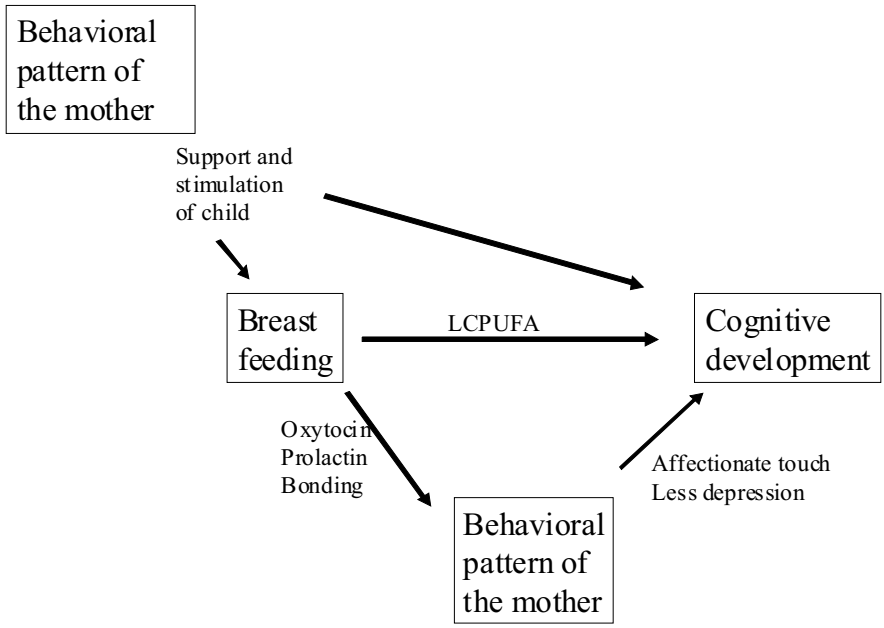

Figure 15-2. Conceptual framework showing how the behavioural pattern of the mother can be either a confounder or a mediator

7. IMPACT AT THE POPULATION LEVEL

Even if the effect of breast-feeding on cognitive development is not large in the individual it will have a large impact at the population level. In their review Drane and Logemann calculated the potential impact of type of infant feeding on the prevalence of developmental disability in the US population.[7] Assuming a normal distribution of IQ in a population and a mean of 95 in the bottle-fed population, they calculated that a 5 IQ-point shift of the population upward to a mean of 100 would result in a decrease from 26% to 16% of children with an IQ less than 85. The estimated cost of special education in the US could be reduced from $4.5 billion to $3.9 billion if the percentage of children that were predominantly breast-fed at four months was increased from 20% to 50%. The authors emphasised that these data were preliminary and should be considered with caution. However, the data point to the potential large effects at the population level of small differences in IQ in an individual. In our study of the impact of breast-feeding on IQ in young adults, we found an overall effect of about 5 IQ-point comparing those breast-fed <1 months with those breast-fed ≥ 7 months. We calculated the percentage of subjects with Full Scale IQ-scores below 90, which can be regarded as a sub-optimal performance, in each group (Figure 15-3). The percentage of subjects with values below 90 were significantly lower with increasing breast-feeding duration; 28% in those breast-fed for ≤ 1 month and only 4% in those breastfed >9 months. After adjustment for potential confounders there was still a highly significant (<0.001) trend.

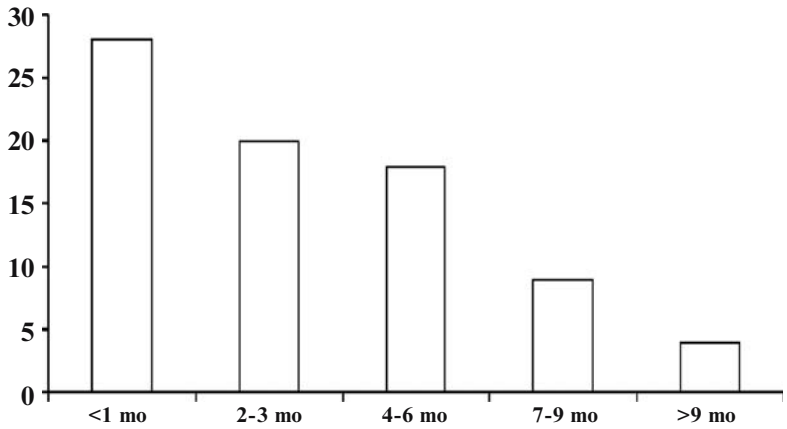

Figure 15-3. Percentage of subjects with full scale WAIS IQ below 90 according to duration of breast-feeding. The differences between the groups were significant (p<0.001) and remained so after adjusting for relevant covariates

8. CONCLUSIONS

The majority of studies examining the effect of breast-feeding on cognitive development have found an advantage of breast-feeding, including when the results are controlled for potential confounders. This was also the case for studies that controlled for maternal IQ. The effects seem to be of the same magnitude (2-5 IQ points) regardless of the age cognitive performance was assessed. Although residual confounding is difficult to exclude, the consistent results, with effects of the same magnitude, in studies from different populations using different methods of assessing cognitive performance and the significant dose-response effects of duration of breast-feeding, points to a causal effect of breast-feeding on cognitive performance.

A plausible explanation is differences in composition between human milk and infant formula, e.g. differences in *n*-3 LCPUFA. These fatty acids, especially DHA, are major components of cell membranes in the CNS and retina. DHA accretion in the brain during the brain growth spurt is affected by feeding. Measures of CNS development, especially visual acuity, has been show to be better in breast-fed infants, associated with intake of *n*-3 LCPUFA, and improved by DHA-supplementation of formula-fed infants. DHA accretion could possibly affect mental development via effects on brain growth, which has been shown to be associated with higher IQ. But the effect may also be caused by effects of DHA on neuronal function as several lines of evidence suggest that cellular functions are affected by lipid composition of cell membranes.[6] The fact that visual acuity is also better in breast-fed infants, associated with red blood cell DHA levels in some studies, and can be improved by DHA supplementation of infant formula also points to an effect of LCPUFA. Factors associated with the feeding situation, i.e. physical or psychological contact between mother and child, or an endocrine effect of breast-feeding on maternal behaviour may also be important. If this is the case it is also a direct effect of breast-feeding. Although some data indicate that these effects could influence cognitive development of the child, the available data does not allow an estimate of the size of an effect of maternal behaviour.

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Chapter 16

DEVELOPMENTAL ORIGINS OF OSTEOPOROTIC FRACTURE

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1. INTRODUCTION

Osteoporosis is a skeletal disorder characterised by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture.[1] It is a widespread condition, often unrecognised in clinical practice, which may have devastating health consequences through its association with fragility fractures. The term 'osteoporosis' was first used in the nineteenth century as a histologic description for aged bone tissue, but its clinical consequences were not appreciated until Sir Astley Cooper recognised that hip fractures might result from an age-related reduction in bone mass or quality over 150 years ago. Since one disadvantage of a fracture-based definition is that diagnosis and treatment will be delayed when prevention is considered optimal treatment, an expert panel convened by the World Health Organisation (WHO) has suggested that both low bone mineral density (BMD) and fracture be combined in a stratified definition of osteoporosis.[2]

Population based data from the United States suggests that while the majority of white women aged under 50 years have normal bone density, osteoporosis becomes increasingly prevalent with advancing age.[3] However, prospective studies indicate that the risk of osteoporotic fracture increases continuously as BMD declines with a 1.5 – 3 fold increase risk of fracture for each standard deviation fall in BMD.[4] There does not appear to be a

G. R. Goldberg et al. (eds.), Breast-Feeding: Early Influences on Later Health,

threshold value for BMD above which the fracture risk is stable, and the risk gradient for this relationship is as steep as that between blood pressure and stroke. Use of this density-based definition allows early diagnosis and therefore early initiation of preventive strategies.

Using the WHO criteria, it has been estimated that most American women under the age of 50 years have normal BMD and that osteoporosis is rare. With advancing age, an increasing number of women have osteoporosis, so that by the age of 80 years 27 per cent are osteopenic and 70 per cent are osteoporotic at the hip, lumbar spine or forearm. Epidemiological studies from North America have estimated the lifetime risk of common fragility fractures to be 17.5 per cent for hip fracture, 15.6 per cent for clinically diagnosed vertebral fracture and 16 per cent for distal forearm fracture among white women aged 50 years.[3] Corresponding risks among men are 6 per cent, 5 per cent and 2.5 per cent. Estimates from Europe suggest that around 23 per cent of women aged 50 years and over have osteoporosis according to the WHO definition. Fracture rates in Britain are somewhat lower than in the United States: for women lifetime fracture incidence rates are 14 per cent, 33 per cent and 13 per cent at hip, spine and distal forearm respectively, while for men the corresponding figures are 3 per cent, 9 per cent and 2 per cent [5] (Table 16-1). Osteoporosis related fractures are estimated to cost around £1.7 billion annually, and the associated morbidity burden is considerable.

Table 16-1. Impact of osteoporotic fractures in British men and women

	Hip	Vertebra	Wrist
Lifetime risk (%)			
Women 50 y	14	33	13
Men 50 y	3	9	2
Mean age (years)	79	67	65
Mortality (relative survival)	0.83	0.82	1.00
Functional impairment (%)	30	10	10

The bone mass of an individual in later adult life depends upon the peak obtained during skeletal growth and the subsequent rate of bone loss. Preventive strategies against osteoporosis may be aimed at either increasing the peak bone mass obtained or reducing the rates of bone loss. There is evidence to suggest that peak bone mass is inherited, but current genetic markers are able to explain only a small proportion of the variation in individual bone mass or fracture risk.[6] It is likely that environmental influences during early life interact with the genome in establishing the functional level of a variety of metabolic processes involved in skeletal growth. This review will cover the normal patterns of skeletal growth during intrauterine life, childhood and adolescence; the environmental determinants

of peak bone mass, including childhood nutrition and exercise; and the role played in establishing osteoporosis risk by influences during intrauterine or very early postnatal life. It will also address the conceptual basis of the fetal origins hypothesis by considering epidemiological studies pointing to the fetal programming of later osteoporosis risk and animal evidence relating to the programming of skeletal growth and metabolism.

2. NORMAL SKELETAL GROWTH

2.1 Peak bone mass

At any age, the amount and quality of an individual's skeleton reflects everything that has happened from intrauterine life through the years of growth into young adulthood. The skeleton grows as the body grows, in length, breadth, mass and volumetric density. For men and women of normal body weight, total skeletal mass peaks a few years after fusion of the long bone epiphyses. The exact age at which bone mineral accumulation reaches a plateau varies with skeletal region and with how bone mass is measured. Areal density, the most commonly used measurement with dual energy x-ray absorptiometry (DXA), peaks earliest (prior to age 20 years) at the proximal femur, while total skeletal mass peaks 6 to 10 years later.[7] However, total skeletal mass does not reflect the considerable heterogeneity in mineral accrual at other skeletal sites. Thus, the skull continues to increase in bone mass throughout life; certain regions such as the femoral shaft and vertebral bodies continue to increase in diameter in late adulthood.

The importance of peak bone mass for bone strength during later life was initially suggested by cross-sectional observations that the dispersion of bone mass does not widen with age.[8] This led to the proposition that bone mass tracks throughout life and that an individual at the high end of the population distribution at age 30 years is likely to remain at that end at age 70 years. Recent longitudinal studies have confirmed this tracking, at least across the pubertal growth spurt.[9]

2.2 Bone growth in utero

The fetal skeleton develops in two distinct components, intramembranous (the skull and facial bones) and endochondral (the remainder of the skeleton) ossification. Intramembranous ossification begins with a layer or membrane

of mesenchymal cells which becomes highly vascular; the mesenchymal cells then differentiate into isolated osteoblasts, which begin to secrete osteoid. The osteoid matrix is mineralised at the end of the embryonic period to form bony spicules that are precursors of the lamellae of the Haversian systems. There is no cartilage model preceding ossification in this type of bone development.

Endochondral ossification is responsible for the formation of the bones that are the main sites of fragility fracture in later life. This form of ossification depends on a pre-existing cartilaginous model that undergoes invasion by osteoblasts and is only subsequently mineralised. The development of this cartilage model can be seen by five weeks gestation with the migration and condensation of mesenchymal cells in areas destined to form the bone.[10] These pre-cartilagenous anlagen reflect the shape, size, position and number of skeletal elements which will be present in the mature skeleton. There is then an ordered differentiation of mesenchymal stem cells into chondrocyte precursors, proliferative chondrocytes, prehypertrophic chondrocytes and hypertrophic chondrocytes. During these stages of differentiation there is expansion of the bony template and production of an extracellular matrix rich in cytokines, which facilitate vascular invasion and mineralisation. The major regulator of the proliferation of chondrocytes is PTH-related peptide (PTHrP) [11], which is secreted by the perichondral cells. Other proliferative stimuli include cytokines of the GH/IGF axis.[12] 1,25 $(OH)_2$ vitamin D_3 [13] and tri-iodothyronine [14] are stimuli for the differentiation of the chondrocytes through different stages. Once the cartilage model has been formed, vascular growth factors embedded in the matrix are released by chondrocyte metalloproteinases. This stimulates angiogenesis and, under the influence of Cbfa1 [15], osteoblasts from the perichondrium invade and lay down matrix which is then mineralised.

During the period of a normal human pregnancy the fetus accumulates approximately 30g of calcium; the majority of this is accrued during the third trimester.[16] To supply this demand, there is a requirement for; (i) an adequate maternal supply of calcium to the placenta; and (ii) increased placental calcium transfer to maintain a higher fetal serum calcium concentration than the mother.[17] This materno-fetal gradient emerges as early as 20 weeks of gestation.[18] There is increased calcium absorption from the gut and also bone resorption to meet this demand. A rise in maternal serum PTHrP and 1,25 $(OH)_2$ vitamin D_3 is thought to drive the maternal supply of calcium to the fetus.[19] Net resorption of the maternal skeleton, liberating calcium, starts early in gestation[20], at a time when the fetal demand is small and this contributes to maternal calciuria during pregnancy.[21] During the last trimester, maternal bone formation increases to balance bone resorption.[22] However, there is an overall decrease in the maternal bone mass

of up to 10% during pregnancy.[21] Active calcium transfer across the placenta takes place in the cytotrophoblasts and involves storage of calcium by calcium binding proteins in the cytoplasm and in the endoplasmic reticulum.[23] Whilst in the mother 1,25 $(OH)_2$ vitamin D_3 is the principle stimulus for calcium absorption, the mid portion of PTHrP is essential at the placenta for the maintenance of the maternofetal gradient.[24] Secretion of PTHrP by the fetal parathyroid glands also enhances fetal renal calcium reabsorption. The rate of materno-fetal calcium transfer increases dramatically after 24 weeks, such that around two-thirds of total body calcium, phosphorous and magnesium are accumulated in a healthy term human fetus during this period. Factors which increase placental calcium transport capacity as gestation proceeds are only partly genetically controlled, and are achieved through regulatory hormones including 1,25 $(OH)_2$ vitamin D_3, parathyroid hormone, PTH-rp and calcitonin. As the majority of fetal bone is gained during the last trimester, one of the major variables affecting bone mass at birth is gestational age. Other factors known to influence neonatal bone mineral content (BMC) include environmental variables such as season of birth and maternal lifestyle. Newborn total body BMC has been demonstrated to be lower among winter births than among infants born during the summer.[25] This observation is concordant with lower cord serum 25 $(OH)_2$ vitamin D concentrations observed during winter months, consequent upon maternal vitamin D deficiency. Other postulated contributors to impaired bone mineral acquisition during intrauterine life include maternal smoking, alcohol consumption, caffeine intake and diabetes mellitus.[26]

2.3 Bone mineral accrual in infancy and early childhood

During infancy, average whole body BMC increases by 389 per cent, and total body BMD increases by 157 per cent.[27] Weight and length are strong predictors of BMC and areal BMD during infancy.[28]

However, because no studies of volumetric BMD have been done during the first year of life, it is not clear whether true volumetric density changes during this period. Gilsanz and colleagues found no significant differences in true volumetric density of the lumbar spine, measured using computed tomography between male or female, black or white children aged greater than 2 years.[29]

2.4 Bone mineral accrual during childhood and adolescence

Cross-sectional and longitudinal studies of bone accretion during childhood have been reported in several Western populations.[30-36] As observed for infants, weight and height emerge as strong predictors of both BMC and areal BMD. Gender and ethnic differences have also been reported. Boys have higher BMC than girls, but these differences are not reflected in areal BMD or volumetric bone density.

Puberty is the period during which the characteristic difference in bone mass observed in adults becomes fully expressed. There is no evidence for a gender difference in bone mass of either the axial or the appendicular skeleton at birth. Likewise, the volumetric bone mineral density also appears similar between male and female neonates. This gender similarity is maintained until the onset of puberty. The most important difference to emerge during pubertal maturation is the greater increase in bone size, consequent upon an increase in the cortical shell in males as compared with females.[37] These gender differences in size contrast with similar values for volumetric bone mineral density between sexes. Studies based on histomorphometry and QCT indicate no difference in volumetric trabecular density at the end of the period of pubertal maturation.

Longitudinal studies using DXA indicate that, during pubertal maturation, BMC and areal BMD at the lumbar spine and proximal femur increase by 4 to 6-fold over a 3 year period (11 to 14 years in girls; 13 to 16 years in boys). The increase in BMD during the corresponding pubertal period appears to be less marked in the diaphyses of long bones such as the radial or femoral shaft, where only 2-fold increases are observed.[38]

There is also heterogeneity in linear skeletal growth. Growth of distal limb segments precedes that of proximal segments. Appendicular growth is more rapid than axial growth before puberty, but decelerates at puberty when axial growth accelerates.[34] This differing tempo in bone growth could have important pathophysiological consequences. Regions growing rapidly or relatively distant from their peak may be more affected by illness than those growing slowly or near completion of growth. Depending on age and pubertal maturation, deficits may occur in limb dimensions (pre-pubertal), spine dimensions (early puberty), or volumetric BMD through interference with the phenomenon of consolidation that follows the marked decline in longitudinal growth. In pre-pubertal children, there is a tight relationship between bone mass at the spine or hip, and statural height.[39] This close relationship vanishes during pubertal maturation, with the appearance of the pattern observed in adults, among whom BMD values are poorly correlated with height. Prospective studies have indicated that the period of maximal

height gain and maximal accrual of bone mineral are dissociated by approximately two years.

3. DEVELOPMENTAL ORIGINS OF OSTEOPOROSIS AND FRACTURE

Epidemiological studies of coronary heart disease performed over a decade ago demonstrated strong geographic associations between death rate from the disorder in 1968-1978, and infant mortality in 1901-1910.[40] Subsequent research, based on individuals whose birth records had been preserved for seven decades, revealed that men and women who were undernourished during intrauterine life, and therefore had low birthweight or were thin at birth, had an increased risk for coronary heart disease, hypertension, non-insulin dependent diabetes, and hypercholesterolaemia.[41] These associations are explained by a phenomenon known as programming [42]; this term describes persisting changes in structure and function caused by environmental stimuli acting at critical periods during early development. During embryonic life, the basic form of the human baby is laid down in miniature. However, the body does not increase greatly in size until the fetal period when a rapid growth phase commences, which continues until after birth. The main feature of fetal growth is cell division. Different tissues of the body grow during periods of rapid cell division, so called 'critical' periods.[43] Their timing differs for different tissues; for example, the kidney has one in the weeks immediately before birth, while the long bones accelerate their rate of growth during the second trimester of gestation. The main adaptive response to a lack of nutrients and oxygen during this period of growth is to slow the rate of cell division, especially in tissues undergoing critical periods at the time. This reduction in cell division is either direct, or mediated through altered concentrations of growth factors or hormones (in particular insulin, growth hormone and cortisol).

It is not in question that the human skeleton can be programmed by undernutrition. Rickets has served as a long-standing example of undernutrition at a critical stage of early life, leading to persisting changes in structure. What is new is the realisation that some of the body's 'memories' of early undernutrition become translated into pathology and thereby determine disease in later life. Evidence has now accumulated that such intrauterine programming contributes to the risk of osteoporosis in later life.

Evidence that the risk of osteoporosis might be modified by environmental influences during early life stems from four groups of studies: (a) bone mineral measurements undertaken in cohorts of adults whose detailed birth and/or childhood records have been preserved; (b) detailed

physiological studies exploring the relationship between candidate endocrine systems which might be programmed (GH/IGF-1; hypothalamic-pituitary adrenal, gonadal steroid) and age-related bone loss; (c) studies characterising the nutrition, body build and lifestyle of pregnant women and relating these to the bone mass of their newborn offspring; and (d) studies relating childhood growth rates to the later risk of hip fracture.

3.1 Epidemiological studies

The first epidemiological evidence that osteoporosis risk might be programmed came from a study of 153 women born in Bath in the UK during 1968-69 who were traced and studied at age 21 years.[44] Data on childhood growth were obtained from linked birth and school health records. There were statistically significant ($p<0.05$) associations between weight at one year and BMC, but not BMD, at the lumbar spine and femoral neck; these relationships were independent of adult weight and body mass index. The data suggested a discordance between the processes which govern skeletal growth, and those which influence mineralisation. They also provided direct evidence that the trajectory of bone growth might be modified *in utero*, an assertion previously only supported by inference from measurements of body height. The association between weight in infancy and adult bone mass was replicated in a second cohort study of 238 men and 201 women aged 60-75 years, who were born and still lived in Hertfordshire in the UK.[45] In this study, there were highly significant relationships between weight at one year and adult bone area at the spine and hip ($p<0.005$); the relationships with BMC at these two sites were weaker but remained statistically significant ($p<0.02$). They also remained after adjustment for known genetic markers of osteoporosis risk, such as polymorphisms in the gene for the vitamin D receptor [46], after adjustment for lifestyle characteristics in adulthood which might have influenced bone mass (physical activity, dietary calcium intake, cigarette smoking, and alcohol consumption). More detailed analyses of the interactions between polymorphisms in the gene for the vitamin D receptor (VDR), birthweight, and BMD, have recently been published from the same cohort study.[47] In the cohort as a whole, there were no significant associations between either birthweight or VDR genotype and BMD. However, the relationship between lumbar spine BMD and VDR genotype varied according to birthweight (Figure 16-1). Among individuals in the lowest third of birthweight, spine BMD was higher ($p=0.01$) among individuals of genotype 'BB' after adjustment for age, sex and weight at baseline. In contrast, spine BMD was reduced ($p=0.04$) in individuals of the same genotype who were in the highest third of the birthweight distribution. A statistically significant

(p=0.02) interaction was also found between VDR genotype and birthweight as determinants of BMD. These results suggest that genetic influences on adult bone size and mineral density may be modified by undernutrition *in utero*. Subsequent studies from the U.S., Australia and Scandinavia have replicated these relationships between weight in infancy and adult bone mass (Table 16-2).

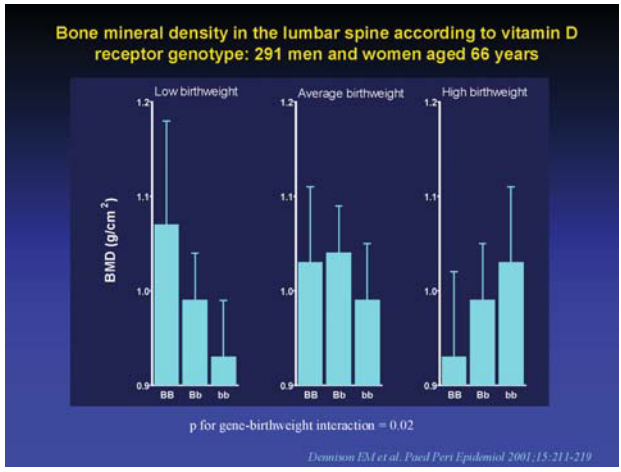

Figure 16-1. Relation between VDR genotype (BB, Bb, bb), birthweight and lumbar spine BMD among 165 men and 126 women resident in Hertfordshire. Data derived from Dennison *et al.*[47]

Table 16-2. Growth in infancy and adult bone mass

Site		Birthweight		Weight at one year	
BMC	Lumbar spine	0.15	(0.10 - 0.20)	0.25	(0.19 - 0.32)
	Femoral neck	0.12	(0.07 - 0.18)	0.20	(0.14 - 0.27)
	Whole body	0.19	(0.10 - 0.28)	0.44	(0.35 - 0.52)
BMD	Lumbar spine	0.12	(0.07 - 0.16)	0.11	(0.04 - 0.18)
	Femoral neck	0.12	(0.07 - 0.16)	0.05	(-0.02 - 0.12)
	Whole body	0.24	(0.17 - 0.30)	0.25	(0.15 - 0.35)

Figures are correlation coefficients with 95% CI. Data are derived from published studies (n=10) relating weight in infancy and adult bone mass

3.2 Physiological studies

To explore further the potential role of hypothalamic-pituitary function and its relevance to the pathogenesis of osteoporosis, profiles of circulating GH and cortisol were compared with bone density among groups of men and women whose birth records had been preserved. These studies revealed that

birthweight and weight in infancy were predictors of basal levels of GH and cortisol during late adult life.[48-50] The levels of these two skeletally active hormones were also found to be determinants of prospectively determined bone loss rate. The data are compatible with the hypothesis that environmental stressors during intrauterine or early postnatal life alter the sensitivity of the growth plate to GH and cortisol. The consequence of such endocrine programming would be to reduce peak skeletal size, perhaps also to reduce mineralisation, and to predispose to an accelerated rate of bone loss during later life.[48-50] Recent studies suggest that interactions between the genome and early environment might establish basal levels of circulating GH, and thereby contribute to accelerated bone loss.[51] Thus, a single nucleotide polymorphism has been discovered at locus GH1-A5157G in the promoter region of the human growth hormone (GH1) gene. This is associated with significantly lower basal GH concentration, lower baseline DND and accelerated bone loss (Figure 16-2). As with polymorphisms in the VDR gene, a significant (p=0.02) interaction was observed between weight at one year, allelic variation at this site and bone loss rate.

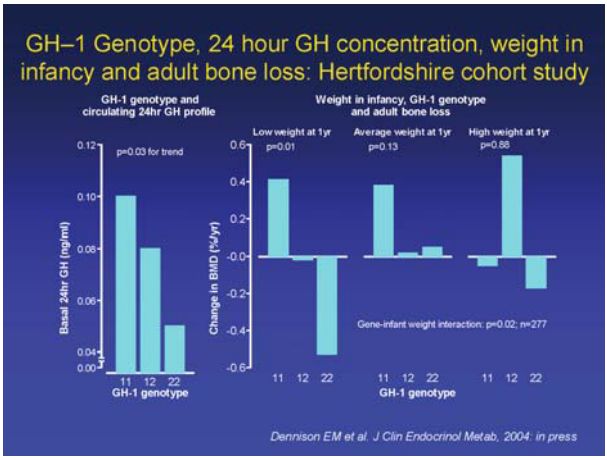

Figure 16-2. GH-1 genotype, 24 hour GH concentration, weight in infancy and adult bone loss: Hertfordshire cohort study. Data derived from Dennison *et al.* [51]

3.3 Maternal nutrition, lifestyle and neonatal bone mineral

The third piece of epidemiological evidence that osteoporosis might arise in part through developmental maladaptation, stems from investigation of a series of mothers through pregnancy; anthropometric and lifestyle maternal characteristics were related to the bone mineral of their newborn offspring.[52].

After adjusting for sex and gestational age, neonatal bone mass was strongly and positively associated with birthweight, birth length and placental weight. Other determinants included maternal and paternal birthweight, and maternal triceps skinfold thickness at 28 weeks (Figure 16-3). Maternal smoking and maternal energy intake at 18 weeks gestation, were negatively associated with neonatal BMC at both the spine and whole body (Figure 16-4). The independent effects of maternal and paternal birthweight on fetal skeletal development support the notion that paternal influences, for example through the imprinting of growth promoting genes such as IGF-2, contribute strongly to the establishment of the early skeletal growth trajectory, while maternal nutrition and body build modify fetal nutrient supply and subsequent bone accretion, predominantly through influences on placentation.

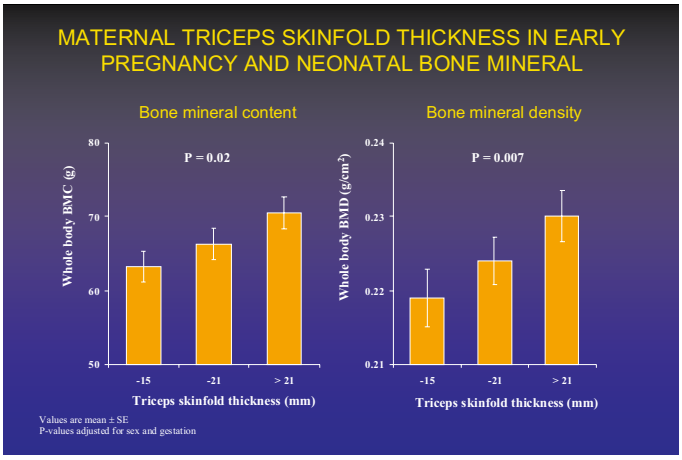

Figure 16-3. Maternal triceps skinfold thickness in early pregnancy and neonatal bone mineral among 144 term neonates. Data derived from Godfrey *et al.* [52]

In the most recent data from mother/offspring cohorts, body composition has been assessed by DXA in 216 children at age 9 years.[53] They and their parents had previously been included in a population-based study of maternal nutrition and fetal growth. The nutrition, body build and lifestyle of the mothers had been characterised during early and late pregnancy, and samples of umbilical venous blood had been obtained at birth. Reduced maternal height, lower pre-conceptional maternal weight, reduced maternal fat stores during late pregnancy, a history of maternal smoking and lower maternal social class were all associated with reduced whole body BMC of

the child at age 9 years. Lower ionised calcium concentration in umbilical venous serum also predicted reduced childhood bone mass (r=0.19, p=0.02); this association appeared to mediate the effect of maternal fat stores, smoking and socio-economic status on the bone mass of the children at age 9 years. Around 25 percent of the mothers had sub-optimal vitamin D status as assessed by serum 25-hydroxyvitamin D concentration. The children born to these mothers had significantly ($p<0.01$) reduced whole body BMC at age 9 years. This deficit in skeletal growth remained significant even after adjustment for childhood weight and bone area.[53] These data suggest that the placental capacity to maintain the materno-fetal calcium gradient is important in optimising the trajectory of postnatal skeletal growth.

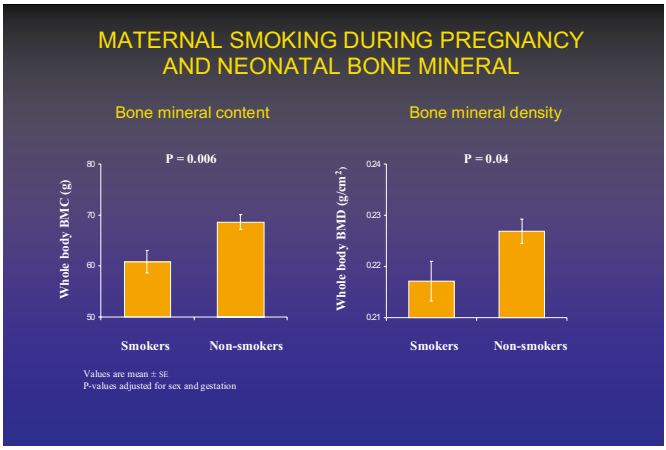

Figure 16-4. Maternal smoking during pregnancy and neonatal bone mineral among 144 term neonates. Data derived from Godfrey *et al*. [52]

4. CHILDHOOD GROWTH AND ADULT HIP FRACTURE

Most of the evidence relating the intrauterine environment to later osteoporosis stems from studies utilising non-invasive assessments of bone mineral. The clinically important consequence of reduced bone mass is fracture, and data are now available which directly link growth rates in childhood with the risk of later hip fracture.[54] Studies of a unique Finnish cohort in whom birth and childhood growth data were linked to later hospital discharge records for hip fracture, have permitted follow-up of around 7000

men and women who were born in Helsinki University Central Hospital during 1924-1933. Body size at birth was recorded and an average of 10 measurements were obtained of height and weight throughout childhood. Hip fracture incidence was assessed in this cohort using the Finnish hospital discharge registration system. After adjustment for age and sex, there were two major determinants of hip fracture risk: tall maternal height ($p<0.001$), and low rate of childhood growth (height, $p=0.006$; weight, $p=0.01$) (Figure 16-5). The effects of maternal height and childhood growth rate were statistically independent of each other, and remained after adjusting for socio-economic status. More important, hip fracture risk was also elevated ($p=0.05$) among babies born short. These data are compatible with endocrine programming influencing the risk of hip fracture. In addition, the observation that fracture subjects were shorter at birth, but of average height by age 7 years, suggests that hip fracture risk might be particularly elevated among children in whom growth of the skeletal envelope is forced ahead of the capacity to mineralise, a phenomenon which is accelerated during pubertal growth.

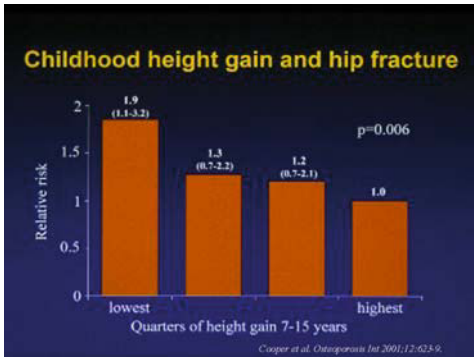

Figure 16-5. Rate of childhood height gain between age 7 and 15 years, and later risk of hip fracture among 3639 men and 3447 women born in Helsinki University Central Hospital between 1924 and 1933.

5. DEVELOPMENTAL PLASTICITY AND OSTEOPOROSIS

Numerous animal experiments have shown that hormones, undernutrition, and other influences that affect development during sensitive

periods of early life permanently programme the structure and physiology of the body's tissues and systems. A remarkable example is the effect of temperature on the sex of reptiles.[55] If the eggs of an American alligator are incubated at 30°C, all the offspring are female. If incubated at 33°C, all the offspring are male. At temperatures between 30 and 33°C, there are varying proportions of females and males. It is believed that the fundamental sex is female, and a transcription factor is required to divert growth along a male pathway. Instead of the transcription factor being controlled genetically by a sex chromosome, it depends on the environment, specifically temperature.

Organ systems in the body are most susceptible to developmental programming during periods when they are growing rapidly. During the first two months of life, the embryonic period, there is extensive differentiation of progenitor cells, without rapid cell replication. Thereafter, in the fetal period, the highest growth rates are observed. Growth slows in late gestation and continues to slow in childhood. The high growth rates of the fetus compared with the child are mostly the result of cell replication; the proportion of cells that are dividing becomes progressively less as the fetus becomes older. Slowing of growth is a major adaptation to undernutrition. Experiments on rats, mice, sheep and pigs have demonstrated that protein or energy restriction of the mother during pregnancy and lactation is associated with smaller offspring.[43, 55-58] In general, the earlier in life that undernutrition occurs, the more likely it is to have permanent effects on body size.[59] Early in embryonic life, growth is regulated by the supply of nutrients and oxygen. At some point shortly after birth, growth begins to track. In humans, tracking is demonstrated by the way in which infants grow along centile curves. Once tracking is established, it is no longer possible to make animals grow faster by offering them unlimited food. The rate of growth has become set, homeostatically controlled by feedback systems. After a period of undernutrition, they will regain their expected size. This contrasts with the effects of undernutrition during intrauterine life, in which skeletal development is slowed and the peak skeletal proportions attained following the completion of linear growth are reduced.

There are three cellular mechanisms for the induction of programming. First, the nutrient environment may permanently alter gene expression; one example of this is permanent change in the activity of metabolic enzymes such as HMG-CoA reductase.[60] Second, early nutrition may permanently reduce cell numbers. The small, but normally proportioned rat produced by undernutrition before weaning, has been shown to have fewer cells in its organs and tissues.[61] Growth retarded human babies have reduced numbers of cells in their pancreas which may limit insulin secretion [62], and reduced size of their airways which may limit respiratory function.[63] Third, certain clones of cells may be altered by environmental adversity during

development; for example, an altered balance of the TH-1 and TH-2 lymphocyte subtypes might predispose to atopic disease in later life.[64] Evidence from the human studies outlined above suggests that skeletal development might be programmed as a consequence of the first two of these mechanisms.

Animal models for the developmental origins of osteoporosis replicate the observations made in humans. In the first such model, the feeding of a low protein diet to pregnant rats produced offspring that exhibited a reduction in bone area and BMC, with altered growth plate morphology in adulthood.[65] Maternal protein restriction also down-regulated the proliferation and differentiation of bone marrow stromal cells as assessed by fibroblast colony formation at 4 and 8 weeks. [66]

6. INFANT NUTRITION AND OSTEOPOROSIS

There have been few studies of the relationship between breast-feeding or early postnatal nutrition and later risk of osteoporosis. In the Helsinki cohort study, 8760 men and women were categorised by duration of breast-feeding; no apparent relationship was found with later risk of hip fracture (Table 16-3). Likewise, little association was found between the duration of breast-feeding in this cohort and bone size or areal bone mineral density. The relationship between infant feeding and adolescent bone mineral content has also been evaluated in follow-up studies of randomised controlled trials of supplementation in infants. Thus, Fewtrell *et al* reviewed 244 pre-term children and 95 term children who had been randomised to banked breast-milk, pre-term formula or term formula. At age 8-12 years, there was no

Table 16-3. Relationship between breast-feeding and hip fracture.

Breast-feeding and hip fracture

Helsinki Cohort Study; 8760 men and women

Duration of breast feeding (mths)	No.	HR (95% CI)	
		Men	Women
Never	1431	1.0	1.0
<2	1249	0.4 (0.1 – 2.0)	0.9 (0.3 – 3.3)
2-4	1676	1.2 (0.4 – 3.8)	0.8 (0.2 – 3.1)
4-6	1199	0.9 (0.3 – 2.8)	-
6-8	1298	0.5 (0.1 – 2.5)	0.3 (0.1 – 2.2)
>8	1826	0.9 (0.3 – 3.3)	0.9 (0.2 – 3.4)

41 hip fractures

significant difference in BMC at the lumbar spine, proximal femur or distal radius, between breast-milk and pre-term formula groups; in contrast between term and pre-term formula, there was a modest ($p<0.05$) benefit observed at the femoral neck.[67]

7. SUMMARY

Undernutrition and other adverse influences arising in fetal life or immediately after birth have a permanent effect on body structure, physiology and metabolism. The specific effects of undernutrition depend on the time and development at which it occurs; rapidly growing fetuses and neonates are more vulnerable. Its effects include altered gene expression, reduced cell numbers, imbalance between cell types, altered organ structure, and changes in the pattern of hormonal release and tissue sensitivity to these hormones. Evidence is now accumulating from human studies that programming of bone growth might be an important contributor to the later risk of osteoporotic fracture. Body weight in infancy is a determinant of adult bone mineral content, as well as of the basal levels of activity of the GH/IGF-1 and HPA axes. Epidemiological studies have suggested that maternal smoking and nutrition during pregnancy influence intrauterine skeletal mineralisation. Finally, childhood growth rates have been directly linked to the risk of hip fracture many decades later. Further studies of this phenomenon are required in order that effective preventive strategies against osteoporosis throughout the life course may be delineated and more effectively applied.

8. CONCLUSIONS

Several modifiable factors influence the growing skeleton and permit it to achieve its full genetic potential. These may act during intrauterine or early postnatal life; childhood; or adolescence. While many important research questions need to be addressed in this area, concerted action in health policy should be directed at: (1) optimising maternal nutrition and intrauterine growth; (2) improving the calcium intake and general nutritional levels of all children; (3) increasing the general exercise level of pre-pubertal and pubertal children; (4) ensuring adequate vitamin D status, not just during infancy but throughout the period of growth. Further research into the interaction between the genome and early environmental risk factors for osteoporotic fracture is urgently required, in order that environmental modification can be targeted to those at the greatest risk. Finally,

intervention studies exploring the role of maternal lifestyle and nutrition on bone mineral accrual among the offspring of these mothers, will provide much needed evidence on this approach to the reduction in fracture risk of future generations.

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Chapter 17

THE LONG-TERM EFFECTS OF BREAST-FEEDING ON ASTHMA AND ATOPIC DISEASE

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1. EPIDEMIOLOGICAL EVIDENCE

In this review, the primary objective is to assess the evidence of whether breastfeeding protects against asthma and atopic disease for the long-term (long-term is defined here as >5 years of age). Two main types of observational epidemiological studies have been used to test this hypothesis. These are cohort studies of random samples of children and cohort studies of children with a family history of asthma or atopy. In each study type, exposure and outcome data are collected either prospectively or retrospectively. The 12 criteria for assessing the adequate measurement of exposure, outcome and statistics of cohort studies in this context are given in Table 17-1.[1]

1.1 Retrospective and prospective studies

In retrospective studies asthma and atopy may be measured currently. Retrospective studies of breastfeeding and asthma have shown no association[2-5], a reduced risk[4, 6-8] or an increased risk.[9] According to the criteria in Table 17-1, late maternal recall of breastfeeding is not acceptable. Only prospective studies are included in this review.

G. R. Goldberg et al. (eds.), Breast-Feeding: Early Influences on Later Health,

Table 17-1. Criteria for assessing the adequacy of cohort studies for the effect of breastfeeding on the development of asthma and allergy (see reference 1)

Measurement	Criteria
Exposure	1. Non-reliance on late maternal recall of breastfeeding
	2. Blind ascertainment of infant feeding history
	3. Sufficient duration of breastfeeding
	4. Sufficient exclusivity of breastfeeding
Outcome	5. Strict diagnostic criteria
	6. Blind ascertainment of outcomes
	7. Consideration of severity of outcome
	8. Consideration of age of onset of outcome
Statistics	9. Control for confounding factors
	10. Assessment of dose-response effects
	11. Assessment of effects in children at high risk of outcome
	12. Adequate statistical power

Prospective cohort studies classify subjects in the study on the basis of presence or absence of exposure. The relevant events, both exposures and the outcomes of interest have not yet occurred when the study is initiated. In some prospective studies, the exposure may have occurred but the outcomes have not yet occurred. The studies that met the criteria in Table 17-1 with children followed to more than five years, are reviewed below after a brief discussion of the excluded studies.

1.2 Studies considered in this review

1.2.1 Excluded studies

For 2 studies it was unclear as to whether they met the criteria in Table 17-1.[11-12] A cohort of 1661 infants recruited between 1972 and 1973 was assessed from ages 9 to 26 years using respiratory questionnaires, pulmonary function tests and allergy skin tests.[11] Following multifactor adjustment breastfeeding showed odds ratios of 1.94 (95%CI: 1.42-2.65 $p<0.0001$) for current asthma at 9 years and 1.83 (1.35-2.47 $p<0.0001$) for current asthma at 9-26 years by repeated measures analysis. This study has a number of limitations. Children were enrolled at the age of 3 years when data on breastfeeding recall were collected from mothers. As with most historical studies, the results of this study cannot be easily generalised to present times when health and lifestyle practices have changed. The cohort was at a time (early 1970s) when breastfeeding rates were low. In the hospital, suckling times and number of feeds were restricted and built up over several days. In

addition, long periods of separation of mother and baby and nightly formula feeds could have limited establishment of breastfeeding. This study might provide useful information about partial breastfeeding which could contribute to one of the important aspects of biological plausibility, i.e. a dose-response. In the cohort, 47% of babies were never breastfed and the modal time of cessation of breastfeeding was just 5-11 weeks. The second study [12] does not meet criteria 1, 3, 4 9, 10 and 11 in Table 17-1.

1.2.2 Included studies

In relation to scientific rigour, to study the effect of breast-milk on the development of asthma and allergy, all of the 12 criteria in Table 17-1 were met in the studies briefly summarised below.

In Finland a long-term study of 256 healthy babies followed from before birth to 17 years showed significant protection by breastfeeding at least for the first 17 years of life.[13] The value of this study lies in the duration of the follow-up and in the comprehensive collection of exposure and outcome data. In this study a clear prophylactic relationship between breastfeeding and atopic disease was found that was not modified by family history of atopic illness.

The Tucson Children's Respiratory Study of 1246 healthy infants was designed to investigate risk factors for lower respiratory tract illness. Established between 1980 and 1984, detailed pediatric assessments were conducted at defined intervals. When the cohort was 6 years of age, recurrent wheeze was less common in non-atopic children who were breastfed as infants, compared to non-atopic children who were not breastfed.[14] At 11 years an association between breastfeeding and asthma differed with maternal asthma status.[15] Among children with a maternal history of asthma, the percent of children who developed asthma was 9% for the never breastfed, 36% for those breastfed for three months or less and 57% for those breastfed for four months or longer. However, breastfed children were significantly less likely to wheeze at age 6, 9 or 11 years if they had not been diagnosed with asthma at any age. A limitation of this study was that formula feeding was prevalent in the United States at the time of study recruitment [16] (more than 50%) and data on formula introduction were not collected, therefore a measure of exclusive breastfeeding was not possible.

In the Dundee Infant Feeding Study, 674 children were followed from birth to seven years.[17] The prevalence of wheeze, breathlessness or cough at age 7 was significantly reduced if a child had been exclusively breastfed for at least 15 weeks ($P<0.05$).

The Western Australian Pregnancy Cohort Study included 2187 children followed to 6 years.[18] There was a significant reduction in the risk of childhood asthma at age 6 years if other milk was introduced before 4 months in either atopic or non-atopic children.[18-19]

The strengths of this study include its representativeness of the general population, its sample size and high response rate.[20] Collection of outcome data (including respiratory history) was prospective, by diary card, at frequent intervals and based on validated questionnaires. [18, 21]

1.3 Summary

Studies that met the criteria for inclusion in this review of the long term effects of breastfeeding on asthma and atopic disease demonstrate a pattern of protection with breastfeeding and an increased risk with formula. In studies that met all recommended standards the odds ratios consistently exhibited an expected direction of effect that suggest biological plausibility and specific protective effects of breast-milk. Although the range of magnitude of effect is not large (1.2-1.5), in population terms this is large enough to be of public health significance.

Inconsistency in the literature is due to researchers measuring different aspects of atopy and asthma in children of different ages and using different ways of measuring those outcomes. The use of multivariate statistical models does not allow for entire control of confounding variables as there are many factors that may influence both the mother's likelihood of breastfeeding and the risk of children developing asthma or atopy.

1.4 Meta analyses

A meta analysis of nine studies examined the association between breastfeeding and the development of asthma. [22] The meta analysis showed that children breastfed for at least three months of age were significantly protected against developing asthma by an estimated odds of 0.80. Other meta-analyses [23-25] suggest a similar protective effect of between 26% - 30% from exclusive breastfeeding during the first 3 months of life against developing asthma, allergic rhinitis and atopic eczema later in childhood.

1.5 Studies of children with a family history

The prospective family cohort study classifies subjects on the basis of presence or absence of the disease in the mother or father or both. As in the population cohort study, the relevant exposure and outcome events, both have not yet occurred when the study is initiated.

A large randomised controlled trial of 519 high risk infants born in two maternity hospitals in South Wales was conducted. Subsequent follow-up[26] until seven years showed that the protection afforded by breastfeeding persisted for wheezing (any breastfeeding 22% wheeze; never breastfed 43% wheeze) ($P<0.001$).

In Canada, researchers assessed the effect of different formulas on atopic disease in a double-blind study of high-risk infants.[27] A protective effect of exclusive breastfeeding for four months or longer against atopic disease until at least five years (OR: 0.42 95% CI: 0.20, 0.89) compared to infants fed cow's milk-based or soy formula was apparent. Whey hydrolysate reduced the incidence of atopic symptoms in high-risk infants (OR: 0.32 95% CI: 0.16, 0.65) and this approach appeared beneficial compared to breastfeeding without maternal dietary restriction, or soy-based formula feeding.[27-28]

2. MAKING SENSE OF THE EPIDEMIOLOGICAL EVIDENCE

A number of key issues complicate the interpretation of the available evidence and are often not taken into full and proper consideration. These issues include: low statistical power; misclassification of information; causal pathway modeling; and effect modification (statistical interaction).

2.1 Low statistical power

Asthma and atopy are complex multifactorial diseases and therefore it is inevitable that most single causes (i.e. breastfeeding) will have relatively small effects on overall disease prevalence. On the basis of published meta-analyses, the true relative risk linking breastfeeding to childhood asthma may be 1.2 to 1.3.[29] In terms of breastfeeding where a large proportion of infants are exposed to early formula feeding, small odds ratios such as this have public health importance. Consequently, studies that appear large by conventional standards may be under-powered and the interpretation of individual non-significant results may be misleading.[30-31]

2.2 Misclassification of information

Both the primary exposure (breastfeeding) and the primary outcomes (asthma, atopic disease, and eczema) are subject to serious potential misclassification, particularly when assessed retrospectively. Study results may be confounded by the early introduction of infant formula as small

amounts of early formula milk may be damaging to the developing infant immune system.

2.3 Causal pathway modeling

A complex disease such as asthma is likely to arise from the action of a series of etiological determinants extended along several complex causal pathways. A given secondary exposure may either be a confounder and therefore we need to adjust for it or may be part of the causal pathway linking the exposure to the outcome of interest and therefore we must not adjust for it. In other words, inappropriate adjustment for determinants lying on a causal pathway of interest may lead to shrunken estimates and inflated standard errors and will reduce an already limited statistical power.

2.4 Effect modification (statistical interaction)

There is the possibility that the association between breastfeeding and asthma and atopy varies at different levels of a key third variable such as the underlying genetic risk of atopy. In fact, evidence would suggest that this may be the case.[15, 32] Such interactions not only complicate interpretation, but also reduce statistical power.

2.5 Resolving the conflict

Given these considerations for the effect of breastfeeding, we would expect relatively small relative risks with large standard errors. This means that, even if a real association between breastfeeding and asthma/atopy exists many studies will generate non-significant estimates, and a non-trivial proportion will produce estimates that apparently go in the opposite direction. Two complementary solutions to this problem are to 1) carry out meta-analyses[22]; and 2) undertake large population-based studies with careful assessment of exposures, outcomes and relevant confounders.

When the exposure is both common and modifiable and the outcome is costly in terms of mortality, morbidity and economics, a small relative risk is of major public health relevance. The putative association between childhood asthma/atopy and breastfeeding unquestionably meets these criteria.

2.6 Causality

Previous studies that have shown a protective effect of breastfeeding on asthma and atopy have fulfilled strict criteria for assessing causality from

observational studies33, that is biological plausibility, consistency of findings, strength of association, temporality (exposure to breastfeeding before atopy develops) and a dose-response relationship.

2.7 Biological plausibility

A large body of literature on the biochemistry of human milk provides biological plausibility for the hypothesis that breastfeeding protects against the development of asthmatic and allergic symptoms. Breastmilk contains a vast array of beneficial and multifunctional compounds including antimicrobial, immunomodulating and bioactive molecules that may have imprinting and subsequent long-term effects.34-40 These molecules exhibit pleiotropic functionality 41, are well adapted to infants' mucosal sites and are not well represented in infant formula. Breastmilk is also a rich source of n-3 fatty acids, which have anti-inflammatory effects. It is not currently known which of the many components in milk may account for the protective effect but several mechanisms are likely.41-44

3. Mechanisms whereby breastfeeding could impact on asthma and atopic disease

There are five mechanisms whereby breastfed children may show a reduced occurrence of asthma and atopic disease.

3.1 Exposure to foreign dietary antigens

A breastfed infant is less exposed to foreign dietary antigen in cow's milk[45] although there are antigens in mothers milk. Some exclusively breastfed infants develop allergic reactions to cow's milk protein (β-lactoglobulin)[46] but the incidence of this is very low (0.5-1.7%) in comparison to the incidence in unselected populations of infants (2%-3%).[47] Studies in partially breastfed infants might help clarify whether the key factors were derived from foreign proteins or protection from breast milk factors.

The young infant's gut is immature, and may poorly exclude multiple allergens or large quantities of allergens that can react with the system of sensitisation. The benefits of exclusive breastfeeding derive not only from elimination of cow's milk protein but from local protection of human milk in the bowel. For example secretory IgA coats the mucosa and blocks entrance of antigens.[48]

Many children do receive proteins from cow's milk formula in the first days of life that may initiate sensitisation in susceptible individuals.[45, 49] Subsequent exposure even to minute quantities of β-lactoglobulin in breast-milk may elicit an allergic manifestation that may be associated with IgE mediated adverse reactions.

3.2 Maturation of gastrointestinal mucosa

Human milk contains factors that promote gastro-intestinal mucosa maturation thereby allowing early 'closure' of macromolecular absorption The most striking interaction between diet and intestinal development occurs immediately following the first feed of mother's milk, and may be due to an expression of genes triggered by milk constituents.

3.3 Gut microflora and reduced sensitivity

By decreasing the incidence of infection and possibly altering the gut microflora that can act as an adjuvant for ingested food proteins, the possibility of sensitisation may be reduced Control of colonisation of mucosal surfaces by organisms is the most important task of the mucosal immune system. In addition to respiratory microbial agents, the commensal (normal, healthy) microbial flora of the gastrointestinal tract stimulate the functional maturation of the immune system.[50] This is accomplished through humoral and cellular mechanisms which control the growth of bacterial, viral and parasitic organisms and non-cellular elements. Microbial products of the gastrointestinal flora may activate the antigen presenting mechanism of dendritic cells, polarising towards a Th1 memory.[51-52] In early life Th1 and Th2 cell populations possess the potential of reversibility towards the alternate cytokine type but the reversibility is lost after long term stimulation by microbes.[53] Possible causal pathways are also discussed in detail in Chapters by Ogra, Kramer and Hanson.

Oligosaccharides in human milk may promote the development of bifidus flora by the provision of substrate for lactobacillus bifidus, the 'healthy' bacteria, while limiting the growth of potentially pathogenic bacteria.[54]

Nucleotides in human milk have multiple functions[55-57] which include effects on gut microflora[58], intestinal growth and development[59] and the response to immunization.[60]

Milk leukocytes survive in the gastrointestinal tract tolerating pH, temperature, osmolarity and resist proteolytic degradation by trypsin. They adhere to the gut epithelium and persist in the intestine for up to 60 hours. It is thought that the ability of milk immunocompetent cells to survive in the gastrointestinal tract, to secrete cytokines and to migrate across the neonatal

intestinal mucosa to the systemic circulation allows them to potentate not only the local response of the gastrointestinal tract but also their systemic immune responses[37].

Human milk lipids contain preformed long chain polyunsaturated fatty acids (n-3) in large amounts, which serve as precursors of biologically potent mediators (e.g. the eicosanoids; prostaglandins, thromboxanes and leukotrienes) as well as vital structural components of membrane systems in all tissues.[61] It is thought that fatty acids are important cell signalling molecules[62] acting rapidly and directly to alter the transcription of specific genes[63] involved in inflammation such as IL-1β. The very long chain fatty acids (EPA and DHA) suppress IL-1β mRNA, a finding that may be important in asthma prevention.[64]

The fatty acid composition of breast-milk from the mothers of children with newly developed atopic dermatitis was observed to be lower than normal demonstrating an abnormal fatty acid status in atopic subjects.[65] This may account for some of the inconsistent results from studies of the effect of breastfeeding on the subsequent development of atopic dermatitis[66] and more recently asthma.[67] Infants need the correct amount and balance of fatty acids for normal immune system development and an imbalance may lead to hypersensitisation. One study investigated the supplementation of pregnant mothers with fatty acids and subsequent fatty acid in cord blood, and found that fatty acid levels were higher in the cord blood of infants of supplemented mothers.[68]

3.4 Immunomodulatory and anti-inflammatory factors in human milk

Human milk has functional immunomodulatory and anti-inflammatory factors that curtail macromolecular uptake Breastfed infants appear to have a more effective immune function, reflected by an ability to mount a targeted response to a potential pathogen.[69] Human milk may have systemic immunomodulating properties[70] which are long lasting and which may protect against a number of diseases[71-75] including allergy.[76] Such clinical and experimental observations suggest that human milk has the ability to modulate the development of the infant's own mucosal and systemic immune systems which may be associated with immunoregulatory agents present in colostrum and mature milk.

Immunomodulating factors in human milk include α-tocopherol, β-casomorphins, prolactin and anti-inflammatory agents. These direct-acting factors protect by non-inflammatory mechanisms, including enzymes that degrade inflammatory mediators. The anti-inflammatory agents in human colostrum and mature milk include lactoferrin, lysozyme, antioxidants,

cytokines, secretory IgA and hormonal factors that down-regulate inflammation[34]. Specifically, lactoferrin and sIgA inhibit endotoxin induced inflammatory cytokine release.[77-78]

Mammalian cells have developed an elaborate antioxidant defence system that includes both non-enzymatic antioxidants (e.g. glutathione, vitamins C and E [α-tocopherol] and β-carotene) and lactoferrin as well as enzymatic activities (e.g. glutathione peroxidase, catalase, and other hemoprotein peroxidases) both of which play a significant part in the anti-inflammatory system of human milk.

3.5 Cytokines and growth factors in human milk

Cytokines and growth factors in human milk may play an important role in modulating the development of asthma The presence of cytokines, hormones and growth factors in human milk play an important role in modulating the development of atopic disease. Cytokines are soluble glycoproteins with established actions on the immune system.[79] They are pluripotent polypeptides that act in autocrine/paracrine fashions binding to specific cellular receptors, operating in networks and orchestrating immune system development and functions.[80] See also workshop summary (Chapter 24). Early milk has an abundance of these components at a time when neonatal organ system immaturity exists, suggesting that the bioactive compounds of milk may be important in neonatal development.[81]

Human milk contains several known growth factors including Epidermal Growth Factor (EGF) and Transforming Growth Factor (TGF)[82]. Both promote the maturation of gastrointestinal and respiratory mucosa restricting the penetration of harmful antigenic material and contributing to the anti-inflammatory effect of human milk.

4. CONCLUSION

Human milk feeding appears to be of considerable relevance to the development of the immune system in infancy and may therefore impact upon the incidence and severity of subsequent asthma and atopy later in life. As evidenced by the studies that met the strict criteria for the study of breastfeeding and atopic disease, all demonstrated a protective effect of breast-milk feeding or conversely, a risk of formula feeding. However, the continuing protective effect of breastfeeding on asthma and atopy later in adolescence and adulthood has yet to be confirmed in larger longitudinal studies. Because achievements in promoting breastfeeding have been based on consistent scientific evidence, they should not be eroded on the basis of a

few studies that show a negative relation to asthma. It is essential that scientists strive to collect better evidence to support or refute causal associations between breastfeeding and childhood illnesses.

Until more rigorous and more direct evidence is collected, links between breastfeeding and the development of allergic illness in adulthood based on studies in which bias and confounding cannot be controlled and mechanisms have not been investigated, should not be incorporated in public health messages. Given the many benefits conferred by breast-milk, breastfeeding should continue to be promoted as the preferred infant feeding method for the first 6 months and up to two years, as recommended by WHO.[83]

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Chapter 18

EVOLUTION OF HUMAN LACTATION AND COMPLEMENTARY FEEDING: IMPLICATIONS FOR UNDERSTANDING CONTEMPORARY CROSS-CULTURAL VARIATION

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1. INTRODUCTION

Artistic reconstructions of ancestral hominids [1] often depict mothers with bared breasts and suckling infants, reflecting assumptions about the importance of lactation in human evolution. However, anthropologists have published no detailed theories about how our ancestors fed young children. In the absence of a scientific model of the evolution of human lactation and complementary feeding, it is difficult to evaluate claims made about the long duration of ancient breast-feeding or the "naturalness" of lactation patterns observed in some human societies. This chapter therefore has two main goals. First, I review several lines of evidence that suggest how changes in birth spacing, foraging strategy and sociality may have increased the selective advantages of a more flexible pattern of lactation and a behavioural shift towards complementary feeding in past environments. Second, I develop a hypothesis that the complementary feeding of young children is a fundamental component of life and socio-behavioural adaptations that evolved among our human ancestors as an ecological strategy for increasing maternal fitness. I suggest that the ancestral habit of introducing

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safe complementary feeding after a period of exclusive breast-feeding is unique to humans. It is linked to the evolution of a species-typical care giving "package", which includes social foraging, food sharing, food processing, and a capacity to invent technological solutions to dietary challenges. I conclude with a brief review of how changes in social organisation, time allocation and diet quality that accompanied the agricultural and industrial revolutions have created an environment in which the evolved tendency to introduce foods to breast-feeding young undermines the health of populations.

1.1 Background

As with most human behaviours, young child feeding practices occur in bewildering variety across cultures [1-5] and change over time.[6-11] Lactation researchers and policy makers have struggled to document this variation by developing indicators for the biologically salient aspects of breast-feeding [12-13] and complementary feeding.[14] Such efforts have been driven by the discovery that some young child feeding practices are causally associated with poor growth, delayed development and increased morbidity and mortality. These include never breast-feeding, partial breast-feeding in the first half of infancy, cessation of breast-feeding before the third year of life [15-16] use of contaminated and often nutritionally inadequate complementary foods [17-19] and passive feeding. [20] Over the last quarter of a century expert groups have proposed a series of recommendations for young child feeding based on observation of clinical outcomes. These have been adopted by global, national and non-governmental health organisations.[21-23].

Clinical concern about the *consequences* of variation in infant and young child feeding practices has meant that fewer studies have attempted to identify their root *causes* in social and environmental conditions or to develop broad interventions to address them.[24-26] Health providers tend to assume that many deviations from clinical recommendations are attributable to unmeasured "cultural" factors, and that these are randomly distributed across societies (i.e. arbitrary), and fixed within societies (i.e. difficult to modify).

Such assumptions are not well tested, and divert attention from important questions about why human breast-feeding patterns are so labile, and why so apparently arbitrary? In developing a conceptual framework in which to understand contemporary variation, it is useful to consider the evolutionary history of human lactation and complementary feeding. Reconstruction of this evolutionary history from comparative biological evidence has the potential to reveal co-evolved links between human diet, life history and behaviour that continue to influence the way people feed infants today.

2. EVOLUTION OF HUMAN LACTATION

2.1 Origin

It may never be possible to ascertain when lactation evolved or what initial selective advantage it conferred because soft tissues are rarely preserved as fossils.[27-28] There is little doubt, however, that lactation was an ancient innovation. Mammary glands of extant monotremes, metatherians and eutherians are sufficiently similar in detail to suggest a monophyletic origin.[29] Analyses of several hundred nuclear gene sequences [30] of the amino acid sequence of [alpha]-lactalbumin protein [31] and of the BRCA1 and IGF2 receptors [32] suggest that divergence of therian mammals and monotremes, and of marsupials and placentals, took place somewhere between 163 and 238 million years ago (MYA), and between 161 and 192 MYA respectively.

These molecular divergence–time estimates are reasonably well supported by a fossil record. A diverse group of mammaliaform insectivores such as *Morganucodon* and *Hadrocodium* appear by at least the early Jurassic (200 MYA).[33-34] Probably descended from egg-laying therapsid reptiles, their cranial-dental morphology suggests they had a fully functioning temporomandibular joint (which in extant mammals develops after the suckling stage in extant monotremes and therians), dyphyodonty (reliance on a single set of specialised permanent teeth that grew after a single set of presumptive "milk" teeth), and several other mammalian synapomorphies (shared, derived traits indicating common ancestry). Dental evidence of highly specialised nipple latching among marsupial alphodontids of the late Cretaceous [35] supports a hypothesis that variation in lactation biology observed among extant mammals is also very ancient.

Lactation almost certainly originated as an increased secretion of carotenoids, antibodies, white cells and other immunity-boosting factors onto eggs or in glandular secretions licked by young.[36-37] Mammary glands are structurally and ontologically similar to tetrapod epitricheal (sebaceous and apocrine) glands, which have the capacity to synthesise carbohydrates, proteins and lipids.[38] It is therefore likely that the addition of nutritive factors to these early secretions triggered selection for an improved capacity of early mammals to quickly store and release energy and nutrients.[39-40] Lactation provided ancient mammals with a new mechanism for scheduling growth and reproduction across the lifespan.[41] The functions of lactation are nowadays three and so important that they are shared by all extant species.[42] They are: (i) immune protection and reduced exposure to dietary pathogens; (ii) a supply of energy and nutrients fine-tuned to juvenile needs and buffered by maternal body stores; and (iii) fertility regulation calibrated to

maximise maternal fitness through promoting optimal litter spacing for infant survival.

2.2 Co-evolution of lactation, life history and feeding ecology

Whatever the precise selective mechanism, the origin of lactation had large implications for the subsequent evolution for mammalian life history and behaviour. Lactation altered the developmental, behavioural and social links between mothers, offspring, and indeed fathers. It opened a developmental window of opportunity for learning through juvenile play and social interaction and made possible the evolution of gestation among therians. Thus, it underpinned the adaptive radiation of mammals.

Many features of lactogenesis [43-44] and immunological activity [37] are remarkably conserved across species. Species differences in other aspects of lactation biology, such as milk energy content, yield, nutrient composition and immune factors probably indicate adaptive modifications that increase fitness in a range of ecological niches with different disease exposure and nutritional challenges.

Within mammal clades, lactation co-evolved with changes in life history strategy and feeding ecology. Lactation length is strongly correlated with adult female mass.[45] It is relatively longer (for body size) among marsupials, bats and primates, and relatively shorter among earless seals and baleen whales. Milk volume, gross composition and peak yield are clearly linked to the behavioural ecology of infant care.[46-47] For example, terrestrial species that nurse continuously (e.g. marsupials) or on demand (e.g. primates) produce dilute milks that are high in carbohydrate and low in fat; terrestrial species that nurse episodically (e.g. felids and canids) produce milks high in fat and carbohydrates, and protein; marine mammals (e.g. phocids) produce relatively concentrated, fatty milks. Species with smaller bodies or relatively faster life histories tend to secrete more nutrient dense and fatty milk at higher rates.[29, 48-49]

Figure 18-1 summarises the behavioural ecological aspects of the post-natal development of feeding in most modern mammals. Juvenile daily intake of energy and specific nutrients increases after birth due to increased milk intake during an initial period of exclusive suckling. Ingestion of foraged foods marks the beginning of a “transitional feeding” period during which milk continues to contribute to nutrition and immune protection but juveniles can increase total intakes beyond peak maternal milk production. As juveniles begin to derive nutrients more efficiently from the environment than from the mother (due to some combination of increased competency, decreased milk production and increased maternal resistance to suckling),

both the absolute and the proportional contribution of milk intake decreases until last suckling occurs. After weaning, independent foraging achieves further increase in total intake. In a minority of species, juveniles may consume provisioned foods foraged by mothers or allo-caregivers both before and after weaning. Milk consumption provides immune benefits during the exclusive and transitional feeding periods. These are tailored at all ages to juvenile development and pathogenic exposure, and persist beyond weaning.

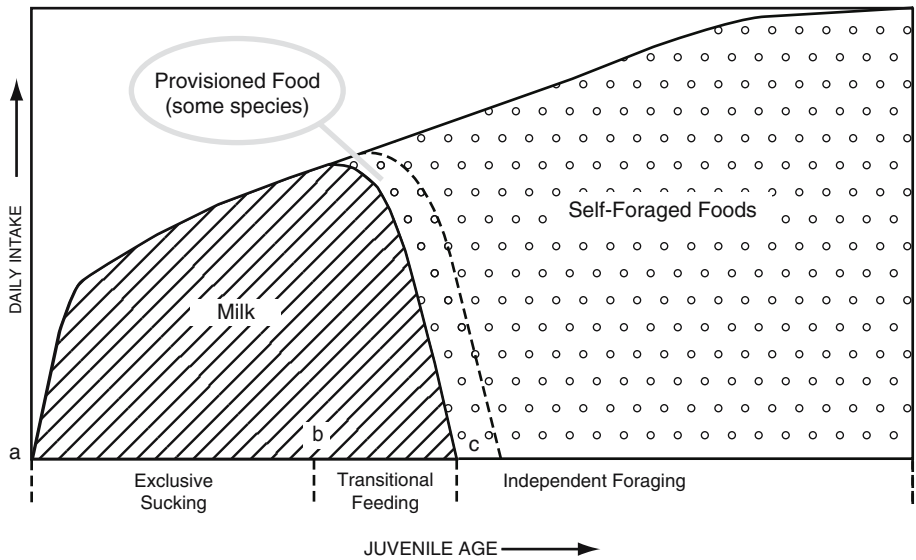

Figure 18-1. Postnatal development of feeding in a typical mammal. Key: (a) birth; (b) ingestion of foraged foods; (c) last suckling (i.e. weaning).

The age at first solid food relative to the age at weaning has been interpreted as an indicator of the function of lactation within the life history and feeding ecology of a given mammal.[45] A longer period of transitional feeding, i.e. first solid food eaten well before weaning, is common among mammals with single, precocial offspring. In these mammals the energetic and nutritional constraints on lactation may be less important in shaping the evolution of life history than the benefits of maintaining contact between mother and young, such as increased opportunities for learning social or foraging behaviour. A shorter period of transitional feeding, i.e. first solid food eaten near weaning is common in polyctocous species with altricial young. In these species the young are very dependent on milk and maternal constraints on lactation may be critical.

A relatively longer duration of exclusive suckling may be interpreted as an evolutionary response to the relative difficulty of acquiring, ingesting or digesting the adult food. An alternative hypothesis is that a relatively abrupt transition from exclusive suckling to weaning can only evolve when the adult diet is more nutrient dense. In some species with "difficult" adult foods juveniles take partially chewed food (e.g. hystricomorph rodents), regurgitated food (e.g. wolves) or modified fecal matter (e.g. koala bears) from the mother and occasionally other adults.

2.3 Transitional feeding in non-human primates

Detailed studies of the diets and foraging behaviour of non-human primate weanlings are few but variation in transitional feeding patterns is apparently wide both within and between species and has yet to be fully described and explained.[50-51] In general, non-human primate infants wean relatively abruptly and begin to forage on foods similar to those selected by the mother, processing them largely for themselves. They therefore fit the general mammalian pattern depicted in Figure 18-1.

Comparative zoological analyses have generated several models to predict duration of lactation (i.e. age at weaning) from other primate life history traits. These include: 1.5 times the length of gestation [52], eruption of first molar teeth [53-54], quadrupling of birthweight [55], and attainment of one-third adult weight. [56] However, no model reliably predicts age at weaning for all species, suggesting that it is quite labile relative to other life history traits.

Weaning age appears to be sensitive to ecological factors that constrain maternal ability to meet the increasing energy needs of growing offspring [55] and the ability of infants to survive without mother's milk. Colleagues and I recently conducted a preliminary test of a hypothesis about variation in the patterning of non-human juvenile primate feeding ecology.[57] We predicted that ecological constraints result in shortened duration of transitional feeding in species where adults exploit a relatively high quality diet. Among 23 species of non-human primate for which data are available, the duration of transitional feeding (estimated as the period between reported age at first consumption of solid food and age at last suckling) is found to increase with maternal body size (Figure 18-2).

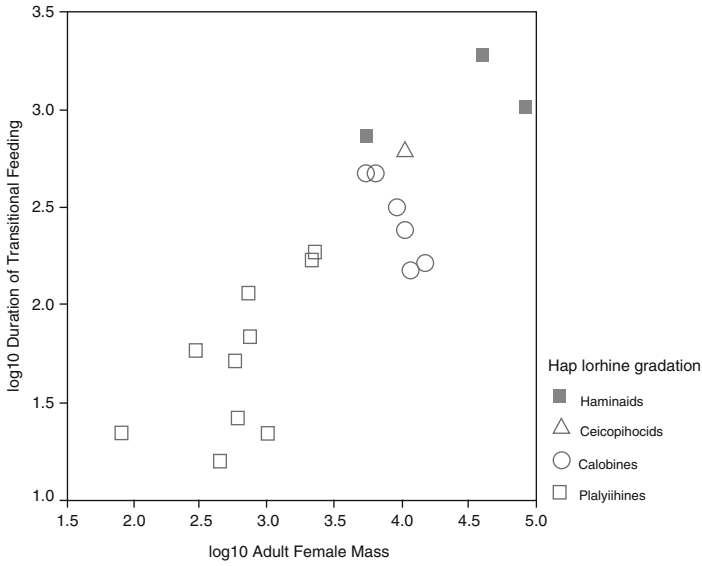

Figure 18-2. Relationship between duration of transitional feeding and body size in a sample of non-human primates (n=23).

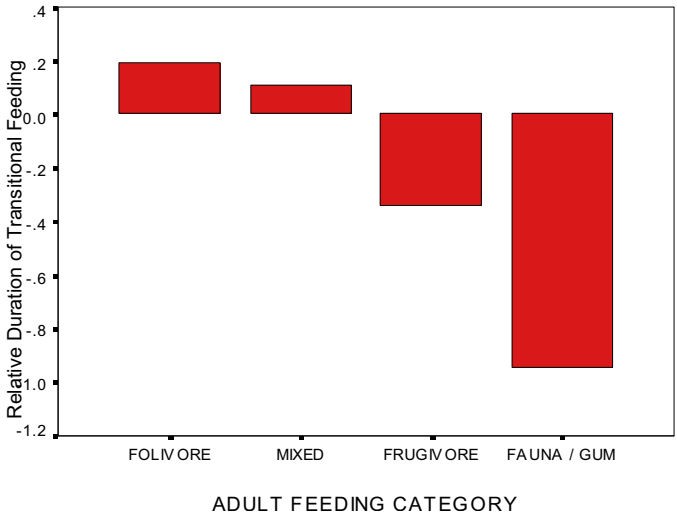

Figure 18-3. Relationship between adult feeding category and relative duration of transitional feeding (calculated as residuals of the log-log plot against body size) in a sample of non-human primates (n=23). $F_{3,23} = 2.611$, $p = 0.087$.

We controlled for large species differences in body size by comparing residuals of the least-squares regression with maternal body size (Figure 18–3). This suggested that transitional feeding was of relatively short duration among gummivores and faunivores, and relatively long among folivores and mixed feeders. These results do not control for phylogeny or group size and other aspects of ecology, but do suggest that diet quality constrains primate weaning. Non-human primate species wean relatively quickly when they have access to better quality foods.

3. SHARED AND DERIVED FEATURES OF HUMAN LACTATION

3.1 Breast milk composition, secretion and delivery

Lactation researchers continue to discover important ways in which human milk differs from that of domesticates such as cows, goats and sheep. Nevertheless, there is nothing particularly unusual about the gross characteristics of human milk in comparison to species with which we share more recent common ancestry. Lactation has been poorly studied among our closest living relatives, the apes, but it is known that gross composition of human milk falls within the range of non-human primates.[58-60] Although human milk contains one of the lowest concentrations of protein and highest concentrations of carbohydrate of any mammal, it is not a remarkable outlier among primates. There is little evidence to suggest that relatively rapid post-natal human brain growth is associated with significantly modified milk.[61] Human milk production is similar to that of other anthropoids when measured on a volume yield and energy output basis scaled for body size. [62] Humans share a general primate pattern of low milk yield, assumed to have co-evolved with low reproductive rates and slow life histories relative to body size.[46,49,63] Unlike other mammals, which may for ecological reasons feed infrequently and have fatty milk, extant primates keep their infants with them at all times, allow frequent suckling and are therefore adapted to produce relatively modest volumes of energy dilute milk that contains relatively high lactose and low protein and fat fractions.[64]

Among primates there is some variation in the number, position and gross morphology of mammary glands.[65] Some theorists have drawn attention to the unique shape, pubertal development and unusual life-long visibility of the human breast, and hypothesised that a derived, secondary function in signalling reproductive potential has evolved.[66-67] However, these

unusual characteristics can be explained more simply. First, the morphology of the human breast probably reflects an evolved structural modification to accommodate suckling by a snoutless baby (Gillian Bentley, personal communication). Second, the pubertal development of this structure involves complex and presumably costly metabolic processes.[68] Scheduling of investment in these processes before ovarian maturation probably resulted in a selective advantage because it was more efficient than repeated resorption and elaboration of breast tissue between successive pregnancies.

3.2 Maternal metabolic accommodation

Several lines of evidence indicate that lactation places significant metabolic demands on non-human primate mothers and that mechanisms exist to accommodate these demands.[69-73] Captive studies in some species suggest that the energetic costs of lactation are met by a suite of energy-sparing adaptations that include breakdown of tissue stores [74], increases in the efficiency of energy utilisation [75], reductions in physical activity [74, 76], and shared care of infants.[77] Field observations show that intake of high-energy foods [78], overall food energy [79-80] and time allocated to foraging [81-82] increase among lactating females, particularly when forage quality is poor.[83] Evidence that wild non-human primate mothers successfully accommodate the costs of protecting their infants against fluctuations in milk supply and quality when conditions are adverse is scant.[71]

Humans may have evolved additional types of adaptation to reduce the maternal cost of lactation relative to that of other primates. These adaptations include appreciable fat storage during pregnancy, relatively slow infant growth, flexible scheduling of weaning and complementary feeding.

3.3 Maternal fat storage

In humans a number of physiological mechanisms reduce the daily costs of lactation when conditions are favourable. The average woman begins lactation with approximately 125 MJ of additional fat accumulated during pregnancy.[84] Depletion of these reserves has the potential to subsidise the energy costs of lactation by ~118.6 MJ (0.325 MJ/d) in the first year of infant life, equivalent to a significant proportion of the total energy required for milk production when fully breast-feeding into the second year. This storage of fat demands the largest proportion (~71%) of additional energy needed to sustain a healthy pregnancy in non-chronically energy deficient women.[84-86] But it is achieved with only modest alterations in maternal energy flux and metabolic partitioning.[87] Many individuals accommodate a proportion

of the daily energy cost of pregnancy by reductions in basal metabolic rate and physical activity. In such individuals, the average daily costs of pregnancy (~0.7 MJ/d) are low (~8%) in relation to the usual dietary energy intakes and requirements of healthy non-pregnant, non-lactating women (~8.78 MJ/d).

3.4 Slow infant growth

In almost all mammals the immediate post-natal linear growth proceeds at a similar rate as *in utero*. Weight gain is more rapid, at least during the suckling period.[39] Indeed, the weight of the fetus appears to be minimised for a given developmental stage. Although many species reach an advanced state of development *in utero*, deposition of offspring body fat reserves occurs almost exclusively after birth. In comparison to great apes, human neonates are relatively large [88] and fat. [89] Human infants grow more slowly [90], and juveniles grow for longer.[91]

Human infants appear to have low energy requirements in comparison to other primates, probably due to this slower growth. It has been estimated that, depending on age and sex, the average unit body mass requirements of human infants range between 0.34 and 0.38 MJ/kg/d (figures recalculated from references [92-93]) and include estimates for maintenance and activity. Even after controlling for Kleiber's allometric relationship between body size (W) and energy requirement ($E \propto W^{0.75}$), such estimates fall below those for free-living yearling baboons and captive large bodied cercopithecines, and within the range of daily intakes observed in much smaller sized (average 2.27 kg) wild yearling baboons.[94] Observation of *ad libitum* intakes among several species of captive large bodied cercopithecine infants results in estimates of average infant energy requirements in the range 0.837-1.255 MJ/kg/d.[95-96] Altmann's pioneering study of free-living yearling baboons (Papio cynocephalus) estimated their minimum total energy requirements for growth and maintenance at 0.871 MJ/d, or 0.383 MJ/kg/d (data recalculated from various tables in reference 97). At this age eight baboons in the sample were consuming a total 2.251 MJ of energy, of which approximately 40 % (i.e. 0.900 MJ/d) was estimated to come from milk.

3.5 Early and flexible weaning

There is no doubt lactation has remained a key life history component throughout hominid evolution. Current expert opinion based on clinical and epidemiological evidence is that infants have not evolved to make efficient use of other foods before six months.[16] Continued breast-feeding clearly remained a strongly selected component of ancestral maternal strategies, as

evidenced by its powerful anti-infective properties.[98] Today, humans benefit enormously from early exclusive breast-feeding and from partial breast-feeding continued into the third year of life, after which the marginal returns on continued breast-feeding diminish.

Nevertheless, weaning appears to have evolved to occur earlier in humans than evolutionary biologists would predict from our recent primate ancestry and current body size. A comparison of human and great ape life history parameters based on demographic data from hunter-gatherer populations shows that humans are distinctive in having a relatively low adult mortality, late age at first birth, long juvenile (i.e. pre-reproductive) period, long life span and high natural fertility (Table 18-1).

Comparative survival analysis of birth intervals in four hominoid species suggests that humans have the shortest ones. Although birth intervals rarely exceed 4 years in natural fertility human populations, half of all randomly-selected closed birth intervals exceed 4, 5 and 8 years in wild gorillas, chimpanzees and orangutans, respectively.[99] Since fertility ends at similar ages in human and chimpanzee females, human birth intervals are shorter and the "species-typical" rate of offspring production is higher.[100] As a corollary, even among hunter-gatherers human infants are weaned after relatively smaller post-natal weight gain.[101]

Nevertheless, as a species we are particularly good at keeping young alive, despite a well-established inverse relationship between birth interval and child survival in humans that is mediated by breast-feeding.[102-104] Infant and weanling survival is much greater among foragers than among apes, and greater still in non-industrial herding and farming economies.[105-108] Thus, ancestral humans evolved an unusual capacity to reduce the length of exclusive and transitional feeding without increasing mortality.

The scheduling of weaning is also unusually plastic among humans.[62] If suckling is initiated, duration of human lactation ranges from a few hours to more than 5 years, spanning the entire range observed for all other mammals.[109] In contrast to non-human primates, humans wean over a wide range of infant sizes. Anthropologists have sought to explain such flexibility as reflecting an evolved maternal capacity to vary reproduction in relation to ecology [110-112], the availability of alternate caregivers [113], and the specific flux of environmental and social factors influencing tradeoffs among the biocultural costs and benefits of weaning to mothers and infants.[114] Observation in contemporary human societies shows lactation behaviour is sensitive to maternal workload and the availability of cooperative childcare and feeding.[115-116] In sum, not only is the human lactation span comparatively short, but human mothers are clearly adapted to exercise more choice in the patterns and duration of breast-feeding than do other primates.

Table 18-1. Average values for hominid life history parameters

	Adult lifespan (1/M)	Age at maturity (1st birth, yrs)	Age at weaning (yrs)	Period of independent growth, α (yrs)	Weaning wt/adult wt, δ	Annual fecundity, *b* (daughters/yr)	α*b*	αM
Human[a]	32.9	17.3	2.8	14.5	0.21	0.142	2.05	0.44
Orangutan	17.9	14.3	6.0	8.3	0.28	0.060	0.52	0.46
Gorilla	13.9	9.3	3.0	6.3	0.21	0.126	0.79	0.45
Chimpanzee	17.9	13.0	4.8	8.2	0.27	0.087	0.70	0.46

a. Source: reference [100] and references therein. a. Pooled data from Ache and !Kung hunter-gatherers.

3.6 Co-evolution of lactation, weaning and life history

These unusual life history characteristics are of more than passing interest, because they suggest that humans have evolved a phylogenetically distinctive response to the fundamental invariants that predict relations between the life history parameters of extant mammals. [100, 117] Specifically, humans differ from other primates in the way in which we conform to Charnov's model predicting cross-species variation in mammalian life histories.[118-120]

Charnov's model proposes that individual growth rates take the form $dW/dt = AW^{0.75}$, where W is body mass and A is the fundamental parameter referred to as the "production coefficient". A takes a similar dimensionless value within each mammal clade, constrains both the rate of juvenile growth and the rate of maternal investment during gestation and lactation, and so links the life history parameters of individual species. The model divides growth into two periods. In the period from conception to weaning, growth is a function of the mother's size. In the period of independent growth from weaning to maturity (α), however, growth is a function of the individual's own body size.

For a given species, the value of α is selected as an evolutionarily stable strategy that responds to the tradeoff between the fitness benefits of beginning reproducing sooner (i.e. having a greater chance of surviving to reproduce) and the fitness benefits of growing longer (i.e. ability to produce more and/or larger offspring at a larger maternal sizes). Thus, α is a measure of "delayed reproduction", and maturity marks a transition where production previously allocated to growth is reallocated to reproduction. The model predicts that any ecological conditions that decrease adult mortality rate (M) will increase adult lifespan ($1/M$) and select for delayed maturity (i.e. increased α) to reap the fitness benefits of larger size. It also predicts that production subsequently invested in offspring increases with age at maturity,

because it is a function of maternal size (W_α). Thus, the size at which offspring become independent feeders (i.e. are weaned) is proportional to, and constrained by, maternal size.

Several observed cross-species life history correlations fit this model well. First, both α and M vary widely across mammal species, but inversely. In other words, larger species live longer and begin reproducing later, and the product (αM) is approximately invariant. Second, the ratio of size at "independence" to adult body size ($W_o/W_\alpha = \delta$) is approximately constant [55-56]; the relationship is very slightly negative, R= 0.991). In other words, weaning weight is constrained by maternal mass (W_α), and size at which offspring are weaned increases in direct proportion to maternal body size. Third, larger, later-maturing species produce larger but fewer offspring. In other words, annual fecundity (the number of daughters produced per year, b) decreases as age at maturity increases with α, and the product (αb) is another approximate invariant. This can be explained by the observation that size at weaning increases faster with maternal size than does the total production that mothers have available to invest (which scales allometrically, $W^{0.75}$, not proportionally).

Primates have a very low A, averaging less than half of that of other mammals (approximately 0.4 vs 1.0). This accounts for their slower growth, lower fecundity for size, and smaller size at a given age of maturity. The ratio of weanling size to adult size, δ, is approximately 0.33 across both mammals in general and primates in particular, but lower in all great apes, and especially low for humans (Table 18-2). In contrast, αb is approximately 1.7 for mammalian taxa in general and for primates, but is less than 1.0 for other great apes. It is exceptionally high (greater than 2.0) for humans.

Compared to other hominins, therefore, humans appear to wean relatively earlier and at relatively small size, to spend longer in "independent" growth before first reproduction, to reproduce at much higher rates during the child bearing years, and to achieve lower adult mortality and longer lifespan. The selective forces that have resulted in such markedly lower δ, higher b, longer α, lower M, and possibly higher value for A require explanation and are currently the subject of intense debate among evolutionary anthropologists. For community nutritionists, the clustering of these anomalous life history traits suggests that shifts in the patterns of exclusive lactation and transitional feeding were intimately involved in the co-evolution of human reproductive scheduling, care giving and dietary practices.

3.7 Complementary feeding

Figure 18-4 presents a model of the evolved pattern of postnatal child feeding reconstructed from current international feeding recommendations

based on a host of clinical studies.[22-23] Initiation of breast-feeding within an hour of birth followed by a 6 month period of exclusive breast-feeding promote optimal growth and development of healthy newborns. Introduction of "complementary foods" (i.e. nutritionally rich and relatively sterile combinations of foods acquired and processed by care givers and fed only to infants and toddlers) is necessary to support increased daily dietary intake after approximately 6 months of age. Family foods (i.e. raw foods and combinations of foods collected, processed and shared by older juveniles and adults) begin to contribute to total dietary intakes during the second half of infancy. During the period of complementary feeding (CF), which continues at least until the third year of life, breast-milk remains an important, sterile source of nutrients and immune protection but complementary and family foods increasingly contribute to total intake as chewing, swallowing and tasting competencies develop. The frequency of suckling and volume of milk consumed diminish gradually, but the age at weaning (d) is extremely variable and there is no upper age limit at which breastfeeding ceases to be of some benefit to children.

This specialised pattern of transitional feeding is unique among primates, and has other corollaries. Detailed studies of the diets and foraging behaviour of non-human primate weanlings are few, but it is clear that provisioning never approaches the levels observed in human populations.[121] Humans are the only primates that wean juveniles before they can forage independently.[91] The targeting and sharing of high yield, nutrient dense foods that entail high acquisition and processing costs is a specialisation of human foragers [122], as is the use of heat treatments and combination of raw foods in "cuisine".[123-124] We are also unusual in the extent to which we recruit and distribute help among conspecifics, including young child feeding and care.[110,125] Indeed, transitional feeding appears to be fundamentally different. Thus, weaning marks a shift to allo-caregiver support, not feeding independence.

The developmental sequence of changes in the physiological characteristics of young children suggests that early childhood evolved in an environment in which the transition to adult foods occurred over several years.[62] Examples include the development of suckling, swallowing and chewing; mechanical changes related to growth of the jaw, the temporo-mandibular joint and tooth eruption; changes in the expression and ratios of gut enzymes and absorption factors associated with the intestinal mucosa; and changes in immunocompetence and renal function. Flexibility in the duration of transitional feeding was likely made possible during the long gathering and hunting phase of human existence by complementary feeding of foods specially collected and processed for the use of infants and young children. Growing human infants outstrip the maternal supply of nutrients at

about 6 months, and may be able to survive without milk at much younger ages and smaller body sizes than do other infant apes. Nevertheless, there is ample evidence to indicate that continued breast-feeding during the period of complementary feeding would have conferred significant fitness benefits for ancestral mothers and babies.

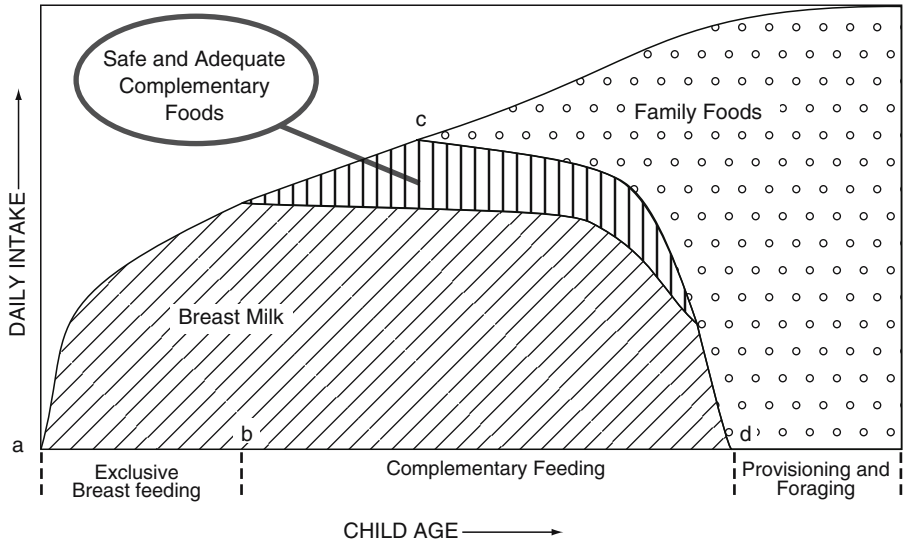

Figure 18-4. Postnatal development of feeding in humans. Key: (a) birth; (b) introduction of complementary foods; (c) introduction of family foods; (d) last suckling. The order of events and phases presented assumes no constraints on maternal and caregiver time allocation or access to food resources and represents an estimate of optimal patterns based on clinical observation. In its general form, we can assume this to be a pattern of care and feeding shaped by natural selection in past environments. Adapted from reference 126.

4. THE COMPLEMENTARY FEEDING HYPOTHESIS

4.1 An evolutionary scenario

How did the behavioural ecology of human lactation come to be so different from that of other mammals, including our closest relatives the apes? We can speculate that the hominid ancestors of modern humans were constrained by similar ecological factors as non-human primates are today.

There would have been a selective advantage to weaning relatively early for body size if the fitness cost to juveniles could have been reduced. There is abundant evidence that human ancestors evolved behavioural strategies for accessing high quality food resources from their habitats. It is plausible to suggest that, some time after the hominid-ape split, the hominids ancestral to humans began to target foraged foods yielding specific key nutrients or to render them more nutritious for weanlings through complex processing. Some of the nutritional constraints on offspring survival and development were thereby relaxed, and new shifts in life history occurred.

First, feeding of older nurslings with nutrient dense, high quality "complementary" foods made possible the reduction of birth intervals without incurring a cost of increased nursling mortality or compromised functional development. Contemporary clinical data showing benefits of improved energy and micronutrient density in the young child's diet suggest the long-term fitness gains for small improvements in ancestral juvenile diets were large. Substitution of self-foraged foods during the juvenile transitional feeding period with complementary foods that more nearly matched infant dietary needs for energy and specific micronutrients therefore provided a dietary mechanism for resolution of the fundamental life history tradeoff between maximising offspring quality or quantity. It resulted in few changes in the composition of breast-milk, and did not diminish the necessity of breast-milk in early infancy.

Second, the behavioural patterns that allowed for the gathering, preparation and sharing of complementary foods for weanlings were "exapted" for the preparation of high quality foods for older, weaned juveniles. Parents who continued to provision offspring for many years after weaning gained a selective advantage. Offspring who evolved to rely on continued feeding of processed, nutrient dense foods rather than on learning to forage raw foods would have benefited from a richer and more reliable food supply during the "independent growth" period, α. Relaxation of ecological pressures to reach adult size quickly selected for extension of a new "childhood" phase in ancestral human development. Continued extension of childhood may have occurred through both later maturation and earlier weaning, and opened a window of opportunity for highly complex social learning.

A third shift in life history may have occurred through consumption of these same foods across the lifespan as "family" foods. Once adults began to prepare and share high quality "family foods" more widely, fitness gains accrued to social group members of all ages.[2] Improvement in maternal diet, and hence birth outcomes and maternal survival, probably produced the strongest selection pressure driving a shift to cooperative foraging and food sharing. Thus, complementary feeding and early weaning triggered and

facilitated the evolution of more general shifts in human foraging, parenting and social behaviour.

The transitional feeding of highly processed foods in addition to breast-milk may be linked to a cluster of derived species characteristics unique to our species that appeared during the last 5-7 M years (Figure 18-5). These include omnivory, social foraging, food processing and sharing, extended childhood, overlapping generations, menopause, fat storage, relatively high reproductive rate and efficient complementary feeding. Life history changes associated with the origin of complementary feeding likely include shorter and more plastic lactation duration, smaller size at weaning, shorter closed birth intervals, and increased weanling survivorship. Other life history changes possibly associated with increasingly efficient complementary feeding probably include shortened exclusive lactation and the evolution of a post-reproductive phase in females.[3]

4.2 Some predictions

This evolutionary scenario is consistent with the patterns of similarity and difference observed for human, non-human primate and mammalian lactation biology. It focuses attention on the fitness benefits of improved nutrition at a critically vulnerable phase in the life history of any primate, the transitional feeding period. It forces us to consider the ways in which the timing and progression to weaning may have been linked to changes in diet and life history among human ancestors. It raises questions about what aspects of the diet are most critical (e.g. which micronutrients) and during what part of the life course the fitness benefits of improved diet would be maximal. It therefore improves upon previous models of the co-evolution of human diet and life history, which too often invoke a rather vaguely defined selective advantage of "improved diet quality".[4]

Drawing upon the salient aspects of this scenario, we can hypothesise that the ability to maintain or increase weanling growth, functional development and survival by substituting moderately processed "adult" foods with nutrient dense, high quality "juvenile" foods was a key adaptive shift among human ancestors. This hypothesis generates several phylogenetic predictions. These are that complementary feeding is: (i) a derived behavioural characteristic, i.e. novel; (ii) universal, i.e. species-typical; (iii) uniquely evolved in the hominin lineage, i.e. a rare adaptation; (iv) recent, i.e. arising since the split with last common ancestor of apes and humans; and (v) co-evolved with diet, life history and "culture". The comparative evidence reviewed suggests these phylogenetic predictions are met.

The "complementary feeding hypothesis" also makes a series of testable mechanistic predictions about the selective advantages of complementary feeding over ancestral alternatives. These are that the behavioural shift to complementary feeding: (i) resolved tradeoffs and conflicts of interest between ancestral infants and their caregivers over the timing of weaning; (ii) increased maternal fertility; (iii) increased offspring survival; (iv) increased development of offspring functional competence; and (v) favoured a complex suite of dietary adaptations among adults and older juveniles. Although it will be difficult to develop indicators of past fitness differentials with which to test these predictions or to find fossil evidence for when complementary feeding began, future work in paleoecology and paleoanthropology has the potential to throw light on the sequencing of steps in the evolution of complementary feeding in relation to other evolutionary shifts.

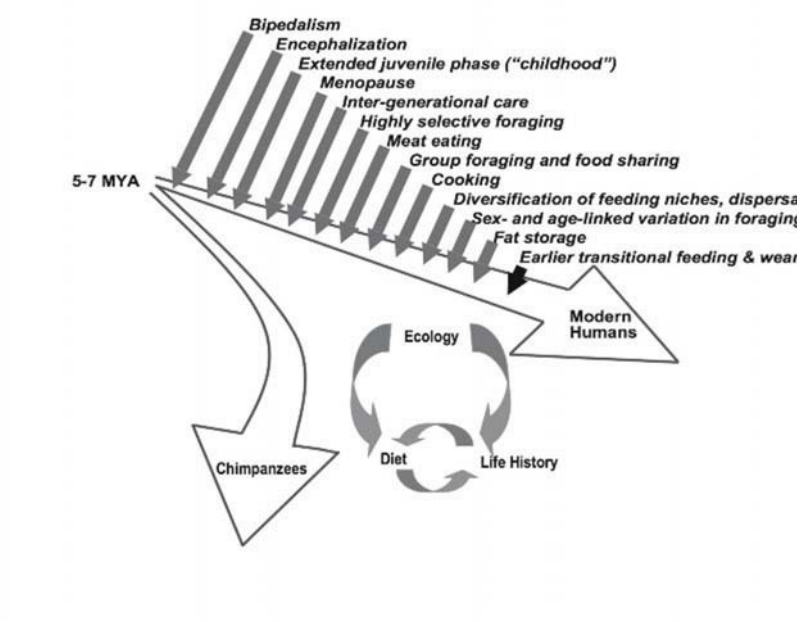

Figure 18-5. Some evolutionarily derived characteristics of human anatomy, behaviour, diet and life history. Any changes in scheduling and fitness consequences of lactation and complementary feeding among hominids must have occurred since the split with the last common ancestor of humans and chimpanzees and co-evolved with significant changes in diet, life history and ecology. Although we know that bipedalism preceded encephalisation, the order and timing of the many evolutionary changes in the human lineage remain poorly understood.

5. RELEVANCE TO CONTEMPORARY ISSUES

5.1 Untimely complementary feeding

The logic of natural selection leads us to assume that in the distant past complementary feeding and flexible weaning did not compromise survival or functional development of the average infant. Indeed, all evidence suggests these behavioural changes were adaptive and sufficiently beneficial to produce lasting changes in human life history scheduling.

In modern contexts, however, a mismatch between actual and recommended young child feeding practices undermines the health of populations. This mismatch results in part from a tendency for families under certain kinds of constraint to introduce other foods to infant's diets sooner than is optimal for maternal and child health outcomes. Why does this happen?

Part of the explanation is to be found in an evolutionary history of increasing behavioural control over the pattern of lactation. This has given contemporary mothers a range of options for minimising the physiological and opportunity costs of lactation. Unfortunately, in some contexts a strong tendency to reduce the frequency of suckling and the duration of lactation may also result in poor health outcomes for child both children and mothers. The evolved flexibility in human lactation patterns and the wide availability of apparently adequate and convenient breast-milk replacements tend to work against efforts to promote exclusive and continued breast-feeding. The greatest degree of mismatch between recommended and actual practices is observed in industrial societies. These are the societies in which the social and opportunity costs of lactation and the safety, convenience and adequacy of complementary foods and breast-milk substitutes such as infant formula are popularly perceived to be highest.

The problem is not peculiar to industrialised societies, however. In noting that humans exhibit an unusually high degree of variation in weaning age, Hartmann and colleagues warned against the assumption that lactation patterns observed in hunter-gatherer and other "traditional" societies reflect evolved, species typical behaviours that optimise the function of the human breast.[42] Indeed, the central tendency for the *earliest* reported age of introduction of liquid and solid non breast-milk foods falls below 6 months for a sample of subsistence societies with natural fertility (Table 18-2). The earliest reported age at weaning falls below 24 months. There are significant problems with the reliability of the data, and it is not possible to ascertain the proportion of individual children fed according to the recommendations in these populations therefore, Nevertheless, we can infer that breast-feeding

patterns do fall short of the current clinical recommendations for many individual children in subsistence societies.

The agricultural and industrial revolutions are recent innovations on an evolutionary timescale. Contrary to popular assumptions, the adoption of agriculture reduced food security, diet quality and general levels of health for a large proportion of the population.[130-131] It also brought radical changes in the ecology of infant care, particularly the opportunity and social costs of maternal time allocation. Mothers in societies that depend mostly on productive subsistence (i.e. various forms of farming and herding) tend to do work that interferes with frequent breast-feeding.[132] Lactation and complementary feeding patterns therefore concord most closely with global recommendations among foraging (i.e. gathering and hunting) societies (Table 18-2). The mean reported minimum duration of breast-feeding exceeds the WHO minimum only in these populations. Across subsistence societies (most of which actually obtain food from a combination of productive and extractive subsistence strategies), the mean duration of breast-feeding increases significantly with the proportional contribution of hunted foods to the diet (Age = 32 +0.346 [% dependence on hunting], $F_{1, 181} = 11.9$, $p= 0.001$).[129] In contrast, caregivers in predominantly farming and herding societies show a tendency towards earlier weaning and untimely introduction of liquid and solid foods (earlier and later than recommended, respectively) (Table 18-2). Nevertheless, the range of variation between subsistence populations in reported child feeding indicators is considerable and these trends are not statistically significant.[129] In summary, untimely complementary feeding is widespread and not easily predicted.

5.2 Promotion of optimal young child feeding

The evolution of complementary feeding and the rise of technology have together rendered human breast-feeding vulnerable to erosion. On the other hand, the fact that our child feeding practices have evolved to be labile should encourage efforts to promote exclusive and continued breast-feeding and safe, timely and adequate complementary feeding. A major challenge for policy makers is how to develop effective methods for promoting such improved young child feeding practices in populations that vary enormously in levels of health risk and other characteristics.[133] The stakes are high because a harsh paradox exists in today's world.[134] While millions survive without ever tasting their mother's milk, many more millions depend on it for a better chance to live. Compared with our foraging ancestors, the differential consequences of infant feeding practices on health and wellbeing signal an increasing divide between the "haves" and the "have-nots" in our species.

In developing country settings, caregivers tend to titrate breast-feeding, complementary feeding and childcare in response to shifts in ecology, subsistence and social environment. Across cultures, underlying attitudes and values about child feeding are often broadly concordant with optimal practice, but caregivers focus more explicitly on tradeoffs between infant/child and maternal/caregiver needs. Cues used to assess the health and development of children and the social and physical constraints on caregivers' ability to provide care or to value investment in children are critical influences on the salient features of young child feeding.[116, 135-137] More often missing are the material conditions conducive for optimal breastfeeding and complementary feeding.

In industrialised nations, the overwhelming evidence that lactation remains a crucial component of a healthy life course for both babies and women who choose to reproduce persuades scientists. Lactation improves life for all, as we can assume it has done for millennia. For some breast-feeding advocates the idea that contemporary humans have the same bodies as those of humans living a foraging existence thousands of years ago is a way to encourage "natural breast-feeding".[138-139] Messages reminding people that mammals are "mothers to us all" are expected to reinforce understandings that breast-feeding is natural and beneficial. Indications that optimal child feeding may have been prevalent in at least a few human societies reassure professionals that they are feasible.[128]

Table 18-2. Comparison of recommended and reported young child feeding indicators for non-industrial populations

Feeding transition:	Clinically recommended age for healthy children (months)[a]	Earliest reported age, months ± SEM (n societies) [b]			
		Pooled	Foragers	Farmers	Herders
Liquid foods introduced	~ 6	3.6 ± 0.9 (18)	- (1)	3.5 ±1.0 (13)	4.7 ±2.6 (4)
Solid foods introduced	~ 6	5.7 ± 0.6 (39)	4.8 ±0.8 (6)	5.5 ±0.8 (29)	7.9 ±2.1 (4)
Weaning from breast	> 24	22.7 ± 0.9 (108)	26.9 ±1.7 (24)	21.1 ±1.1 (72)	23.9 ±2.7 (12)

Sources: a. Reference 127; b. Reanalysis of indicators of age at introduction of complementary foods and termination of breastfeeding from 172 ethnographic and demographic reports published between 1873-1998, following methods in references 128-129.

Beyond the world of lactation researchers and public health practitioners, however, most people think of children as not necessarily needing breast-milk to survive; in many situations they are essentially correct. Many lay people living in such societies now question whether lactation is still useful for humans. It is therefore very important to disseminate new knowledge about the lowered mortality and illness rates among fully breast-fed children in developed and emerging economies. But is also important that this be done in ways that resonate with people's attitude's, beliefs and values and their common sense observations.

The challenge is formidable. For example, there is evidence that use of the "argument from biology" may be counter-productive in the promotion of breast-feeding because some people fear to be stigmatised as "primitive".[139] Breast-feeding is increasingly portrayed in popular culture as a behavioural anachronism that limits opportunities for both mothers and babies, and interferes with their ability to live comfortably in an industrialised and capital-driven society. The breast is no longer regarded as primarily an organ of nutrition, immune defence and fertility regulation. Even some influential pediatricians contend that the technologically fed baby may have more desirable outcomes, rather like a doped athlete. There now exists in most societies a counter-veiling, dominant and popular cultural model that lactation is an artifact of our past, and that breast-feeding, like hunting and gathering, can be improved upon.

To some extent, this kind of shift in cultural thinking is yet another legacy of our success in complementary feeding. The industrial production and commercial marketing of formula companies have played a role in this, but broader cultural forces are at work. Threats to a culture supportive of breast-feeding are ubiquitous and linked with seemingly inevitable shifts in most people's worldview. Such ideas have "gone global" because they reinforce the sense of modernity felt by the growing populations of the world's urban centers. In so far as they contribute to poor health outcomes for infants and mothers, many observers fear such cultural changes may lead inevitably to the erosion of breast-feeding. Everywhere, industrialisation and modernity are associated with the highest prevalence of untimely supplementary feeding and formula feeding. There is no doubt that ongoing changes in culture and technology exert a powerful influence over contemporary patterns of human lactation. The challenge for promotion is not to allow our evolutionary heritage, which has served us well until recently, to undermine the health of our children.

6. CONCLUSIONS

Comparative biology suggests humans have evolved a uniquely flexible strategy for feeding young. Overall, the costs of human lactation seem especially low. The pattern of postnatal feeding is distinctive. Compared to other mammals, humans tend to have a low weaning weight relative to birthweight and individuals of our species are weaned at a wider range of weights and ages relative to adult body size and age at maturity. The use of both complementary foods and family foods appears to be unique to humans. Nevertheless, the three adaptive advantages of lactation (infant immune protection, infant nutrition, maternal fertility regulation) are retained in humans, and there is no cogent evidence for additional derived features of human milk or mammary glands. Humans need breast-milk for optimal growth and development, just like any other mammal.

Complementary feeding is a species-typical, derived behavioural characteristic that evolved uniquely in a branch of the hominid lineage leading to humans. It probably evolved as a facultative strategy for resolving tradeoffs between maternal costs of lactation and risk of poor infant outcomes. Comparative data are consistent with the hypothesis that complementary feeding is an adaptation that increased maternal fertility by accelerating the transition to weaning without decreasing offspring survivorship. It is plausible that complementary feeding of foods specially collected and processed for the use of infants and young children evolved during the long gathering and hunting phase of hominid existence. Among mammals, only humans have evolved the capacity to keep young alive without consumption of any maternal milk. This biocultural innovation now threatens to erode breast-feeding practice below physiologically healthy and previously adaptive thresholds.

NOTES

1. The term “hominid” refers to a clade of bipedal apes, including Australopithecines and members of the genus Homo, some of which may have been ancestral to modern humans. The wider grouping “hominin” includes the great apes and humans.
2. It is interesting to consider the advent of “fast foods” as merely a recent extension of a general evolutionary trend towards widespread preparation and consumption of highly nutrient-dense meals.
3. Provisioning of descendent kin such as daughters and grandchildren could result in higher fitness returns than continued reproduction. This proposal differs from the version of the “grandmother hypothesis” proposed by Hawkes and colleagues.[140]

4. To date, few anthropologists have called for a focus on hominid strategies to garner foods of special value to weanlings (a rare example is Binford's argument regarding scavenged bone marrow.[141]

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Chapter 19

MACY-GYÖRGY PRIZE LECTURE: MY MILKY WAY

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1. LIFE WITH AND WITHOUT SECRETORY IGA

After having been breast-fed for 17 months (as if my mother knew that I was deficient in IgA), I have kept in close contact with maternal milk up into my present advanced age.

After the course in biochemistry during my medical studies in 1955, I was invited to stay on to help with the laboratory course and eventually to do research. The head of the department was Professor O Mellander known for his work defining the major subfractions of casein in human milk. There was also a somewhat senior medical student, Bengt Johansson, who was isolating human milk proteins, including one, which bound iron, now known as lactoferrin. During my subsequent course in bacteriology/immunology I was given the opportunity to stay on as an assistant in the Department of Bacteriology. The head of that department was Professor Ö Ouchterlony, who was known for his double-diffusion-in-gel method permitting separation of the precipitates for each component in an analysed fluid using an antiserum to that fluid. So I used that method to analyse human milk using antisera of my own production since there were no others at that time. It quickly turned out that the patterns obtained became very complex because of the presence of multiple components and the sensitivity of the method. Due to that sensitivity it also turned out that the "purified" proteins

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of that time were quite impure. Thus another more discriminating method was needed.

Five years earlier Professor P Grabar together with CA Williams had published the method of immunoelectrophoresis.[1] So in 1958 I went to Professor Grabar at the Service de Chimie Microbienne at the Pasteur Institute in Paris to learn and work with that method. Immunoelectrophoresis functioned well with human milk, but again it turned out that most preparations of proteins, which seemed pure in the hands of the biochemist turned out to contain numerous components when analysed with this sensitive immunological method. Therefore I developed with the technician C Wadsworth a variant we called comparative immunoelectrophoresis.[2] With this technique it was possible to identify some of the proteins in human milk, such as α-lactalbumin and lactoferrin. It was also obvious that among the immunoglobulins in milk IgG and IgM were very minor components compared to serum. Instead IgA was the predominant immunoglobulin (Figure 19-1a). After absorption of my sheep antisera against milk proteins with human blood serum a precipitate still remained at the site of the milk IgA precipitate (Figure 19-1b).[3] That this additional structure, labelled MS3, really resided in the milk IgA was shown by comparative immunoelectrophoresis (Figure 19-2a). This was the Secretory Component (SC).

The characteristic strong IgA precipitate, containing the MS3 was also present in the IgA that Johansson and I isolated from human colostrum.[4] Using comparative immunoelectrophoresis we also demonstrated that the milk IgA contained two antigenic determinants corresponding to what we now call the kappa and lambda light chains present in all immunoglobulins (Figure 19-2b). All these studies were part of my PhD thesis in 1961, the same year as I completed my MD.[5] But it was a disappointment that the external examiner regarded the presence of a different IgA in milk than serum as "biologically improbable" although nothing could be said to be wrong with the analyses. The committee did not disagree with the statement. Then I left immunology and became a paediatrician, something that I always have been thankful for because it opened another world and ever since I have mainly worked with paediatric problems, using my basic background in immunology.

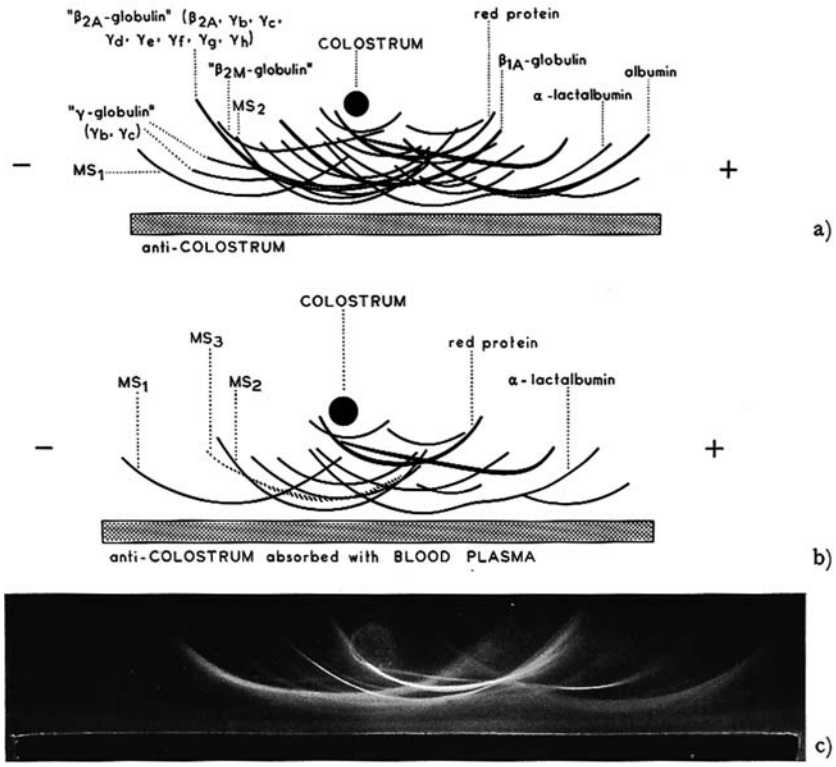

Figure 19-1. a) Immunoelectrophoretic analyses of colostrum using an anti-colostrum antiserum. Some identified proteins are indicated as well as different antigenic determinants identified in the minor immunoglobulins IgG and IgM, and the major milk immunoglobulin IgA. They were at that time still called γ-globulin, β2M-globulin and β2A-globulin. b) The same analysis after the anti-colostrum antiserum made in sheep had been absorbed with human blood plasma. MS = "milk specific". MS3 corresponds to the Secretory Component (SC) and remained at the site of β2A precipitate. c) Photograph of Figure 19-1a, showing the strong β2A precipitate to the left.

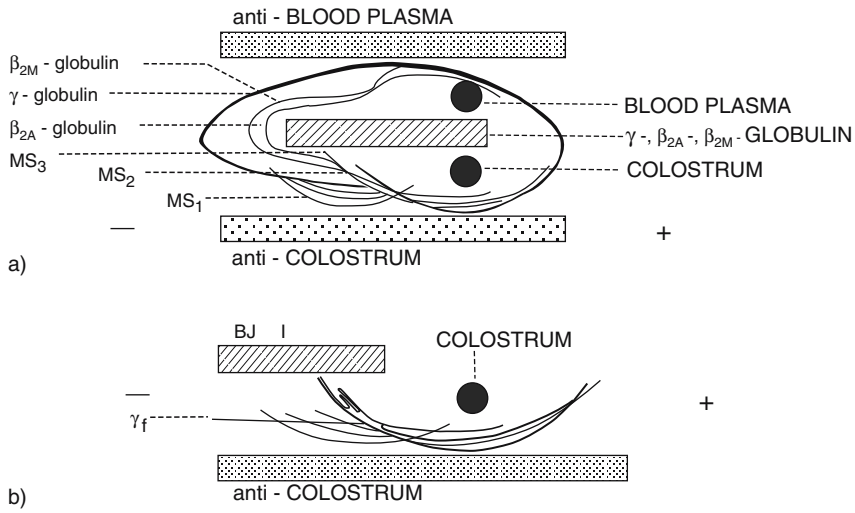

Figure 19-2. a) Comparative immunoelectrophoretic analysis analysing the relationship between the immunoglogulins of serum and colostrum using a preparation of serum immunoglobulins (γ-globulin, β2M- and β2A-globulins) and antisera against blood plasma and colostrum. Whereas the IgG and IgM seemed identical in serum and milk, there was a "spur" on the milk IgA precipitate showing that it contained an extra structure specific for the milk IgA, corresponding to MS3, or SC. b) Similar analysis identifying the κ light chain in SIgA using a Bence-Jones protein I, labelled BJ I in the figure.

Two years later I spent a very stimulating year in Henry Kunkel's laboratory at the Rockefeller Institute in New York. In the ongoing work two of us in the lab, John Rockey and I, used our own sera as controls, but it turned out that both of us had a selective deficiency of IgA.[6] This initiated many subsequent studies to try to explain why some lacking this kind of antibody, which makes up some 80% of all of man's antibodies [7], have obvious symptoms of this immunodeficiency with frequent infections, whereas others do not. The presence of additional deficiencies of IgG2 and IgG3 subclass antibodies gave part of an explanation in some [8, 9], in others we found compensatory increases of other immunoglobulins.[10]

On the other hand immunodeficiencies in mothers, including IgA deficiency, could be used to demonstrate the fact that their newborns at birth already had a weak immunoglobulin production of their own including IgA and IgM, suggesting a very early response partly possibly due to stimulation with anti-antibodies from the mother.[11] The stimulus in the IgA deficient mothers could have been IgG and/or IgM anti-idiotypes; in the

hypogammaglobulinemic mother anti-idiotypes in the IgG given therapeutically. Experimental data supporting this possibility were recently summarised.[12]

2. BREAST-FEEDING IN REALITY

When leaving New York I travelled with my family through USA down to Mexico in 1963. In New York we had met a Mexican Professor of Pediatrics and he invited us to visit his hospital in Mexico City. There I saw for the first time severely undernourished and infected children and that made a lasting impression.

My continued work included on one hand basic studies of milk SIgA antibodies, their specificities, avidities, their appearance in the enteromammaric link, the effects of vaccines etc, using animal models as well as man.[13] On the other hand opportunities to study breast-feeding in developing countries became possible, first with Dr JR Cruz and colleagues from INCAP in Guatemala and later with Professor Fehmida Jalil and her colleagues at the Department of Social and Preventive Paediatrics, King Edward Medical College in Lahore, Pakistan. From the latter cooperation, numerous studies resulted, illustrating how very old traditions still continuing interfered with breast-feeding, including a delay in onset. Thus only about 45% of the village mothers and 65% of the mothers in the periurban slum had started breast-feeding 48 hours after delivery.[14] Pre-lacteal feeds were regularly given and we could show that they were usually heavily contaminated with microbes. In this setting even partial breast-feeding compared to no breast-feeding protected against neonatal septicaemia with a high OR.[15] It was also noted that the habit of giving extra water during the hot season was linked to an increase in the prevalence of diarrhoea.[14, 16] The extra water given was not only unnecessary, but resulted in less breast-feeding and impaired growth.[16]

Actually, the newborn has a poorly functioning complement system and the neutrophils are relatively inefficient.[17] All these functions are needed for the trans-placental IgG antibodies from the mother to defend the neonate efficiently against microbes in blood and tissues. The fact that neonatal septicaemia/meningitis are such dangerous infections may result from this deficient neonatal host defence and illustrates that breast-feeding of the neonate is needed for adequate protection. This provides a very strong argument for breast-feeding: the trans-placental antibodies are just not sufficient for optimal protection of the neonate. The added defence via the milk providing non-inflammatory protection at the mucosal level is also needed. [18]

The very timely paper recently by Labbok *et al.*[19] stresses the many advantages of breast-feeding, a procedure which is much underused today and which could make a great and affordable difference in many societies if it was properly applied. The important defence against neonatal septicaemia/ meningitis via breast-feeding mentioned above is a good example. In some settings optimal use of breast-feeding for the first 6 month will require that politicians and others realise that mothers need much better support from the society and the work place.

It is no easy task to promote breast-feeding, but the fact that breast-feeding in addition to its efficient protection against infections and enhancement of healthy growth also has a strong contraceptive effect [20] made me approach The Holy Father in Rome. Promoting breast-feeding would not only protect many children and provide optimal growth, but also prevent many births in a natural and acceptable way. In 1991, I was given the opportunity to present the background to my proposal of promoting breast-feeding in 10 minutes during the week-long meeting on "Resources and Population" by the Pontifical Academy in the Vatican. However, the Chairman of my session, Professor Max Perutz, fell ill the evening before and I was asked if I could take his full hour. I did and a very active discussion followed showing that the contraceptive and other protective effects of breast-feeding were not familiar to the group. I was also interviewed on Vatican Radio, which transmits in 32 languages. The Pontifical Academy published all the presentations from the "Study week", including my essay. [21] However, to make promotion of breast-feeding via the Vatican take more effect I tried to bring about a meeting there concentrating on the positive aspects of breast-feeding, and also to discuss the possibility of a meeting with the ISRHML there. Finally a meeting came about without my direct doing in 1995 in cooperation between the Royal Society and the Pontifical Academy and some of us in the field summarised the evidence for the advantages of breast-feeding.[22] However, much more work is needed through that and many other channels to have breast-feeding adequately promoted. That should not be difficult considering all the short and long term advantages of breast-feeding, including the remarkable reduction in infant and child morbidity and mortality attainable in poor countries and the many substantial advantages also in developed countries, especially as to the long term positive effects on acute and certain recurrent infections, allergy, obesity etc.[17] In a way it seems strange that we have not been more successful in convincing politicians and society about the advantages of breast-feeding in many poor, as well as well-to-do regions.

The limited success of promotion suggests that other ways must be tried, preferably to indicate how resources for better infant and child health could be found. Since my first visit to Costa Rica in 1980 I had been impressed by

the remarkable standard of that country when it came to health and education compared to other similar countries, including its neighbours. Costa Rica had formally and legally eliminated its army in 1949. Especially through the Head of the National Children's Hospital in San Jose, Dr Edgar Mohs, who was Assistant Minister of Health and Minister of Health for two terms, a very efficient reorganisation of the health care system was introduced. Stress was put on primary health care, breast-feeding promotion, vaccination programs, availability of potable water and sewage control to reach a majority of the population. This had striking effects on health parameters, including decreasing infant mortality levels at that time already below those of Washington DC. Typically the fertility rate came down some years after the reduction in infant mortality, as noted in many areas of the world.[20]

I was very impressed by this example of using our knowledge and reaching such remarkable results by employing available, although scarce, resources efficiently. This example should be made known so I started to propose Costa Rica for the Nobel Peace Prize because of its remarkable example. To use resources not for arms, but for health! When Oscar Arias became President of Costa Rica I proposed him as a good representative of his country, which in democratic order had eliminated the army. After 5 years of campaigning the Nobel Peace Price was awarded to Costa Rica represented by Oscar Arias with the motivation I had given.[23]

I am certainly aware that it may not be possible to eliminate armies, but it should be possible to invest less in arms and more in health in the world. I even think it is necessary if we are at all to manage the enormous problems of the large, poor populations in several countries and provide a liveable future. More arms and wars do not seem to be an answer; rather they seem to increase the risk of terrorism, unrest and misery. Again, the poorest pay the most by poor health, terrible living conditions and scant possibilities of a reasonable life course. One of the free, available and efficient remedies to give children a better start in life, probably with long-term positive effects, is breast-feeding!

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Chapter 20

EXCLUSIVE BREAST-FEEDING AND HIV INFECTION

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1. MOTHER TO CHILD HIV TRANSMISSION

Women can transmit HIV to their infants prenatally, at delivery or postpartum through breast-feeding. Without any interventions, 30-45% of infants of HIV-infected mothers become infected themselves.[1] In many African countries, antiretroviral drugs, usually nevirapine, are becoming increasingly available for mothers and infants at delivery. Although programmatic data on transmission rates in nevirapine-treated populations are only beginning to become available, it appears that transmission rates by 4-6 weeks postpartum are approximately halved [2] and that total transmission after a variety of low cost interventions in several African trials was 24%. [3] This means that the breast-feeding route of transmission to approximately 16% of infants [4] is becoming the major route of mother-to-child transmission in Sub-Saharan Africa. Unfortunately, even in urban areas of low income countries with potential access to delivery antiretrovirals, replacement feeding is associated with high risk of infant morbidity and mortality from other infections.[4-6] This dilemma has caused enormous amounts of discussion among researchers and international agency staff and enormous anxiety and stress among women and health care staff in low income countries.

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2. REDUCTION OF HIV TRANSMISSION ASSOCIATED WITH EXCLUSIVE BREAST-FEEDING

Therefore, there was great relief and hope a few years ago when it was reported from South Africa that exclusive breast-feeding (EBF) appeared to protect infants from postnatal transmission compared with infants mixed-fed with breast-milk and other foods.[7] Since then EBF has been promoted in many places for HIV-infected women who do not have access to safe and nutritionally complete replacement foods [8], even though the South African study was the only evidence. Recently, however, a larger study in Zimbabwe found very similar results.[9] The hazard ratio for HIV transmission between the ages of 6 weeks and 18 months was, compared with EBF, 1.61 (0.72 – 3.64) for predominant breast-feeding (breast-milk plus non-milk liquids), and 2.60 (1.21 – 5.55) for mixed feeding (breast-milk and non-human milk or solids). The study also found that 68% of postnatal transmission occurred after 6 months, suggesting that EBF to 6 months followed by early cessation might be a strategy for reducing postnatal HIV transmission. Both the Zimbabwean and South African studies were designed as randomised controlled trials of vitamin A supplementation and women selected their own feeding mode. The limitations in interpreting the associations of EBF with decreased HIV transmission in such non-randomised studies are well recognised.

2.1 Possible mechanisms whereby EBF could protect against postnatal HIV transmission

Is the relationship between EBF and reduced HIV transmission causal, and, if so, what is the mechanism? Three main mechanisms have been postulated: 1) mixed feeding damages the intestinal barrier permitting breast-milk virus to cross into the infant's circulation; 2) mixed feeding stimulates immune cells and stimulated immune cells are more susceptible to HIV than are resting cells[10]; 3) mixed feeding induces subclinical mastitis in the woman's breasts, resulting in increased viral load in the milk. All of these potential mechanisms were considered in the South African study.

2.1.1 Gut permeability

Permeability of the small intestine can be measured fairly non-invasively by a dual sugar permeability test. The test was carried out in the South African infants at 1, 6, and 14 weeks of age using lactulose and mannitol as the test sugars.[11] The test was sensitive enough to detect increased gut

permeability in infants given no breast-milk but the mixed-fed infants did not have increased permeability at any of the three time points. Admittedly, the lactulose/mannitol test measures permeability to low molecular weight molecules in the small intestine only, which is not necessarily the same route whereby HIV might cross a damaged intestine. Nevertheless, the results do not support the hypothesis that intestinal damage is key and indicate instead that in mixed-fed infants breast-milk is able to protect against relatively modest damage resulting from food antigens.

2.1.2 Immune stimulation

Although presumably it is immune stimulation at the level of the infant gut that is important in breast-milk transmission of HIV, we were unable within the South African study to measure gut immune stimulation non-invasively as was required for sequential investigations in mainly healthy infants. We chose to measure urinary neopterin, an indicator of whole body cellular immune activation [12], in urine samples collected for lactulose/mannitol tests. We found no association between feeding mode and urinary neopterin.[11]

2.1.3 Subclinical mastitis

Over the last few years our research group has investigated subclinical mastitis – its prevalence, causes, and consequences - in several populations around the world. Subclinical mastitis, which we measure as a raised milk sodium/potassium (Na/K) ratio [13], is common in early lactation; bilateral (both breasts) raised Na/K ratio tends to decrease with time postpartum but prevalence of unilateral subclinical mastitis remains high, 12-20%. [13-15] Subclinical mastitis has several causes including local and systemic infection, poor maternal antioxidant micronutrient status, and poor lactation practice.[13] Subclinical mastitis has been associated in all populations in which we have investigated it with poor infant weight gain. Importantly for the present discussion, milk Na/K ratio was correlated with milk HIV RNA viral load among HIV-infected women in both Malawi and South Africa.[14, 16] Although numbers were small, there was a suggestion that subclinical mastitis was associated with increased mother to child HIV transmission.[14, 16]

In the South African study the associations among feeding mode, subclinical mastitis, and milk viral load were complex and varied with time postpartum.[14] Since the results of this study, which was designed for a different purpose, were inconclusive, we set up the Breast-feeding and Postpartum Health study in Zambia specifically to investigate causes and

consequences of subclinical mastitis among women in an HIV-prevalent area.

2.2 Non-causal associations between EBF and reduced HIV transmission

Since the two trials showing reduced HIV transmission with EBF were not randomised controlled trials of feeding mode – and it is well acknowledged that such trials would have almost insurmountable ethical and logistic difficulties – it is possible that the relationship seen is not causal. The most common non-causal mechanism postulated is that women with more advanced HIV disease, usually as indicated by low blood CD4 cell counts, are not only more likely to transmit to their infants [3, 14], but are also more likely not to feel able to EBF. Both the South African and Zimbabwean studies have investigated this hypothesis using their available maternal health data. In South Africa maternal health data was available from recruitment at 28-32 weeks gestation and at delivery; no association was found between maternal health and feeding mode.[7] In Zimbabwe maternal health data considered were CD4 cell count at delivery, severe anemia, and maternal mortality by 18 months, an indication of rapid progression of HIV disease; these were not found to be associated with feeding mode.[9] For both studies the maternal health data were fairly remote in time from when the feeding data was collected.

3. BREAST-FEEDING AND POSTPARTUM HEALTH STUDY

The authors' Breast-feeding and Postpartum Health Study conducted in Lusaka, Zambia, may be able to shed some light on the association between EBF and risk factors for HIV transmission. Study subjects were 198 HIV-infected and 189 HIV-uninfected women from a middle class area of Lusaka where antenatal HIV prevalence was 32%. The clinic and referral hospital are both Baby Friendly and study midwives received additional training through the World Health Organization courses on breast-feeding, and on HIV and infant feeding counselling. Staff strongly supported EBF by all women who chose to breast-feed, which was virtually all women regardless of HIV status. Study subjects received 11 postpartum visits at home or in the clinic by midwives up to 16 weeks postpartum. Any breast-feeding or health problems thus received rapid attention from a trained professional.

Breast-feeding practice in the cohort was very good with 83% of women initiating breast-feeding within an hour of delivery and only two women experiencing onset of full lactation more than 3 days postpartum. Nevertheless, and in spite of support from the project staff, EBF rate, defined as the percent of women feeding breast-milk and nothing else from birth to that study visit, was 94% at day 3 but declined throughout the follow-up to reach 37% by week 16. Interestingly, HIV-infected primiparous women stopped EBF sooner than HIV-infected multiparous women or HIV-uninfected women of any parity. The reason for this is unknown and would benefit from some qualitative research in order that health care staff can understand how best to support this vulnerable group of women.

Figure 20-1 shows the prevalence of unilateral and bilateral subclinical mastitis for HIV-infected and uninfected women at each of the postpartum visits. As we have seen previously in South Africa [13], bilateral subclinical mastitis is common in early lactation and decreases with time, whereas unilateral subclinical mastitis remains common even into full lactation. Compared with HIV-uninfected women, HIV-infected women showed a slower decrease in bilateral subclinical mastitis and persistently higher prevalence of unilateral subclinical mastitis. When milk Na/K ratio was expressed as an average value for each woman across the 11 time points, both maternal HIV infection (P=0.001) and primiparity (P<0.001) were associated with higher mean natural log Na/K ratio.

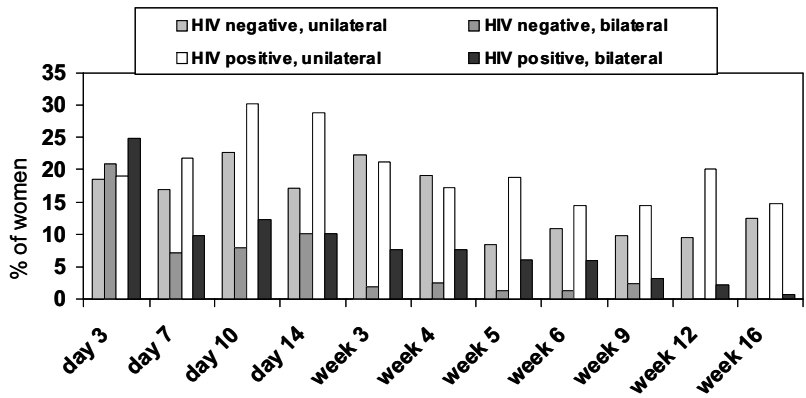

Figure 20-1. Proportion of HIV-infected and uninfected women with unilateral or bilateral milk Na/K ratio > 1.0 at each visit

We collected several indicators of maternal health: reported symptoms at each visit, midwife observations, and plasma acute phase proteins, C-reactive protein and α_1-acid glycoprotein (AGP), at 34 weeks gestation and 6 weeks postpartum. These generally correlated with each other but AGP at 6 weeks proved to be the measure most closely associated with outcomes of interest. Ln AGP at 6 weeks was negatively correlated with duration of EBF ($r=-0.19$, $n=306$, $P=0.001$) and positively correlated with mean overall ln Na/K ratio ($r=0.23$, $n=319$, $P<0.001$) and mean log HIV RNA in the milk aqueous phase at week 6 ($r=0.28$, $n=89$, $P=0.007$). These correlations indicate that postpartum maternal systemic illness was associated with subclinical mastitis, milk viral load, and shorter duration of EBF. Interestingly, similar correlations were not seen with AGP at 34 weeks gestation. In order to detect associations between maternal illness and stopping EBF, it may be necessary to have maternal health data from a time close to when women are making these feeding decisions.

4. CONCLUSIONS

The association between EBF and mother-to-child HIV transmission requires further research in order to determine how to promote optimal feeding practices for HIV-infected women. The Breast-feeding and Postpartum Health Study in Lusaka, because of the extensive maternal health and infant feeding data that are collected from HIV-infected and uninfected women, may be able to provide important insights. Preliminary results suggest that HIV-infected primiparous women are a particularly at risk group and will require clinical and breast-feeding support if they are to breastfeed exclusively and avoid subclinical mastitis with its associated dangers for increased HIV transmission and poor infant growth. Since maternal postpartum systemic illness appears to be an important factor for stopping EBF, subclinical mastitis, and milk HIV viral load, it will be important to determine whether prophylaxis against or improved management of maternal infections can improve infant growth and decrease mother-to-child HIV transmission.

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Chapter 21

BREAST-FEEDING PRACTICES IN RELATION TO HIV IN INDIA

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1. INTRODUCTION

1.1 Role of breast-feeding in infant and child health

The benefits of breast-feeding on infant and child health are both well established and substantial. Breast-feeding provides complete nutrition for the newborn for at least 6 months and can provide a significant proportion of nutritional needs up to 1 year of life.[1] Breast-feeding also confers significant protection against infectious morbidity and mortality from gastrointestinal infections [2,3] lower respiratory infections [4], and otitis media [5] due to the presence of numerous immunological factors in breast-milk. [6] Breast-feeding is especially important in developing countries where use of replacement foods is not advisable due to poor sanitation, unsuitable preparation and prohibitive costs.

Recent studies confirm the impact of breast-feeding on morbidity and mortality. A pooled analysis of 6 studies in developing countries revealed that mortality rates were significantly higher in non-breast-fed infants through the first 8 months of life, with the greatest protection in disadvantaged populations with lower education.[7] Deaths from diarrhea were 10 times higher in infants who were never breast-fed or where breast-feeding

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was stopped.[8] With regard to morbidity in resource poor settings, risks of both diarrheal and acute respiratory illness are associated with type of infant feeding. [9] Infants who were not breast-fed had a 17-fold increased risk of hospitalisation for pneumonia compared to breast-fed infants.

Over the past several decades countries throughout the world have put tremendous effort into promoting breast-feeding within the population. In developing countries, during the 1990s (based on 37 countries with trend data) continued improvements were made in exclusive breast-feeding (see definitions below)[a] for the first four months of life, with rates increasing from 48% to 52%. Estimates show that amongst infants aged six months or younger in the developing world, the prevalence of no breast-feeding was around 5%. The prevalence of continued breast-feeding was 86% and 68% for infants and children aged 6–11 and 12–23 months, respectively.[10]

1.2 Impact of HIV/AIDS on breast-feeding

The gains in breast-feeding rates appear to be threatened by the rising epidemic of HIV/AIDS. Transmission of the human immunodeficiency virus via breast-feeding was first recognised in the mid to late 1980s when evidence emerged that HIV was present in breast-milk [11] and case reports were published of infants who contracted HIV from mothers [12,13] or HIV-positive wet nurses [14] during breast-feeding. At this same time, governments in developed countries recommended that women not breast-feed if they were infected with HIV. The situation was different in resource-poor settings and UNICEF and WHO urged continued support of breast-feeding in settings where infectious diseases were the primary cause of infant deaths, regardless of maternal HIV status.[15]

The most recent estimates indicate the rate of transmission of HIV through breast-feeding to be 8.9 per 100 child years of breast-feeding based

[a] **Exclusive breast-feeding**: The infant has received only breast-milk from his/her mother or a wet nurse, or expressed breast-milk and no other liquids, or solids with the exception of drops or syrups consisting of vitamins, mineral supplements, or medicines. A child may be exclusively breast-fed with expressed human milk from his mother, a breast-milk donor or from a milk bank. **Predominant breast-feeding:** The infant's predominant source of nourishment has been breast-milk. However the infant may also have received water or water-based drinks. **Partial breast-feeding:** Giving a baby some breast-feeds, and some artificial feeds, either milk or cereal, or other food. **Replacement feeding:** The process of feeding a child who is not receiving any breast-milk with a diet that provides all the nutrients the child needs. During the first six months this should be with a suitable breast-milk substitute — commercial formula, or home-prepared formula with micronutrient supplements.

on data from Africa.[16] There are currently a number of on-going studies, primarily in Africa, examining the feasibility and impact of different infant feeding alternatives, such as exclusive breast-feeding and exclusive replacement feeding, on the HIV-free survival of children. There are also several clinical trials currently underway to test the efficacy of anti-retroviral drugs given either to the mother or the child or both during breastfeeding, as a means to prevent postnatal transmission. However, definitive results of these studies are not expected for a few more years.[17]

UNAIDS/WHO/UNICEF currently recommends for HIV-positive women the avoidance of all breast-feeding when replacement feeding is affordable, feasible, acceptable, sustainable and safe. Otherwise exclusive breast-feeding is recommended for the first months of life, followed by early breast-feeding cessation.[18] For the majority of HIV-positive women living in developing countries, it is rare to be able to fulfill the above stated requirement for affordable, feasible and acceptable replacement foods.[19-20] Commercial formula can cost more than $USD100 for 6 months [21] making it out of reach for most families. In addition, the nutritional adequacy of alternative locally available replacement milks is either unknown, or they are found to be substantially lacking in essential nutrients.[22]

The additional risk of promoting breast-milk substitutes within a population is the issue of "spillover". Spillover occurs when women who are HIV-negative or of unknown HIV status switch to breast-milk substitutes because of fear about HIV transmission through breast-milk, reduced efforts to promote breast-feeding by governments and health professionals due to this same fear, and the increased acceptability of breast-milk substitutes when a subpopulation of women begins using artificial feeding. In a study conducted in four African countries (Botswana, Kenya, Namibia and Uganda), authors concluded that there was a major decline in the protection and promotion of breast-feeding resulting from the HIV/AIDS pandemic and mother-to-child HIV transmission (MTCT) policies.[23] Therefore, fear of HIV infection can undermine overall support for breast-feeding and reduce the commitment to breast-feed among women who are not infected.

1.3 Factors influencing mother to child transmission through breast-milk

There are a number of maternal and child factors that can influence the rate of transmission of HIV through breast-milk. Most notable appears to be maternal viral load and T lymphocyte counts. Studies have shown that high maternal viral load measured during pregnancy [24] or after delivery [25] and low CD4/CD8 ratio [26] are associated with an increased rate of transmission through breast-feeding. Therefore, newly infected women as well as those

later along in their infection are most likely to transmit the virus to their child.

Studies also have found that inflammatory conditions such as mastitis, assessed clinically [27] or biologically [28], and breast abscesses [29] increase the risk of mother to child transmission (MTCT) through breast-feeding. Oral lesions or thrush in the baby may increase the risk for transmission as well, due to the greater number of portals of entry for the virus and exposure to blood.[30]

Patterns of breast-feeding are another major contributor to HIV transmission. Recent studies have emphasised the importance of exclusive breastfeeding by demonstrating the detrimental impact of mixed feeding[b] (breast-milk with other milks). Work conducted by Coutsoudis and colleagues in South Africa found comparable risks of transmission between exclusive breast-feeding and exclusive formula-feeding, and that at 3 months exclusive breast-feeding resulted in a 44% reduction of HIV transmission over mixed feeding.[31] Early reports from research in Zimbabwe appear to indicate similar findings, however, confirmation of this result is still needed.

As noted above, breast-feeding is nearly universal in most developing countries; however, exclusive breast-feeding for four to six months is rare. These patterns of infant feeding combined with patterns of infection and health of the mother and child will have a large impact on the rates of MTCT in a population.

2. HIV IN INDIA

In 2003, India had the second highest number of HIV cases in the world with over 5 million HIV-infected individuals.[32] Although the overall HIV prevalence is low (1.0% for ages 15-45), with a population of over one billion, the HIV epidemic in India is expected to have a major impact on the overall spread of HIV in Asia and the Pacific, and worldwide.

There is substantial variation in HIV prevalence across India's 32 states. Based on data collected from over 650 sentinel surveillance sites throughout the country, four states, Maharastra, Tamil Nadu, Andhra Pradesh, and Karnataka, have been identified as high prevalence states.[32] This definition is based on having HIV prevalence rates exceeding 5 percent among high-risk groups and exceeding 1 percent among antenatal women (as a proxy for prevalence in the general population). The other significantly affected areas are two smaller states in the northeast, Manipur and Nagaland, which lie

[b] Mixed feeding is defined here as the consumption of other non-breast-milk products (such as infant formula, other animal milks, water, etc.) in conjunction with breast-feeding.

along major trade routes for illegal drugs and have high rates of injection drug use.

In India, recent surveillance suggests that the epidemic is no longer limited to "high risk" groups such as commercial sex workers and people with sexually transmitted diseases (STDs). In recent years, the HIV epidemic is shifting to the general population of young married women and children, with about 25% of all HIV infections occurring in women, thereby increasing the potential of pediatric HIV infection in the future.[32] In the last 5 years, the percentage of all HIV cases attributed to mother to child transmission has increased nearly tenfold from 0.33% of total cases in 1999 to 2.8% in 2004. Recent statistics indicate that over 130,000 infants have been infected through this route. With an estimated 27 million pregnancies annually, this is expected to lead to several thousand HIV-infected babies being born every year in India.

2.1 Infant feeding patterns in India

Breast-feeding is nearly universal throughout in India. Based on data from the National Family Health Survey, 95 % of all children born during the four years prior to NFHS-1 are breast-fed; the median length of breast-feeding is slightly more than two years.[33] Most women do not immediately initiate breast-feeding after childbirth and many women discard colostrum from the breast. Only 37 % of children who were ever breast-fed received colostrum. Only 26 % of children who were ever breast-fed started breast-feeding within one day of birth. Supplementation with other foods also begins early in most populations, as only one-half of children under four months old were found to receive mother's milk exclusively at the time of the survey. Approximately 70% of children at age 7 and 11 months receive breast-feeding with supplementation.

2.2 Infant feeding patterns of HIV-positive mothers in Pune, Maharastra, India

There is a growing body of work in the area of mother-to-child transmission in India, yet much of it has not been published. The majority of the data presented here are based on research conducted on a population of women attending an urban out-patient antenatal clinic (ANC) of a government run public hospital in Pune, Maharastra, India from 2000-2004. These women were participating in either the pilot phase or the actual trial designed to examine the efficacy of low dose nevirapine given to infants for 6 weeks as a means of reducing maternal to infant HIV transmission.

Most recent estimates of HIV prevalence in this ANC in Pune are 3-4%. The demographic characteristics of HIV-positive women appear to be similar to those of HIV-negative women attending this urban ANC. Nearly all reported being married (99%), most were 25 years or younger (86%), and housewives (83%). However, HIV-positive women tended to have less education compared with their HIV-negative counterparts.[34]

The HIV counselling and testing program is well established in this clinic. During their antenatal clinic visit, small groups of women (8-9) participate in a group education session on HIV using specially designed posters and visuals to aid their understanding. These women then go on to individual counsellors where the materials are reviewed again and the women are given the chance to undergo HIV testing. An extensive post-test counselling process was created so that women receive considerable and repeated counselling if found to be HIV-positive. Counselling on infant feeding choices is conducted at the post-test counselling and again after delivery in the post-partum ward.

Over the four years of this study, we found a significant increase in the percentage of HIV-positive women in our population intending to and ultimately choosing to breast-feed. Intention to breast-feed has risen from 47% in 2000-2 to 87% in 2003-4. The percentage of women who actually initiated breast-feeding rose from 60% in 2000-2 to 88% in 2003-4.[35]

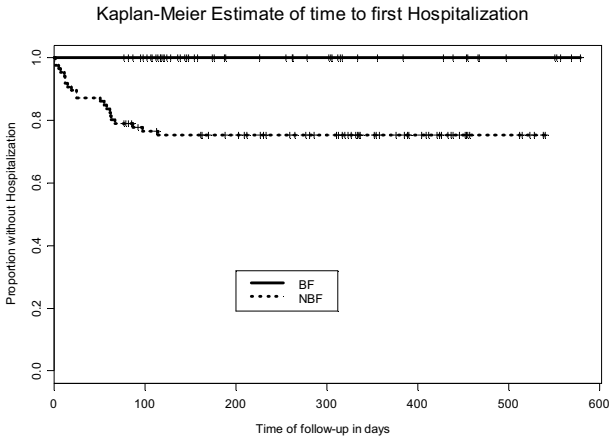

Figure 21-1. Kaplan-Meier estimate of the time to first hospitalisation by infant-feeding practice for infants born to HIV-infected mothers (adapted from Phadke *et al* [36]) BF=breast-fed, NBF=not breast-fed. The difference in the rate of hospitalisation between BF and NBF infants was highly significant ($P < 0.0001$).

We attribute some of this change toward breast-feeding to modifications of the counselling which occurred in late 2002, where counsellors presented

local data detailing the risks of replacement feeding on infant health. Data from an observational study conducted at this hospital documented a substantial increase in hospitalisations in replacement-fed infants compared with their breast-fed counterparts.[36] From a sample of infants born to HIV-positive women, researchers found 27 hospitalisations and 4 deaths occurred in the 86 infants who were replacement fed compared with no hospitalisations and no deaths in the 62 breast-fed infants. The difference in the rate of hospitalisation between replacement and breast-fed infants was highly significant ($P < 0.0001$). The Kaplan-Meier analysis demonstrates a high rate of early postpartum hospitalisation for replacement-fed infants (Figure 21-1). Armed with this information, using an evidence-based counseling approach, counsellors informed their patients of the increased morbidity risks for infants that may result from replacement feeding. This choice continues to be supported with additional data from Pune further documenting a three-fold increase in morbidity especially with pneumonia, acute gastroenteritis and sepsis among infants who are not breast-fed.[37] There is also some evidence that rates of breast-feeding among HIV-positive women are increasing throughout the country.[35]

Throughout India, however, a large number of HIV-positive women choose to replacement feed. Based on data from our sample, despite extensive counselling, preparation of the modified animal milks was extremely variable. The predominant mixture consisted of diluted cow's milk combined with sugar. None of the mothers who replacement-fed their child reported regular use of micronutrient supplements of any kind as recommended by WHO when this option is followed. No woman in our sample reported use of infant formula for her infant because of the prohibitive cost.

Mixed feeding with animal milks was also a common practice among our HIV-positive mothers. Sixty-two percent of HIV-positive mothers and 55% of HIV-negative mothers reported some mixed feeding during the first 14 weeks post partum. A higher proportion of HIV-positive mothers (29%) provided animal milks to their child than found in the HIV-negative group of mothers (17%), especially during the first week after birth. This was found to be directly related to morbidity experienced by the mother in the first week postpartum. After the first week, mixed feeding rates were low in both groups.[35]

There was considerable difficulty expressed by the women regarding cessation of breast-feeding. By six months fewer than 10% of the women reported that they had completely stopped breast-feeding. None of the HIV-negative mothers stopped breast-feeding by 6 months, in keeping with traditional practices.

2.2.1 Biological factors affecting mother to child transmission

The complex of maternal factors affecting mother to child transmission in this population is not well understood. In general, HIV-positive women attending this clinic in Pune appear relatively healthy, are able to engage in sexual activity and subsequently become pregnant. Although the immunological status of the women in our sample is not yet available, preliminary data from another study of 40 HIV-positive women from this clinic demonstrated the median CD4 count to be 400 cells/mm^3 (range 36-1063) and a median plasma viral load of 12,564 copies/ml (560-252,714).[38] Data from West Africa showed that breast-feeding transmission was considerably lower in women with CD4 counts > 500 cells/mm^3[39]; therefore, these Pune data suggest that there may be a substantial portion of Indian women from this clinic where mother-to-child HIV transmission will be low.

Other biological factors of importance include lesions on the breast or in the infant's mouth that would facilitate transmission of the virus. From our sample, few (14%) women reported any breast morbidity during the first 14 weeks, most of which occurred in the first 6 weeks. We found no difference in reported breast morbidity between HIV-positive and HIV-negative women. The most commonly reported symptom was tender breasts and nipples. During the first 14 weeks, only 8% HIV-positive mothers reported cracked nipples, one of which also included bleeding nipples. No clinical assessment of sub-clinical mastitis has been conducted on this population. Likewise, we are not aware of studies examining the rates of oral thrush in infants in this population.

Data collection on the nutritional status of women participating in the trial in Pune is on-going. However, some preliminary data reveal that nutritional deficiencies, especially anaemia, are of concern. Anaemia may occur as a pre-existing condition, as a side effect of medication, or as a result of HIV-related illnesses or HIV itself. Results from an analysis of 430 HIV-positive and 10467 HIV-negative women from this antenatal clinic and delivery room indicate low hemoglobin levels for all women. In general, HIV-positive women had higher rates of anemia, particularly those presenting in labour without prenatal screening.[40]

2.2.2 Socio-cultural factors affecting mother to child transmission

In this urban antenatal clinic of women who recently learned of their HIV-positive status, few had yet experienced substantial stigma and discrimination as result of having HIV. However, a majority stated that there is significant stigma associated with not breast-feeding. There are basically three infant feeding options available for HIV-positive women in this

setting: exclusive breast-feeding and early weaning, use of infant formula, or use of modified animal milks with micronutrient supplementation. Heat-treated expressed breast-milk was once considered as a possible alternative for HIV-positive women. However, this was found not to be feasible because of the enormous difficulty associated with expressing and preparing the milk for consumption, and the stigma associated with not putting the child to the breast. For the majority of women in our sample, exclusive breast-feeding and early weaning was still by far the best option despite the risk of transmission and social pressures to mix feed. This was followed by use of modified animal milks, which was considered difficult because of the cost and stigma associated with the practice of replacement feeding. The least likely option for these women was infant formula, because of the prohibitive cost.

Those women who chose to replacement feed despite these obstacles did so with substantial household support, especially from their husbands.[41] Despite this support and the extensive counselling received, nearly 30% of women who had chosen to replacement feed still ended up partially breast-feeding because of social and familial pressures. It is therefore likely that replacement feeding, without concomitant lactation inhibition, will not drastically reduce rates of HIV transmission.

Stigma and discrimination related to HIV status continue to be problems for the majority of HIV-positive women throughout India who do not have the benefit of participating in a large trial with dedicated staff and resources. Recent pilot data from Mumbai suggests considerable stigma and discrimination faced by HIV-positive women in the health care setting, the community and within their own family.[42] Data from focus group discussions show that stigma, in the form of neglect, refusal of care and pressure to medically terminate their pregnancy were common. Within the family, these women faced rejection, isolation, and blame for having contracted the disease, despite the fact that for the vast majority, the husband was likely the initial carrier. As stigma and discrimination can significantly affect health-seeking patterns, it is critical to address these issues within the health care setting.

In Pune, efforts to improve care of HIV-positive patients and reduce stigma are currently underway. An extensive nurses training program has been initiated within the hospital with plans of expanding to neighboring hospitals and districts. Such efforts, as well as counselling and testing programs that seek to remove the burden of blame from women and encourage greater participation of husbands, are necessary in the battle for adequate care for HIV-positive individuals.

3. CONCLUSIONS

The documented patterns of infant feeding by HIV-positive mothers in Pune, India from 2000-2004, highlight the complexities of making an informed and healthy choice under suboptimal conditions. The local realities prevent access to voluntary counselling and testing, anti-retroviral drugs and safe, adequate infant feeding alternatives for a majority of HIV-positive women in the world. We, therefore, need to identify which infant feeding options under these circumstances would save the most lives, be the most feasible, least costly to society and have the fewest negative effects.

In an attempt to reduce the risk of HIV transmission through the use of replacement milks and without sufficient knowledge of the adequacy of these alternatives, health care providers have put forth suggestions of alternative feeding strategies that appear feasible within the local cultural context. However, over the past five years this has led to a significant proportion of women choosing to replacement feed, placing infants at increased risk of morbidity and mortality and women facing stigma and discrimination because of their feeding choices.

If breast-feeding is the chosen feeding regimen, exclusive breast-feeding for up to 6 months and rapid weaning (cessation) thereafter appears optimal for infant health and reduced risk of HIV transmission. Continued support within the health care setting immediately post-partum for the mother and infant is critical. Given that the strong social pressures to provide infants with fluids such as water, additional research is necessary to identify the differential risks of HIV transmission associated with mixed feeding with water compared with more complex foodstuffs.

Although the HIV prevalence rates are still low in most of India, a mere 1% increase in the HIV prevalence in adults would result in an additional 5 million infected people. Efforts at the national level have expanded tremendously in the past few years to deal with the problem. The antenatal counseling, testing and antiretroviral treatment to prevent MTCT has now been expanded to 225 antenatal clinics, and is the largest national antenatal screening program in the world.[43]

As these efforts continue to expand, it is critical to place additional focus on improving conditions for women. Pregnant women face multiple challenges if diagnosed with HIV. Because of their already low social status, women are the most vulnerable to further ostracism, stigma and discrimination, and violence within their families and the community. These women are often malnourished and suffer from a number of physical and mental stresses. In addition, they feel the burden of responsibility for transmitting the virus directly to their child during pregnancy, delivery or through breast-feeding. Through counselling and support services improving

the health and nutritional status of these women, substantial gains can be made in the reduction of maternal-to-child transmission of HIV in India.

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Chapter 22

BREAST-FEEDING AND HIV-1 INFECTION: MATERNAL HEALTH

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1. INTRODUCTION

Breast-feeding is promoted worldwide for child health and survival, and is entrenched in most cultural practices in developing countries as the natural and expected way of feeding infants. Unfortunately, breast-feeding contributes significantly to mother-to-child transmission (MTCT) of HIV-1.[1] Current recommendations for infant feeding in the context of HIV-1 infections have been based on risks to the infant. Possible risks or benefits to the mother have not been sufficiently explored.

It is expected that most HIV-1 infected women in developing countries will opt to breast-feed their infants due to cultural pressures or lack of affordable replacement feeds. Thus, it is important to understand the impact of breast-feeding on HIV-1-infected women's health.

Maternal health and survival affect child survival. It has been estimated that the risk of a young child dying is increased by 3-8 times if the mother dies.[2] Poor maternal health impacts negatively on the child's health since the mothers are often the primary care-giver. Therefore promotion of maternal health is an important priority both for mother and child.

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2. POSSIBLE INTERACTIONS BETWEEN BREAST-FEEDING AND HIV-1 DISEASE

2.1 Nutrition interactions

Both breast-feeding and HIV-1 infection are characterised by increased nutritional demands that can lead to accelerated HIV-1 disease progression. During breast-feeding, maternal daily energy requirements increase by 600 kcal and daily requirements for other essential nutrients in the diet also increase.[3 4] In addition, HIV infection is characterised by a negative energy balance due to either a high catabolic state or reduced dietary intake at all stages of infection.[5-8] This implies that in order to meet the daily requirements, HIV-1-infected persons need to increase nutrient intake. Daily energy intake should be increased by 10% for asymptomatic and by 20 to 30% in symptomatic HIV-1-infected adults, in order to meet the recommended daily requirements.[9] As immune status deteriorates in HIV-1-infected individuals, other conditions that jeopardise nutrient intake such as anorexia, diarrhea, malabsorption, infections, oropharyngeal lesions and depression set in, and compound malnutrition even further. It is therefore plausible that when breast-feeding and HIV-1 infection co-exist, the overall nutrient requirement would be pushed even higher and could result in severe malnutrition, especially in populations where food is limited. However, to date, direct effects of breast-feeding on HIV-1 disease course have not been clearly illustrated.

2.2 Hormonal interactions

Mothers who breast-feed have elevated serum prolactin reaching up to 10-fold higher concentrations in the early post-partum period. In contrast, women who do not breast-feed have concentrations that return to normal within a few days after delivery.[10 11] Prolactin is an immunomodulatory hormone that may play a role in HIV-1 disease progression, but the direction is not clear.[12-14] It has been hypothesised that although normal physiological levels of prolactin may stimulate lymphocyte activity, too high or too low prolactin concentrations may be immunosuppressive.[14] In a non-pathological state such as breast-feeding, where prolactin is associated with insulin stimulation, increased insulin receptors and an increased appetite, the overall effect may lead to improved nutritional outcome if intakes are adequate.[10] It is not clear if lactation induced hyperprolactinaemia, can exacerbate disease progression in HIV-1 infected individuals, with poor nutritional intake.

Other hormonal changes that occur during breast-feeding include higher serum oxytocin during suckling and a modest increase in thyroid stimulating hormone.[15] The influence of these hormones on HIV-1 infection have not been fully explored.

2.3 Immunological interactions

During breast-feeding the mother produces large amounts of immunologically active cells and substances in breast-milk. These include: lymphocytes, macrophages, secretory IgA, leptin and cytokines. Some of the cytokines which are abundant in breast-milk such as TGFβ and IFNγ are thought to down regulate proliferation of HIV-1.[16 17] It is not clear how these local changes in the breast affect the general immune responses in a breast-feeding mother. However it has been reported that there is concurrent increased immune factors in the peripheral circulation in the early postpartum period which may lead to improved immunological responses.[18-20]

3. PREVIOUS STUDIES

From 2001 to 2004, three studies that sought to determine the effect of breastfeeding on maternal health among HIV-1-infected women were published. The first study was a randomised clinical trial conducted between 1992 and 1998 among HIV-1-seropositive ARV naïve women living in Nairobi Kenya. In this study, although women randomised to breast-feed were comparable to those randomised to formula-feed in terms of their baseline CD4 cell counts and plasma HIV-1 viral load, women in the breast-feeding arm had a 3.2-fold increased risk of mortality in the 2-year postpartum period (95% CI 1.3, 8.1). These women also had a significantly greater decline in weight compared to the formula-feeding women (mean 0.17 versus 0.00 kg/month; p=0.03). These results suggested that breast-feeding might lead to adverse effects on an HIV-1 infected mother.[2] However, this study was primarily designed to determine infant outcomes, and maternal outcomes were assessed as secondary analyses. Thus, data were not available for other indicators of maternal disease progression such as CD4 cell counts, viral levels, morbidity pattern, or even the cause of death.

The second publication described an observational study of a cohort of HIV-1-infected postnatal women in South Africa. This was also a secondary analysis and there were no additional risks of mortality associated with breast-feeding. Mortality was very rare in this cohort, 2/410 (0.5%) among

women who ever breast-fed versus 3/156 (1.9%) among those who never breast-fed. Therefore this study lacked adequate power to demonstrate differences in the two feeding groups. Being an observational study, there could also be a possibility of selection bias.[21]

The third study, conducted in Dar es Salaam, Tanzania was an observational study to determine the association between breast-feeding and disease progression among HIV-1-infected women. This study had the advantage of determining a dose-response relationship between breast-feeding duration and maternal disease progression. Results from this study showed that amount of breast-feeding was not significantly associated with the risk of poor health (defined as anemia, weight loss, CD4 cell decline, or death) in this cohort of HIV-1-infected women.[22]

Overall, there are conflicting results from these studies, which make it difficult to draw conclusions. The South African and the Tanzanian studies did not note adverse effects of breast-feeding and differ from the Kenyan study. One explanation for the observations in the Kenyan study may be that formula-feeding women may have had closer interactions with the clinical team, and as a result they may have received better care and health education than the breastfeeding women. Today, there are several epidemiological and ethical challenges that hinder the conduct of ideal studies that would address the associations between breastfeeding and HIV-1 disease.

4. AN ON-GOING STUDY ON MATERNAL HIV-1 DISEASE PROGRESSION

Our research collaboration between the University of Washington and the Kenyan Research Institutes (University of Nairobi and Kenya Medical Research Institute) have led to the conducting of several mother-infant studies of various kinds. One of our current on-going studies is set up to determine disease progression among HIV-1 infected postnatal women and the effects of breast-feeding on HIV-1 disease progression. HIV-1 positive women willing to live in Nairobi (the capital city of Kenya) for at least 2 years after delivery and who give informed consent are enrolled in the study at around 28 weeks gestation and followed up for 2 years after delivery. Women are given multivitamins and haematinics throughout pregnancy and during lactation. Short course zidovudine is provided to all women for prevention of mother to child transmission of HIV-1 according to the "Thai-CDC" regimen. Women choose their preferred mode of feeding their infants after thorough counselling. Serial blood specimens are taken for measurements of CD4, CD8 cells and HIV-1 viral load. Adverse postnatal outcome is defined as development of severe immunosuppression (CD4 cells

<200 cells/ml) in the postnatal period, or death. In a sub analysis of 186 breast-feeding women who had sufficient time to be followed for 1 year after delivery, the median duration of breast-feeding was 8 months and 53% of women were exclusively breast-feeding their infants at 6 months. Twenty one (11%) women had CD4 cell counts less than 200 cells/ml at baseline. Out of these, 4 women died due to HIV-1 related illnesses. There were no deaths in the first year after delivery among women who were not immunosuppressed at enrolment. Further data collection and analysis are underway (at the time of writing) to determine the effects of breast-feeding on HIV-1 disease progression in this cohort.

5. CONCLUSIONS

Breast-feeding still remains the predominant and important way of feeding infants in developing countries. In the context of HIV-1 infection it is still not clear whether breast-feeding is detrimental to a mother's health or not. Although breast-feeding contributes to almost half of all mother-to-child transmission of HIV-1 in developing countries, most HIV-1-infected women still choose to breast-feed their infants for various reasons. It is not clear whether breast-feeding is protective, safe, or detrimental to the health of HIV-1-infected women. It is plausible that since breast-feeding and HIV-1 infection are both associated with increased nutritional demands and physiological changes, coexistence of the two states may adversely affect the health of HIV-1 infected mothers especially in situations where food intake is limited. However, there is conflicting information on the impact of breast-feeding on the health of an HIV-1- infected woman. It is important to develop strategies to integrate maternal care into prevention of mother-to-child transmission programs. Maternal health affects infant health, therefore, addressing maternal health is important not only for women, but for the whole family and society at large. Further evidence is required before recommendations on infant feeding can be made in relation to the mother's health.

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Chapter 23

EARLY BREAST-FEEDING CESSATION AND INFANT MORTALITY IN LOW-INCOME COUNTRIES

Workshop summary

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1. INTRODUCTION

The importance of breast-feeding for infant and child health and survival in less developed countries has been the subject of a number of studies over the last 25 years.[1-9] However, the epidemic of AIDS in low-income countries, together with the discovery of an important risk of mother-to-child transmission of HIV-1 through breast-milk,[10] has prompted renewed interest in this subject. Indeed, in order to weigh the mortality risks associated with different infant feeding practices against the risk of mother-to-child transmission of the virus, it is essential to have precise estimates of relative risk of death of non-breastfed infants within narrow age intervals.

Available 'decision guides'[11-13] mostly use relative mortality risks published by a WHO working group [14], based on 6 retrospective or prospective studies, among which 3 contributed to risk estimations during infancy (Table 23-1).

The mortality risk was not analysed by mode of breast-feeding (i.e. exclusive, predominant or partial), because such data were not available for all studies included. In addition, exclusive breast-feeding is rare in many

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settings and sample sizes are therefore insufficient to assess mortality risks associated with this mode of feeding. This is unfortunate since a cohort study in South Africa suggested that exclusive breast-feeding up to 3 months postpartum may be associated with a lower risk of postnatal HIV-1 transmission to the child than when other fluids or foods are added.[15]

As for all observational epidemiological studies, those dealing with the association between child mortality and breast-feeding may be subject to a number of methodological problems. The objective of this workshop was to illustrate some of these, using examples from the literature, and mostly consider confounding, reverse causality and bias by indication.

Table 23-1. Odds ratios of death from infectious diseases among non-breast-fed infants, by country and age (adapted from the WHO pooled analysis[14])

Age (mo)	Brazil	Pakistan	Philippines	Pooled
0-1	7.2 (3.3, 16) [a]	21.3 (7.9, 58)	2.5 (1.0, 6.3)	5.8 (3.4, 9.8)
2-3	3.8 (2.3, 6.1)	11.8 (3.1, 45)	5.1 (1.9, 14)	4.1 (2.7, 6.4)
4-5	2.5 (1.4, 4.5)	1.6 (0, 10.3)	2.6 (1.1, 5.8)	2.5 (1.6, 3.9)
6-8	2.4 (1.2, 4.7)	3.5 (0, 27)	1.5 (0.8, 2.6)	1.8 (1.2, 2.8)
9-11	1.9 (0.7, 5.3)	-- [b]	1.2 (0.6, 2.5)	1.4 (0.8, 2.6)
All	3.2 (2.3, 4.2)	7.9 (3.8, 16)	1.9 (1.3, 2.7)	

[a] 95% confidence interval in parentheses

[b] No deaths in age interval

2. CONFOUNDING

Confounding is the phenomenon of a spurious relationship between two factors, which is not causal but rather explained by both factors' association with a third factor. A classical example in breast-feeding epidemiology is that of the association between child or adult 'intelligence' and former breast-feeding for which the mother's educational level is a major confounder. Indeed, there is a strong positive relationship between mother's education and her offspring's performance during intelligence tests. In developed countries, where highly educated women breast-feed more often and for longer, mother's education tends to create a spurious positive relationship between offspring's cognition and breast-feeding. Conversely, in developing countries less educated women breast-feed for longer, thus tending to obscure any existing relationship between breast-feeding and intelligence.

The classical solution to confounding is adjustment, that is, taking into account pre-existing differences between breast-feeding and non-breast-feeding women in multivariate analyses. However, adjustment is virtually always incomplete, due to difficulties in identifying all confounding factors

and in measuring them with precision. Therefore, when a relative risk decreases greatly following adjustment, there is a serious danger that the remaining excess risk is also due to confounding.

The only situation in which confounding may be formally excluded from causing a significant relationship in observational studies is when it actually should *decrease* the strength of a relationship (e.g. mother's education cannot explain a positive association between breast-feeding duration and child intelligence in less developed countries since it is likely to create a negative relationship).

Following similar lines of thought, confounding by socioeconomic status is likely to obscure the relationship between breast-feeding duration and mortality risk in low-income countries. Women living in the city, having some degree of formal education or a professional occupation tend to have children at lower risk of death. This is not because of their lower duration of breast-feeding but is most likely explained by factors such as better child nutrition, immunisation and health care seeking behaviours.

Sanitation, in particular, is associated with both socio-economic status and child survival. A large-scale retrospective study in Malaysia showed that the quality of water supply and toilet facilities modified the relationship between child survival and breast-feeding. In households without piped water and toilets, exclusive as well as any breast-feeding during a given age interval was associated with greatly improved infant survival during the subsequent age interval.[4,16] Conversely, in households with both modern facilities, the benefit associated with exclusive breast-feeding was modest, while that associated with any breast-feeding was close to zero (Figure 23-1).

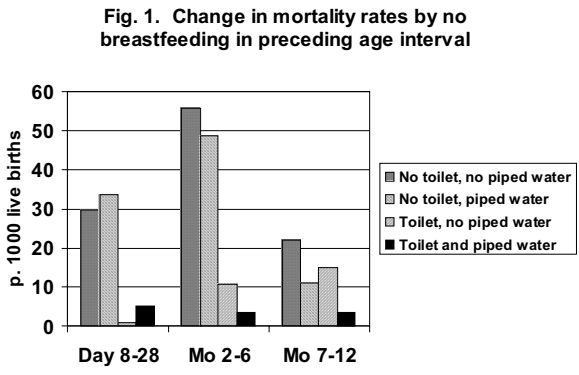

Figure 23-1. Adapted from reference 4.

3. REVERSE CAUSALITY

Reverse causality occurs when one confounds the cause and the effect within a truly causal relationship. In breast-feeding and child mortality studies, it may occur in (badly designed) analyses if dead children are assigned their date of death as date of 'weaning'. Obviously, such children did not die because they were weaned, rather they stopped breast-feeding because they died. An estimation of the bias created by such a definition of date of weaning was enormous: the number of deaths attributable to non-breast-feeding since birth was 74.7 (SD 15) per 1000 live-births using this definition, instead of 16.0 (SD 14) when a proper definition was used.[5]

4. BIAS BY INDICATION

Bias by indication, also known as self-selection bias, is related to reverse causality, but it is more difficult to detect. It occurs when the suspected risk factor, here breast-feeding cessation, is somehow linked to the outcome, here child death. It has long been recognised as a major potential source of bias in studies on breast-feeding and child mortality.

An example was provided by a hospital-based study from Rwanda, Central Africa. Children no longer breast-fed at admission had a much higher risk of death from diarrhea, measles, and respiratory infectious diseases than breast-fed children.[17] However, as argued by others, severely ill children tend to refuse the breast, at least during the final stage of illness, so breast-feeding cessation may not be the cause of death but rather an indicator of the severity of the disease.[18] This classical risk of bias has been avoided in most recent studies by defining breast-feeding status of dead children prior to the onset of the disease leading to death,[6 9] or, if that information was not available, at 7 days prior to death [14] (e.g. some studies included in the WHO pooled analysis).

However, illness may also be a reason for *voluntary* weaning from the breast. A prospective community-based study in peri-urban Guinea-Bissau, West Africa, showed that child illness and maternal illness were both reasons cited by mothers for ceasing breast-feeding (7 and 9% of 945 weaned children, respectively).[19] Furthermore, these children were breast-fed for significantly shorter durations: medians were 19 and 18 months, respectively, compared to 23 months for those weaned because they were 'healthy' or 'ready' ($P<0.001$). Not surprisingly, more children weaned because of their own illness tended to die than children weaned because they were healthy (RR: 2.8, 95% CI: 0.98, 8.3).[20] There also tended to be an excess risk of death among children weaned because of maternal illness or

new pregnancy (RR: 3.1, CI: 0.90, 10, and 2.7, CI: 0.94, 7.9, respectively). The latter became significantly greater when all women who gave birth within 9 months following weaning were considered to wean because of pregnancy, i.e. including those who reported a different reason (RR: 3.3; CI: 1.45-7.3).

These results suggest that the greater mortality risk associated with early weaning in observational studies may be due to 'self-selection' (or rather maternal selection) of weaker subjects more likely to die. Thus, their higher mortality risk would not be explained by weaning but by their frail state, which also led to weaning.

This interpretation was favoured by the authors of a recent analysis of demographic and health study data from 14 developing countries.[21] They found that child or maternal illnesses were common reasons for not initiating or for ceasing breast-feeding, and that child mortality was significantly higher among children weaned for these reasons, compared to those weaned for other reasons. However, children never breast-fed for reasons other than morbidity had low cumulative mortality at 24 months of age (40 per 1000), compared to average infant and under-five mortality rates in sub-Saharan Africa (e.g. 80 and 213 per 1000, respectively, in rural Senegal in the 1990s),[22] and children never breast-fed were a small minority in the samples from which they were taken. These observations are probably explained by high economic status and level of education among women who never breast-feed in Africa and Asia. Thus, whatever the reason these women reported for weaning, a major underlying reason may have been their easy access to formula, clean water, electricity and high quality health care.

An analysis of 1992 DHS data from Malawi used Cox regression to assess infancy and childhood relative mortality risks associated with breast-feeding cessation by reason for cessation.[23] Weaning due to child or maternal illness was associated with a relative infant mortality rate of 4.5 compared to breast-fed infants ($P<0.0001$). Interestingly, children weaned during infancy due to other reasons had even higher mortality risks than those weaned because of child or maternal illness (10.1), despite adjustment for a number of socioeconomic characteristics. This result does not support the Brahmbhatt & Gray hypothesis that pre-existing child and maternal illness is responsible for the higher mortality risk of early weaned children in observational studies.

The technique of defining breast-feeding status as that prevailing prior to the disease leading to death avoids the biases induced both by ill children 'refusing the breast' and by those intentionally weaned because of current illness. However, it is not known whether some mothers may also wean their children early because of *frequent*, though not current, illness. Such bias by indication would not be prevented by defining breast-feeding status prior to

the illness leading to death, neither would the bias induced by mothers weaning early as a consequence of their own illness.

A cohort study in Senegal, West Africa, investigated reasons for prolonging breast-feeding as well as reasons for early weaning in a rural, homogenous population.[24] Since a large majority of mothers (64%) intended to cease breast-feeding at 2 years postpartum, those who weaned earlier than that were asked about their reasons for early weaning while those still breast-feeding beyond that time were asked about reasons for prolonging breast-feeding. Child and maternal illness each accounted for only 2% of early weaning events. The main reasons were that the child ate family food well (60%) or was 'tall and strong' (46%), together with maternal pregnancy (35%). Conversely, the major reasons for prolonging breast-feeding (mean duration: 28.1 months) were that the child was 'little and weak' (33%), currently or frequently ill (24%), or that the family suffered a food shortage (25%).[24]

Thus, it seems that in rural Senegal maternal behaviour tends to select frequently ill or malnourished children for prolonged breast-feeding and well-nourished healthy children for early weaning. Some mothers explained directly that they used prolonged breast-feeding as a strategy to improve child survival. Such behaviour is likely to bias the association between breast-feeding and survival towards an *underestimation* of the benefits of prolonged breast-feeding, particularly in the age range 18-30 months. Indeed, breast-feeding cessation prior to 18 months of age is very seldom found in this setting (4.3%),[24 25] and is mainly explained by mother-centred reasons (e.g. maternal death or migration for labour without the child).[24]

This result is important to bear in mind when interpreting mortality risks of non-breastfed children in the WHO pooled analysis. One African dataset was taken from the same rural Senegalese population as the one studied above and despite the earlier weaning of healthy children, the odds ratio of dying of 20-23-mo-old weaned children was 1.8 (95% CI: 1.1, 3.0).[14] Thus, this excess risk is probably an underestimate.

How can child illness be a cause for both early and delayed weaning in the same population and why do children weaned because of pregnancy tend to die more often? In rural Senegal, child illness caused early weaning either when the child was hospitalised and thus separated from the mother (a rare event), or when he or she was ill with diarrhoea while the mother was pregnant.[24] Indeed, there is a strong belief in this population that a pregnant mother's milk may cause diarrhoea in her suckling child, and this is the main reason reported by pregnant women for stopping breast-feeding earlier than they originally planned to. This belief has also been reported from many other populations.[26-29] Therefore, when pregnant womens' breast-fed children present with diarrhoea, they are usually weaned abruptly despite

their increased dependency on breast-milk during this illness. This behaviour may well be the reason for the increased mortality rate of children weaned during their mother's pregnancy, an increase which has been documented in several studies.[20 30]

If this hypothesis is correct, the technique of defining breast-feeding status prior to onset of the disease leading to death will induce an underestimation of the mortality risk associated with weaning.

5. DISCUSSION

Studies included in the WHO-coordinated pooled analysis of mortality risk associated with not breast-feeding exhibit considerable heterogeneity, particularly for early infancy.[14] Possible reasons for this were discussed among participants in the workshop, including differences in the relative importance of causes of death (some of may be more sensitive to not breast-feeding than others), educational and economic status differences across settings, access to non-human milks, but also maternal motivations for weaning at various ages.

The excess risk of non-breastfed children was particularly high among neonates in Pakistan (RR: 21.3; 95% CI: 7.9, 58, Table 23-1).[14] Similarly, a case-control study controlling for socioeconomic status and age found an odds ratio of 18 for neonatal sepsis in non-breast-fed infants.[31] Reasons for avoidance of breast-feeding were not investigated, so bias by indication cannot be formally ruled out (L.Å. Hanson, personal communication). However, since deaths in the first week of life were excluded from the WHO pooled analysis, it seems unlikely that early infection may have been the reason for non-initiation of breast-feeding in neonates who later died.

Maternal motivations for ceasing breast-feeding are important for the reliability of estimates of the protection against child death provided by breast-milk. Former studies have mainly investigated reasons for ceasing breast-feeding, while reasons for prolonging it have been neglected.[19-21] In Guinea-Bissau, some mothers may well prolong breast-feeding due to child illness although others wean ill children earlier than average, but no data are currently available to test this hypothesis (M.S. Jakobsen, personal communication).

During recent years, high rates of survival of non-breast-fed African infants have been reported from intervention studies aiming at the prevention of mother-to-child transmission of HIV. [32 33] However, these figures arise from capital city mothers receiving free formula and careful instructions in hygiene and in proper preparation of milk. In addition, their children are subject to close monitoring and benefit from prompt free health care.

A study conducted in Nairobi, Kenya, is of particular interest because HIV-positive pregnant women were randomised to formula-feeding or breast-feeding in an attempt to reduce self-selection. There was no significant difference in mortality by 2 years of age between breast-fed and formula-fed children (24 vs. 20%).[32] Unfortunately, such research programmes are too far removed from local conditions to inform public health policies in less developed countries.

Given the serious doubts about the safety of early weaning in poor settings, it would be unethical to monitor health and survival of bottle-fed infants without intervening (with training, medical care, etc). Therefore, despite their obvious methodological limits, retrospective studies of children whose HIV-positive mothers have benefited from free donations of formula in recent loosely-controlled public health interventions may be the only approach which is both feasible and ethical to obtain estimates of relative mortality risk of non-breast-fed infants in sub-Saharan contexts.

6. CONCLUSION

Current estimates of the child mortality risk associated with not breast-feeding are based on limited data, that is, 3 studies for infancy and 6 for the second year of life. Notably, no data could be included for African infants, because of the very low frequency of early breast-feeding cessation on this continent.

The crucial importance of improving the prevention of mother-to-child transmission of HIV-1 in less developed countries justifies a critical questioning of the true benefits of breast-milk in these settings. However, the risk of bias by indication due to early weaning of ill children is greatly reduced by defining breast-feeding status prior to the illness which led to child death, and may even induce an underestimation of relative mortality risk of non-breast-fed infants. In addition, child illness and malnutrition were frequent reasons for delayed weaning in one African setting, providing an important risk of underestimation of relative mortality risks of weaned children during the second year of life.

The interpretation of reported maternal reasons is difficult and the collection of only one reason per child is probably an oversimplification of a complex decision-making process. Also, the analysis of maternal motivations for weaning or not breast-feeding should include an evaluation of the economic, sanitary and educational level of the family.

NOTES

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Chapter 24

MEASURING TRACE IMMUNE FACTORS IN HUMAN MILK

Workshop summary

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1. INTRODUCTION

There is increasing interest in measuring cytokines and other trace immune factors in human milk in order to understand different aspects of the impact of breast-feeding on maternal and infant health. Results generated from different laboratories are often difficult to compare because of variations in methods or lack of sufficient explanation of methods. The research areas using the information are varied and include investigation of how breast-milk protects infants from infection, how it promotes infant gut and immune system development, how breast-feeding may influence the development of allergies, and how breast-milk immune factors protect the breast from infection. Research into milk cytokines is not yet as advanced as research into plasma cytokines and it seems important at this stage to consider how researchers into plasma cytokines have proposed standardising and comparing methods across laboratories [1-4] and to see how their ideas can be extrapolated to the measurement of cytokines and other trace immune factors in breast-milk. In the workshop the various issues of concern in measuring milk immune factors were presented and participants discussed their own successes, failures and suggestions in small working groups. This chapter summarises the issues and provides recommendations for validating and reporting laboratory methods in order to permit better interpretation of results on trace immune factors in human milk.

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2. WHAT METHODS ARE AVAILABLE?

Various different methods for investigating cytokines in milk are possible, depending on the research question being asked. For example, assays can be established to measure cytokine mRNA, intracellular or secreted protein, or bioactivity. It is important to realise that these measure different points of the processes of cytokine production and effect and thus results should not necessarily be expected to correlate well. For example, tumour necrosis factor-α (TNFα) is synthesised as a prohormone which requires processing to make the active form [4] and regulatory processes at this stage may result in lack of correlation between amounts of mRNA and functional protein. TNFα may also be bound to soluble receptors that inhibit its activity [2, 5] so that the amount of protein, measured by e.g. ELISA, will not necessarily correspond to bioactivity. In such cases, although techniques may require greater work-up and validation, the regulatory processes themselves may provide insights into the biological role of the milk cytokine.

In the literature to date, the most frequently used assays for trace immune factors in human milk appear to be ELISA assays of the protein. Researchers into plasma cytokines have considered the problems of conducting and interpreting such assays and have written summaries of how to handle the issues.[1-4] However, breast-milk, probably because of its more complex matrix, appears to be harder to manage than plasma for these assays.

Most researchers use ELISA reagents or kits developed for plasma or other aqueous matrices. In many cases the milk lipid layer interferes with the kit function so that measured levels of cytokine, assay detection limits, or assay variability may be different from those indicated by the kit manufacturer. A frequently used 'solution' to this problem is to centrifuge the milk and assay only the aqueous layer. However, since there is evidence that at least some trace immune factors, e.g. interleukin-10 [6], leptin [7], and transforming growth factor–β [8], are found in the lipid fraction, and that the lipid fraction has important immunoprotective properties [9], this solution should not be used without first ascertaining that the cytokine of interest is actually mainly in the aqueous phase. The compartmentalisation of milk immune factors into the different milk fractions may be important for their function and our understanding of the factors' functions will only progress if we account for the localisation of the factors within the milk.

If whole milk is to be used, methods which have been used to disrupt the lipid layer in order to assay cytokines within it are treatment with bile salts [6, 8] or sonication.[7] Preliminary work is needed to be sure these treatments do not damage the cytokine itself, as the author's laboratory has discovered for bile salt treatment of TNFα. Such treatments may greatly

increase the already often large variability in the assays of milk cytokines.[8] If milk fractions are to be used, it is important to describe in enough detail in publications what fractionation methods were used and what the resulting fractions contained.

For example, not all milk cells can be assumed to be in the cell pellet since milk macrophages in particular are known to often contain enough lipid that they float in the lipid layer [10] or to be in an active state such that they stick to the walls of the sample tube. Furthermore, the cell pellet cannot be assumed to contain only cells since it may also contain other particulate matter.[6] It is important to process milk samples soon after collection especially when wishing to work with the cellular fraction or labile cytokines.

The author's laboratory has some experience, to date entirely unsuccessful, of measuring TNFα in breast-milk from British and Zambian women (unpublished). TNFα appeared to be predominantly in the aqueous fraction. Three different commercial ELISA kits were used (Biosource Europe, Nivelles, Belgium; Neogen, Lexington, KY, USA; eBioscience, San Diego, CA, USA), two of them claiming to measure bound as well as free TNFα, and the standard bioassay based on toxicity towards WEHI 164 cells.[11] Recovery of recombinant human TNFα (Sigma, Poole, UK) spiked into samples was variable and consistently zero for one of the kits. The bioassay, as is frequently the case [2], was more sensitive than the immunoassays and could detect TNFα in all UK milk samples although many of the samples were below the detection limits of the immunoassays. The levels of TNFα measured by the different assays varied 100-fold and, importantly, did not correlate with each other. Note that few laboratories compare several methods for their milk cytokine assays and so will not be aware of how widely results can differ among kits. We decided to use the bioassay for our Zambian milk samples since we determined that it was biological activity that really interested us. Unfortunately the Zambian samples contained a factor, as yet unidentified and not inhibitable with anti-TNFα antibody, which caused rapid death of the WEHI 164 cells so the assay could not be used.

There was discussion at the workshop on whether to use ELISA kits such as in the above work or to develop an in-house method using antibody pairs. Delegates had had good results with both kits, e.g. from ALPCO Diagnostics (Windham, NH, USA) or eBioscience (San Diego, CA, USA), and antibody pairs, e.g. from R&D Systems (Minneapolis, MN, USA), Endogen (Pierce Bioctechnology, Rockford, IL, USA), or Southern Biotech (Birmingham, AL, USA). There is usually a trade-off between cost and time since kits are more expensive but require less assay work-up. However, development of an assay starting with an antibody pair takes time and an experienced

technician, both of which have cost implications. The author's laboratory has had good results for some cytokines with Pelikine compact ELISA kits (Sanquin Reagents, Amsterdam, Netherlands) which provide all reagents except buffers and coated plates and which are a sort of compromise between most commercial kits and starting from scratch with an antibody pair. However, as above, there appears to be no way of knowing *a priori* which type of assay will work for a particular cytokine or research question.

Neither the author nor any delegates present at the workshop appeared to have experience in investigating breast-milk cytokine production by measuring mRNA. Proteomic techniques may prove useful in the future but methods are still being developed.

3. ASSAY DEVELOPMENT AND QUALITY CONTROL

Methods for assay development and quality control are similar to those for any laboratory assay. To determine whether the immune factor of interest can be measured in a breast-milk matrix, known amounts of a cytokine standard can be added to the milk and recovery measured in the assay. To determine whether the milk matrix affects quantitation of the cytokine, the milk sample can be serially diluted and the resulting plot of the concentration against dilution should be parallel to that of the standard dilution. If samples will be frozen and thawed or otherwise manipulated, it is important to determine how these treatments affect the amount of cytokine measured by the assay.

For most cytokines an international standard is available (e.g. from the National Institute of Biological Standards and Control, Potters Bar, UK) and should be used as an external control to validate the assay. A quality control sample should be run with each day's assay or on each ELISA plate. This quality control can be made by aliquotting and freezing a large or pooled milk sample. A new aliquot should be thawed for each day's assay. The interassay coefficient of variation (standard deviation/mean) for the quality control sample results should be presented in publications. Do not be surprised if your coefficient of variation is considerably higher than the one the ELISA kit manufacturer quotes for plasma – that is simply a function of working with human milk.

4. ANALYSIS AND PRESENTATION OF RESULTS

Breast-milk is well known to vary with the time of day or time within a feed that the sample was collected. Therefore, many researchers standardise these aspects of sample collection. An alternative for situations, such as field studies, where this is not feasible and spot milk samples must be used, is to standardise the immune factor data against another component of the milk, such as total protein or potassium, which is fairly constant in the aqueous phase. In addition, work from several laboratories has shown that many milk immune factors vary with milk sodium level, which rises during clinical and subclinical mastitis.[12] Although in some cases controlling for milk sodium may cause differences of importance to the hypothesis to disappear, for other research questions such control may reduce within group variability and permit the differences of interest to be revealed. It may be most informative to present data both per unit volume and standardised against another milk component.

Levels of most cytokine or other trace immune factors in milk, plasma or other fluids are not normally distributed. This is a result of their basic biology in which an initial infectious or traumatic insult will result in a cascade of cytokines in a multiplicative, rather than additive, fashion. If the sample size is large enough, this generally results in a log-normal distribution of cytokine levels and parametric statistics can be used after appropriate transformation of the data. However, in smaller clinical or mechanistic studies (fewer than about 100 samples), the data distribution cannot really be determined and so non-parametric statistics should be used.

When presenting results for log-transformed data, it is usual to present geometric means and 95% confidence intervals. When presenting for non-normally distributed data, it is usual to present medians and 25^{th} and 75^{th} percentiles, either in tables or in standard box plots which will also show outliers. Note that because of the usual distributions of cytokine data, it is rarely appropriate to present arithmetic means and standard deviations.

5. CONCLUSIONS

In order for studies of trace immune factors in milk to progress and, hopefully, eventually result in improvements in understanding and managing care of the breast-feeding mother and her infant, it will be necessary to combine and compare measurements of the factors across different research groups. At the moment this is difficult, not only because many different methods are being used but also because many publications do not provide sufficient details of assay work-up and validation to permit comparisons

between assays. The box summarises the workshop discussions in the form of guidelines that should be considered when developing, conducting and reporting assays for trace immune factors in human milk.

Developing, conducting and reporting assays of trace immune factors in breast-milk

1. Choose an assay type depending on the research question
2. Determine which milk fraction contains the immune factor of interest
3. If using milk fractions, characterise the fractions according to method used to separate them and components obtained
4. Calibrate the assay against an international standard
5. Determine possible interfering factors in the assay
    a. by spiking the milk with the standard and calculating percent recovery
    b. by comparing the plot generated by serially diluting the sample with that generated by diluting the standard
6. Determine the effects of freezing, thawing or other manipulations on the results obtained from milk samples
7. Determine the detection limits of the assay and the inter- and intra-assay variability
8. Consider comparing several different assays to further validate they are measuring true immune factor levels
9. Report methods and validation in sufficient detail in publications

NOTES

By mentioning specific companies providing biological reagents, the author does not guarantee any product.

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Chapter 25

ABSTRACTS OF POSTER PRESENTATIONS

A1. COMPARISON OF VOLUME OF MILK PUMPED FROM THE RIGHT AND LEFT BREASTS IN MOTHERS OF VERY LOW BIRTHWEIGHT INFANTS

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Background: Lactating mothers have anecdotally reported clinical indices such as one breast being consistently fuller than the other, milk flowing more freely, or infants "preferring" one breast that suggest one breast may regularly produce more milk than the other. During breast pumping, mothers also often note different milk volumes from the right and left breasts but these differences have not been studied extensively. This study sought to describe the direction and magnitude of differences between the milk output of the right and left breasts in mothers of VLBW infants who were exclusively pumping and had not yet fed their infants at breast. **Methods**: Thirty-five mothers of VLBW (<1500 g) infants who had established lactation were studied during six complete milk expression sessions. Mothers pumped using a hospital grade, electric, dual pump (Symphony, Medela, Inc.), which was programmed with one of three different suction patterns for each milk expression. The volume of milk obtained from each breast was measured using an electronic scale (±0.1g) to weigh the milk. The differences between the volume pumped from the right and left breasts (right – left = difference) were calculated in ml and as a percentage of the total volume pumped. **Results:** The overall mean difference between the right and left breast volumes was 6.6 ml (SD=12.1), with a maximal difference of 94.2 ml. The difference in volume represented 11.4% of the total volume pumped. Greater volumes were more frequently pumped from the right breast (R>L in 60-69% of the cases, depending on the suction pattern). There was a subgroup of women (n=9, 25.7%) that consistently demonstrated significantly larger volumes (M=19.3 ml; SD=11.6) in the right breast, and one woman (2.9%) that consistently demonstrated larger volumes in the left breast (M=-17.6 ml). **Conclusions:** These findings support clinicians' and mothers' observations that there are often remarkable differences in the volume produced by each breast and, for some mothers, this difference is consistent and large. **Funding:** Medela, Inc

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A2. THE BURDEN OF PUMPING: MOTHERS PROVIDING MILK FOR THEIR VERY PRETERM (<30 WKS) INFANTS

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Background: The birth of an infant is perhaps the most stressful time in a woman's life. When that birth occurs prematurely, the transition to motherhood is cut short. Plans to breastfeed are altered and require a shift from frequent feeding to mechanical milk expression. Current recommendations for mothers delivering prematurely include frequent pumping of at least 8 times per day. **Purpose:** This study examines the maternal burden of milk expression as it relates to time spent per session and per day, as well as, average daily milk volumes attained. **Method:** This is an analysis of data obtained from a multi-site, randomised control trial evaluating the effectiveness of a new hospital-grade electric breast pump (Symphony, Medela, Inc., McHenry, IL). Mothers received early (within 24 hours of birth) and frequent lactation assistance regarding milk expression methods. Subjects recorded frequency of pumping sessions and volume of milk expressed from each breast during the early weeks post delivery. **Results:** 116 mothers of very preterm infants (< 30 wks) maintained daily pumping records during the early postpartum period. Mean ($\pm$ SD) pumpings (n) and duration (min) per day were 4.5 $\pm$ 1 and 79 $\pm$ 27, respectively. Mean ($\pm$ SD) 24-hour milk volume (ml) at 2, 3, 4, and 6 weeks post delivery, 368 $\pm$ 160, 499 $\pm$ 138, 624 $\pm$ 86 and 612 $\pm$ 64, respectively. **Conclusions:** Providing expressed breast-milk for the very preterm infant requires considerable time-dependence upon a mechanical pump. Despite current recommendations of frequent pumping of at least 8 times per day many mothers are successful in maintaining adequate milk volumes on fewer daily pumpings. These data will be useful to clinicians who provide counselling to this vulnerable population.

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A3. HOURLY RATE OF MILK SYNTHESIS IN WOMEN

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Background: Babies breastfeed according to their appetite (demand feeding) consume variable amounts of milk at breastfeeds that occur at irregular intervals during the day. Thus it is necessary to measure every breastfeed over at least a 24-hour period to determine the milk intake of a breastfed baby (i.e. 24-hour milk production). The aim of this study was to determine whether a simpler milk expressing method could be used as an alternative for measuring the 24-hour milk production in lactating women. **Method:** We examined the rate of milk synthesis in 10 women by expressing each breast for 10-15 minutes, every hour for up to 7 hours and recording hourly milk production. These values were compared with the average hourly intake of the baby for each breast determined from 24-hour milk production measurements. **Results:** Repeated measures ANOVA showed that the hourly rates of milk production from the 24- hour milk production measurements were significantly less than the hourly rates recorded between zero and one hour. However, there were no significant differences ($p > 0.05$) between the hourly rates of milk production from the 24- hour milk production for each breast and the hourly rates recorded between 1 and 2, 2 and 3, 3 and 4, 4 and 5, and 5 and 6, hours. The mean (± SEM) hourly rates for the 24-hour milk production were 18.4 ± 2.0 g/h and 15.5 ± 1.7 g/h for the right and left breasts, respectively. The overall mean (± SEM) hourly milk productions for the hourly milk expressions from 2-6 hours were 18.0 ± 0.5 g/h ($n = 38$) and 14.0 ± 0.7 g/h ($n = 47$) for the right and left breasts, respectively. **Conclusion:** These findings demonstrate that when the mothers express their breasts at hourly intervals they produce a relatively constant volume of milk per hour from the second to the fifth hourly intervals and that this was similar to the average volume of milk produced per hour over a 24-hour period of breastfeeding. It is suggested that this is the mother's intrinsic rate of milk production. The measurement of intrinsic milk production may provide an alternative to 24-hour milk productions for the clinical assessment of milk synthesis in both breastfeeding mothers and in mothers expressing their milk for pre-term babies.

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A4. COMFORT AND EFFECTIVENESS OF THE SYMPHONY BREAST PUMP FOR MOTHERS WITH VERY LOW BIRTH WEIGHT (VLBW; <1500G) INFANTS: A RANDOMISED CLINICAL TRIAL

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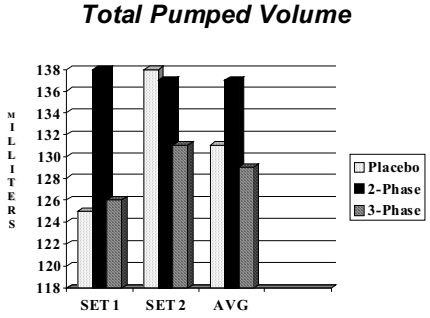
Background: Mothers of VLBW infants must initiate and sustain lactation with a breast pump, and many remain pump-dependent for weeks or months. A pump that is comfortable, effective, and convenient is important for maintaining an adequate milk volume (MV) during the infant's NICU stay. The purposes of this study were to compare the comfort and effectiveness of: the Symphony pump (SP) and the Classic pump (CP), and 3 different suction patterns in the SP for mothers of VLBW infants. **Methods:** 35 mothers initiated lactation with the CP within 48-hrs post-birth and recorded MV separately for the right and left breasts for all milk expressions thereafter. When daily MV totalled 350 ml for 5 consecutive days, women began a 6-day blinded randomisation of 3 suction patterns within the SP. for which they used a pattern once daily in the NICU while an investigator collected data. The CP was used for all other daily milk expressions. Mothers used the 3 patterns in a randomised order before trialling them a second time in a randomised order. One pattern was identical to the CP suction curve, serving as a placebo. The other patterns featured 2 and 3-phase suction curves. Mothers completed a Likert-type questionnaire following each randomisation, rating that pattern and comparing it to the CP. **Results:** Baseline MV and pumping times (mean ± SD) were # pumps per day 5.3±1.5; # min per day 98.4±31.1; 24 hr vol (ml) 639.0±251.8; per pump vol (ml) 126.0±55.9. The figure shows mean pumped MV per pattern. Percent of MV removed at 5, 10, and 15 minutes was statistically similar among the 3 patterns. Questionnaire data revealed mothers preferred the SP to the CP on all measures of comfort and effectiveness, with consistent preferences for the 2-phase curve within the SP. Only 2 mothers reported that the placebo curve in the SP was *similar* to CP. At the study's end, 91.4% of mothers chose either the 2-phase or the 3-phase curve for in-home use. **Conclusions:** The SP performs comparably to the CP with respect to MV removed and rate of milk flow. Mothers preferred the SP to the CP, regardless of pattern in the SP, but preferred the 2-phase to comparison patterns. **Acknowledgement**: Funded by Medela, Inc., McHenry, IL.



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A5. MILK FLOW RATES CAN BE USED TO IDENTIFY MILK EJECTION IN WOMEN

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Background: Milk removal from the breast is highly dependent on milk ejection and as such, milk ejection is a crucial component of human lactation. However, there are currently no methods suitable for the routine assessment of milk ejection in women. Ultrasound is an objective method of determining milk ejection by monitoring acute increases in milk duct diameter but requires an ultrasound machine and a highly trained sonographer. We hypothesised that changes in milk flow rate were associated with changes in milk duct diameter and may therefore provide a technique suitable for the routine, clinical assessment of milk ejection. **Methods:** 23 mothers expressed milk from one breast for a period of 15 minutes on 2-4 separate occasions using an experimental electric breast pump (Medela AG). Breastmilk flow rates were recorded at 5 second intervals by connecting a tube from the breastshield to a bottle placed on a balance connected to a computer. Milk ejection was determined by an acute increase in milk duct diameter in the contra-lateral breast, as monitored by ultrasound (Acuson XP10), and was compared to milk flow rates. All data are mean±SE. **Results:** Mothers expressed 93.6±5.8 g milk (range 10.7-231.2 g). Milk flow rates ranged from 0–4.6 g per five second period. Increases in flow rates were positively associated with increases in duct diameter, as determined by ultrasound ($P<0.05$). Furthermore, within each milk ejection, maximum duct diameters were positively related to greater volumes expressed per 5 second period ($P<0.001$). The number of milk ejections per expression period was the same when determined by either ultrasound (4.5±0.3) or by flow rates (4±0.2). **Conclusions:** These data show a direct relationship between milk ejections (increases in duct diameter) and acute increases in milk flow and that the total number of milk ejections for the expression period was the same for both techniques. Therefore it was concluded that changes in milk flow can be used to identify milk ejection in women. Since, increases in milk flow can be easily monitored in mothers expressing breastmilk using an electric breast pump, we suggest that this technique could be used in a clinical setting to assess milk ejection in women.

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A6. THE EFFECT OF VACUUM ON THE REMOVAL OF MILK USING AN ELECTRIC BREAST PUMP

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Background: Effective breast pumps are very important for mothers of premature babies and those who need to express to increase their milk production. However, the effect of the strength of vacuum on the rate of removal and total yield of breast-milk is unknown. **Methods**: Breastfeeding mothers (21) and expressing mothers (2) measured their 24-h milk production from each breast by test weighing their babies before and after each feed or measuring milk expressed from each breast. In addition, small milk samples were collected immediately before and after milk removal from each breast. The measurement of the fat content of these samples allowed calculation of the storage capacity of the breast and the amount of milk available in the breast when the sample was taken. Using an electric breast pump, on four separate days, the mothers expressed milk for 15 min after the first milk ejection using their strongest comfortable vacuum (A), 75 % of that vacuum (B), –125 mm Hg (C) and –75 mm Hg (D). The milk removed was collected on a continuous weighing balance and milk ejections were monitored using ultrasound. **Results**: The storage capacity of the expressed breast was 178 g (SD 61), and the maximum breastfeed or expression during the 24-h measurement of milk production was 124 g (SD 50). The maximum comfortable vacuum ranged from -98 to -270 mm Hg. There were differences between the vacuums in the total milk expressed, the percentage of available milk removed and the time until 80 % of the milk was removed. **Conclusion:** There was a wide range in maximum comfortable vacuum. Stronger vacuum resulted in expression of more of the available milk, approaching the maximum breastfeed or expression, but rarely removed all of the available milk. Softer vacuums took longer to remove the milk. However, mothers will be unlikely to continue with breast expression if it is uncomfortable. Therefore, some mothers may need to express for longer using softer vacuums.

Test	Vacuum (mm Hg)	Total milk expressed (g)*	% available milk removed*	Number of milk ejections	Time until 80% total milk expressed (min)*
A	-191±56	119±56	70±30	4.3±1.9	6.7±2.9
B	-143±42	91±45	60±25	4.8±2.9	7.6±3.6
C	-125	82±37	45±19	4.6±1.6	8.3±3.6
D	-75	74±47	39±18	4.4±2.3	10.0±2.6

Mean ± SD. Differences between vacuums * $P<0.0001$

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A7. ULTRASOUND IMAGING OF THE EFFECT OF FRENULOTOMY ON BREASTFEEDING INFANTS WITH ANKYLOGLOSSIA

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Background: Infants with ankyloglossia have an abnormally short lingual frenulum. Ankyloglossia has been associated with breastfeeding difficulties such as attachment difficulties, sore nipples and poor infant weight gain leading to early weaning. Treatment for ankyloglossia is frenulotomy (clipping of the frenulum). The aim of this study was to determine the changes in the sucking dynamics of infants with ankyloglossia post frenulotomy. **Methods:** Infants for this study were referred to a paediatric surgeon for frenulotomy by health professionals. Sub-mental ultrasound scans (ACUSON XP10) of the oral cavity of 11 infants were performed during a breastfeed both before and at least 7 days after frenulotomy. In addition, the milk intake of the infants during the breastfeed was measured by the test weigh method. **Results:** The distance from the tip of the nipple to the hard soft palate junction (N-HSPJ) was significantly greater ($P<0.05$) before frenulotomy (7.99 ± 2.80 mm) than after frenulotomy (6.49 ± 1.87 mm). In addition, infants prior to frenulotomy displayed an accentuated upward curve of the posterior tongue with a disorganised piston-like motion. These features resolved in 73% of infants 7 days after frenulotomy. The rate of milk transfer during a breastfeeding increased significantly from 3.3 ± 3.6 mL/min before frenulotomy to 7.2 ± 5.1 mL/min after frenulotomy ($P=0.031$). Furthermore all women reported an increased comfort level during breastfeeding. **Conclusions**: In this study ultrasound imaging has demonstrated that frenulotomy performed on infants with ankyloglossia changed the sucking mechanics of the infant during breastfeeding. The sucking mechanics of infants post frenulotomy more closely resembled that observed in normal breastfeeding infants. In addition, the infants became more efficient in removing milk from the breast and all infants continued to breastfeed as mothers experienced less pain. Ultrasound is useful in assessing the contribution of ankyloglossia to breastfeeding difficulties.

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A8. ULTRASOUND IMAGING OF THE SUCKING MECHANICS OF THE BREASTFEEDING INFANT

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Background: A recent study has found the main milk ducts in the breasts of lactating women to be small (1-2 mm), superficial, compressible and branch without evidence of lactiferous sinuses. Since it is believed that during breastfeeding the infant 'strips' the milk from the lactiferous sinuses the aim of this study was to reinvestigate milk removal by the infant. **Methods:** Sub-mental ultrasound (Acuson, XP10) scans of the interior of the mouth of eight fully breastfed infants (1-16 weeks) were performed during a breastfeed. Intra-oral pressures were measured simultaneously via a milk–filled supply line (SNS) connected to a pressure transducer. **Results:** When the infant's tongue was lowered (down position) milk ducts in the nipple and milk flow into the oral cavity were observed. As the tongue was raised (up position) neither milk ducts in the nipple nor milk flow were observed. Peak vacuum occurred when the tongue was down and was associated with milk flow. Nipple diameter was greater in the tongue down position (9.6±1.1mm) compared to the tongue up position (6.4 ±0.1mm; $P<0.01$). The distance from the tip of the nipple to the hard/soft palate junction (N-HSPJ) was greater when the tongue was up (6.8 ± 1.5mm) compared to when it was down, (5.6 ± 1.5mm; $P<0.01$). The maximum distance of the tongue from the hard palate was 4.2±1.2mm (range 2.6-6.7mm) and coincided with peak negative intra-oral pressure (range 146 – 288 mm Hg). Stronger vacuums resulted in a shorter N-HSPJ, wider nipple diameter and greater downward movement of the tongue ($P<0.05$). **Conclusion:** Negative pressure generated by the infant as the tongue moved down resulted in opening of milk ducts in the nipple and milk flow from the breast. Therefore, we conclude it is the negative pressure exerted by the infant during sucking that plays a major role in milk removal from the breast rather than a stripping action by the tongue.

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A9. THE USE OF UTRASOUND TO CHARACTERISE MILK EJECTION IN WOMEN USING AN ELECTRIC BREAST PUMP

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Background: Stimulation of milk ejection is critical to release milk from the breast during breastfeeding or expression of breast-milk. Ultrasound imaging has identified multiple milk ejections during breastfeeding as acute increases in milk duct diameter. The pattern of milk ejections during a pumping session in relation to milk removal has not yet been investigated. Therefore, the aims of this study were to use ultrasound to investigate the milk ejection responses of the milk ducts to two markedly different vacuum patterns of an electric breast pump, and determine if there is a relationship between changes in duct diameter and milk flow. Methods: For 11 mothers, one breast was expressed for 10 minutes with an electric breast pump, while one milk duct of the other breast was scanned. Two different vacuum patterns Classic (47 cycles/min) and 3-Phase (pre-milk ejection, 120 cycles/minute and expression phases A for 2 minutes post-milk ejection, 20 cycles/minute and B 2-10 minutes post-milk ejection, range 55-78 cycles/minute) were tested, and the milk expressed was collected in 30-second intervals. **Results:** Multiple milk ejections were detected in 95% of the expression periods and were associated with increases in milk flow. The number of milk ejections during the expression period did not correlate with the total volume of milk removed for either pattern or the percentage of available milk removed. The volume of milk expressed during the first milk ejection represented 41.3 ± 21.5 % and 46.7 ± 26.6 % of the total volume of milk expressed for the Classic and 3-Phase patterns, respectively. **Conclusions**: Multiple milk ejections occur during a 10 minute expression period and are associated with acute increases in milk flow. However, the number of milk ejections did not relate to the volume of milk expressed as previously reported for breastfeeding. Furthermore, almost half of the total volume of milk expressed was removed during the first milk ejection. Milk ejection characteristics and volumes of milk expressed did not change in response to different expression patterns therefore other factors such as nipple/breast anatomy should be considered to optimise milk removal during pumping.

Milk ejection characteristics and volumes of milk expressed during a 10 minute expression period with an electric breast using two markedly different expression patterns.

	Classic Pattern	3-Phase Pattern
Increase in milk duct diameter (%)	33.80 ± 5.67	36.58 ± 5.98
Number of milk ejections	3.27 ± 2.05	3.72 ± 1.19
Time to milk ejection (s)	123.8 ± 68.5	84.7 ± 54.5
Total volume of milk expressed (mL)	89.5 ± 9.3	95.2 ± 13.2
% of available milk expressed	49.1± 5.6	52.7 ± 6.0
Volume milk expressed first milk ejection	38.4 ± 20.4	42.1 ± 25.3
% of available milk expressed first milk ejection	21.9 ± 3.96	23.87 ± 4.37

Classic Pattern: 47 cycles/min, 3-Phase Pattern: pre-milk ejection, 120 cycles/min; Expression phase A 20 cycles/min 2 min post-milk ejection, and B 55-78 cycles/min 2-10 min post-milk ejection

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A10. ULTRASOUND IMAGING OF THE SUCKING MECHANICS OF INFANT FEEDING FROM THE BREAST AND AN EXPERIMENTAL TEAT

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Background: The aim of this study was to compare the infant sucking mechanism during feeding from both the breast and via an experimental teat whose design is based on the anatomy of the human nipple. **Methods:** Sub-mental ultrasound (Acuson, XP10) scans of the interior of the mouth of seven fully breastfed infants (1-16 weeks) were performed during a breastfeed and during a feed of expressed breast-milk using an experimental teat (solid silicone containing four milk channels). Intra-oral pressures were measured simultaneously via a milk–filled supply line (SNS) connected to a pressure transducer. **Results:** During feeding from both the breast and the experimental teat, milk flow and peak negative intra-oral pressures were observed when the infant's tongue was lowered (tongue down). In addition, stronger vacuums were positively related to nipple/teat diameter and tongue movement ($P<0.05$). The distance from the nipple to the hard/soft palate junction (N-HSPJ) was greater when the tongue was up compared to down during feeding from the breast. There were no significant changes in the position or diameter of the experimental teat. There was significantly more expansion of the mother's nipple compared to the experimental teat when the tongue was down. **Conclusions:** Milk flow from both the breast and the experimental teat was associated with peak negative pressure during infant sucking. Infants used a similar sucking mechanism to remove breast-milk from the experimental teat as the breast. The smaller variation in the teat N-HSPJ and diameter with tongue movement may reflect the physical characteristics of the experimental teat.

Intra-oral pressure and ultrasound measurements for infants monitored during a breastfeed and feed with an experimental teat (mean ± SD)

	Breast		Experimental Teat	
	Tongue up	Tongue down	Tongue up	Tongue down
Pressure (mmHg)	–68.2 ± 24.9	–204.6 ± 69.5	–71.9± 32.5	–170.1± 68.6
N-HSPJ (mm)	6.8 ± 1.6	5.6 ± 1.6*	6.3 ± 1.7	6.0 ± 1.0
Nipple diameter (mm)	6.5 ±0.08	9.4±1.1*	6.2±0.6	6.8±1.0
Tongue movement (mm)		4.3±1.3		4.7±1.1

N-HSPJ - distance of the tip of the nipple to the hard/soft palate junction. *Significant difference between the tongue up and tongue down position ($P<0.01$).

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A11. MATERNAL MILK VOLUME (MMV) AND MILK TRANSFER (MT) FOR LOW BIRTH WEIGHT (LBW; <2500G) INFANTS IN ECUADOR: COMPARISON OF ELECTRIC BREAST PUMP AND HAND EXPRESSION

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Background: In developing countries LBW infants often do not receive exclusive mothers' milk feedings during the early weeks post-birth due to inadequate MMV. Our previous studies have suggested that MMV is higher for electric breast pump use than for hand expression in this population. The purpose of this study was to compare MMV, MT, and dose of mothers' milk (% of total enteral feedings = mothers' milk) received during the hospital stay for electric breast pump and hand expression groups. **Methods**: Women were randomly assigned to use the electric pump (Lactina, Medela, Inc, USA; n =7) or hand expression (n = 9). MMV was quantified for all daily in-hospital milk expressions. Infants were fed their mothers' milk when available and formula when MMV was inadequate. Dose of mothers' milk was calculated for each infant by dividing the total ml of mothers' milk received by the combined volume of formula + mothers' milk. After breastfeeding was initiated, MT was measured by test-weights (BabyWeigh Scale, Medela, Inc). All comparisons were performed with Mann-Whitney U tests. **Results**: See Table. **Conclusions:** Our findings reveal significantly greater MMV and dose of mothers' milk for the electric breast pump group, and are consistent with our previous findings from Nigeria and Kenya. MT was clinically inadequate for both groups at both time points, and is consistent with findings suggesting a vulnerable period for adequate MT in premature infants. Our study is limited due to inadequate in-hospital refrigeration space, so our MMV measure represents the portion of daily MMV that mothers expressed during visits to the hospital rather than a true 24-hour MMV measure. **Acknowledgement**: This project was partially supported by Medela, Inc., McHenry, IL, USA, and the Rush University Medical Student Research Fund.

Table (mean ± SD)

Measure	Electric Pump	Hand Express	P
Birthweight	1261 ± 93	1314 ± 210	NS
Gestational Age	33.7 ± 2.4	34.3 ± 1.9	NS
MMV (ml)	87.9 ± 34.8	47.3 ± 20.4	.016
Dose of Mothers' Milk (%)	70.8 ± 18.7	40.5 ± 18.1	.008
Mean MT for hospital stay (ml)	9.0 ± 5.5	8.4 ± 2.6	NS
MT day of hospital discharge (ml)	9.0 ± 4.6	12.6 ± 9.4	NS

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A12. FORMATIVE RESEARCH TO DEVELOP APPROPRIATE INFANT FEEDING RECOMMENDATIONS FOR HIV POSITIVE WOMEN IN SOUTH-WEST NIGERIA

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Background: The discovery that HIV can be transmitted through breastmilk threatens to reverse historic gains in child health afforded by breastfeeding, including reduced incidence of acute diarrhoeal diseases; acute respiratory infections, childhood malnutrition and an overall reduction in infant morbidity and mortality. With the aim of formulating infant feeding recommendations for HIV infected mothers, while at the same time minimising "spill over" of artificial feeding to infants of HIV uninfected women and women of unknown HIV status, the specific objectives of the study were to examine a) cultural norms about infant feeding; b) knowledge about HIV and MTCT; and c) acceptability, affordability and safety of replacement feeding options. **Methods:** This study was conducted in a semi-urban area of Nigeria (Ile-Ife). The study used qualitative and quantitative approaches. For the qualitative component, 6 focus group discussions were conducted with groups of mothers, fathers and grandmothers. Qualitative data were analysed using the Text Based Beta software. For the quantitative component, a survey was conducted with 120 fathers and 120 mothers with children between 0-12 months. Subjects were selected using a multi-stage sampling technique. Data analysis was by SPSS. **Results:** All participants agreed that newborns must be breast-fed, however, exclusive breastfeeding rate was 51.8%. General knowledge about HIV/AIDS was high but knowledge about MTCT was low. 83.3% of mothers do not think an HIV positive mother should breast feed her baby. While 50% of fathers say a man will support his wife if she is HIV positive and does not breast feed, 35% say he will reject her. Although Infant formula was considered expensive, it was mentioned as the safest way of feeding infants of HIV positive mothers. Wet nursing was considered an option by 18.3% of fathers and 49.2% of mothers. Most respondents had never heard about giving goat, camel and sheep milk to infants, a few would give these milks to their infants if told to do so in the hospital. Heat-treating breast-milk is believed not to be practicable. **Conclusion:** Based on our results, short exclusive breastfeeding followed by abrupt cessation and replacement with infant formula or home-prepared cow's milk appears to be the best option for feeding infants of HIV positive women. HIV positive mothers will however require education, social and financial support to purchase infant formula or the home-prepared milks.

Travel award: T Abiona was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A13. IMPACT OF PEER COUNSELLING ON EXCLUSIVE BREASTFEEDING AMONG LOW-INCOME, INNER-CITY USA WOMEN: A RANDOMISED CONTROLLED TRIAL

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Background: Breastfeeding support and education is increasingly being provided by public health and other health workers to pregnant and nursing mothers in the USA. In spite of this breastfeeding initiation rate, exclusive breastfeeding (EBF) and general breastfeeding (BF) duration still remain low among low-income groups, which are over represented by minority communities. **Methods:** Pregnant women attending the prenatal clinic at Hartford Hospital, Hartford, CT and intending to breastfeed their infant were recruited and randomly assigned to receive either EBF peer counselling (PC) support, pre- (3 times), peri- (as long as mother-infant pair remained hospitalised) and postnatally (9 times: 3 in week 1; 2 in week 2; and 1 per week from weeks 3 to 6) or to a control group (CG) where they received only the routine BF support provided by the hospital staff. Two PCs who had successfully breastfed were trained by a certified lactation consultant following the WHO/UNICEF 40 hrs BF counselling training course and the Hispanic Health Council BF training manual. Mother-infant pairs were followed via phone interviews weekly in the first month pp and biweekly thereafter until the infant was 3 months old. **Results:** Baseline socio-demographic characteristics were similar in both groups except that 82% of PCs compared to 61% of CGs were of Hispanic origin ($p=0.003$), and 38% of PC compared to 21% of CG spoke only Spanish ($p=0.009$). At hospital discharge 90% in PC compared to 76% in CG had initiated BF, with the percent EBF being 56% and 46%, respectively ($p=0.068$). At 1 month pp, 32% in PC and 8% in CG EBF during the previous 24 h ($p<0.001$). At 3 months the corresponding EBF figures were 26% and 3%, respectively ($p<0.001$). Likewise, the percent EBF throughout 3 months was significantly higher ($p<0.001$) for PC (25%) than CG (3%). Women in PC (53%) were more likely than their counterparts in CG (33%) to remain amenorrheic at 3 months pp ($p=0.026$). **Conclusion**: Our results indicate that timely and well-structured BF support provided by community based peer counsellors is effective at improving EBF rates among low-income, inner-city women in the USA.

Travel award: AK Anderson was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A14. MATERNAL OBESITY AND BREASTFEEDING SUCCESS IN DANISH WOMEN

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Background: In American populations in which the rate of initiation of breastfeeding (BF) and the duration of BF do not meet public health goals, maternal prepregnant overweight (BMI = 25.0 - 29.9 kg/m^2) and obesity (BMI ≥ 30.0 kg/m^2) are associated with poor lactational performance. It is not known if these associations exist in populations, such as that in Denmark, where BF is nearly universal. **Methods:** The subjects were 9490 mother-infant dyads from the Danish National Birth Cohort who ever breastfed (> 98%). In this prospective, observational study, we used logistic regression, adjusted for potentially confounding variables, to investigate the association between maternal prepregnant BMI and successful BF initiation. Successful initiation was defined as still BF 5 d postpartum. We used Poisson regression, adjusted for potentially confounding variables, to investigate the association of prepregnant BMI and discontinuation of exclusive BF (EBF) and any BF (ABF). **Results:** By 6 mo postpartum, > 98% of women had terminated EBF and 33% had terminated ABF. Among those who had terminated EBF, the mean duration was 15.0 wk. Among those who had terminated ABF, the mean duration was 14.7 wk. Overweight [odds ratio (OR) = 1.5, $P < 0.01$] and obese (OR=1.8, $P < 0.01$) women were less successful at initiating lactation than normal-weight women (BMI = 18.5 - 24.9 kg/m^2). We found higher rates of EBF discontinuation among women who were overweight [relative risk (RR) = 1.11, $P < 0.0001$] or obese (RR = 1.16, $P < 0.001$) compared to normal-weight women. We also found higher rates of ABF discontinuation among women who were overweight (RR = 1.15, $P < 0.005$) or obese (RR = 1.41, $P < 0.0001$) compared to normal-weight women. **Conclusions:** These results suggest that excess maternal prepregnant BMI, even in a population of women who are very successful breastfeeders, may reduce the duration of BF. **Supported by** NIH training grant HD07331, Hatch grant NYC399405 and The Danish National Research Foundation.

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A15. ECONOMIC ANALYSES OF THE COSTS AND SAVINGS OF BREASTFEEDING PEER COUNSELLING WITHIN THE WIC PROGRAM

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Background: Breastfeeding peer counselling, while more common in developing countries, is not widely practiced in the United States. This, in part, may be due to the absence of published, US-based economic analyses evaluating the costs and potential savings of implementing peer counselling models. **Methods:** In order to evaluate the potential costs and savings associated with inclusion of breastfeeding peer counselling as a routine benefit in the Special Supplemental Nutrition program for Women, Infants and Children (WIC), we used data from 3 sources: a) our previous randomised trial evaluating an inner-city US-based peer counselling program; b) post-rebate food package costs from the WIC program; and c) estimated postnatal medical savings attributable to increased breastfeeding rates. The costs included in these analyses include salaries for peer counsellors and postpartum lactation consultants (LC) (1 LC/7000 breastfeeding initiators). In these analyses, peer counsellors provide "hands-on" support during the perinatal hospitalisation and phone/office support in the prenatal and postpartum periods. **Results:** Our findings suggest that 75% of pregnant WIC clients are considering breastfeeding (BF), and that BF peer counsellors would increase WIC's breastfeeding initiation rate from the current level of 57% to 69%. The savings attributable to implementing the peer counselling program include the medical cost savings associated with a 7.8% decrease in the incidence of otitis media within the first 6 months postpartum ($34 million), and savings in WIC food package costs in the first 3 months postpartum ($12.6 million). Options are presented for 3 peer counselling service packages: a break-even package ($33/client), a universal coverage package ($40/client) and a minimal cost package ($24/client). All options reflect the cost per WIC client stating a prenatal interest in breastfeeding. **Conclusions:** These results have major implications for WIC's breastfeeding promotion efforts.

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A16. FACTORS AFFECTING THE DURATION OF EXCLUSIVE BREASTFEEDING AMONG HIV-INFECTED AND UNINFECTED WOMEN IN LUSAKA, ZAMBIA

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Background: Exclusive breastfeeding (EBF) is associated with decreased risk of mother-to-child HIV transmission compared with mixed feeding of breast-milk and other foods. Further investigation into why women stop EBF before the recommended 6 months is needed in order to support EBF, especially by HIV-infected women. **Methods:** Women (177 HIV-infected, 177 HIV-uninfected) from Lusaka, Zambia were visited regularly from 34 weeks gestation to 16 weeks postpartum for collection of information on their and their infant's health, infant growth, and infant feeding practices. Maternal haemoglobin was measured at 34 weeks gestation and day 3, day 7 and week 6 postpartum, and acute phase proteins at 34 weeks gestation and 6 weeks postpartum. **Results:** Two thirds of the women breastfed their infants within 30 minutes of birth and only 2 women had their milk 'come in' later than 3 days postpartum. 94% exclusively breastfed on day 3 and 37% at week 16. In univariate analyses, factors associated with earlier cessation of EBF were primiparity, low haemoglobin at 6 weeks, high $_1$-acid glycoprotein (AGP) at 6 weeks, lower infant weight at 6 and 16 weeks and lower infant length at 6 weeks. In multivariate analyses the factors with significant associations were primiparity, AGP at 6 weeks, and infant length at 6 weeks. **Conclusion:** Even with strong support for EBF and in the face of epidemic levels of HIV, women may stop EBF early because of poor maternal health or poor infant growth. Programmes supporting EBF may need to include support for maternal health also. The impact of such postpartum maternal health support on the rates of EBF and mother-to-child HIV transmission requires further investigation. **Funding:** The Wellcome Trust

Travel award: M Chisenga was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A17. SIMPLE ANTENATAL EDUCATION TO IMPROVE BREASTFEEDING PRACTICE: A RANDOMISED CONTROLLED TRIAL

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Background Breastfeeding rates in Singapore are among the lowest in Asia. This study investigates if simple antenatal education can improve breastfeeding practice. **Methods** 310 women were recruited in the National University Hospital after 36 weeks of pregnancy and were randomised into three groups antenatally. Group A received a booklet about the benefits and techniques of breastfeeding, watched an educational video on breastfeeding, and had one session with a lactation counsellor who taught them nipple preparation and breast-milk expression techniques. Group B received the breastfeeding booklet and watched the video only. Group C, the control group, did not receive any form of special intervention before delivery. Routine antenatal, intrapartum and postnatal care were given thereafter. Breastfeeding data were collected by questionnaires administered at 2 and 6 weeks postpartum. **Results** At six weeks postpartum, exclusive breastfeeding was practised by 28.4%, 23.5% and 17.7% of mothers in Groups A, B and C, respectively. Partial breastfeeding was practised by 42%, 48% and 42.5% of mothers in Groups A, B and C, respectively. 29.6%, 28.6% and 39.8% of mothers did not breastfeed at all in Groups A, B and C, respectively. Comparing Groups A and C, Group A was twice as likely to breastfeed exclusively ($p=0.045$, OR 2.16, 95% CI 1.01 – 4.63). Comparing Groups B and C, there was a trend towards exclusive breastfeeding for Group B even though this was not statistically significant ($p=0.113$, OR 1.85, 95% CI 0.86 – 3.96). **Conclusion** A simple one-encounter form of antenatal intervention significantly improved breastfeeding practice at six weeks postpartum. Healthcare workers should bear this in mind when taking care of pregnant women. Efforts should be made to educate and prepare mothers for breastfeeding *before* they deliver.

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A18. THE INVESTIGATION OF BREASTFEEDING IN NORWAY IN THE YEAR 2000. COMPARABLE NATIONAL SURVEYS OF FEEDING ROUTINES IN NORWEGIAN MATERNITIES, CARRIED OUT IN 1973, 1982, 1991 AND 2000

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Aims: To monitor changes over time in routines related to infant feeding in maternity ward on a national level, as perceived by staff responsible. **Background and methods:** In 1973, 1982, 1991 and 2000, similar questionnaires were sent to all Norwegian maternity wards asking for information about "common practice" in specific areas, such as scheduled vs. unrestricted feeding, weighing of the baby, the use of supplements, time mother and baby spent together. The respondents were usually the head midwife or nurse, and we had a response rate of 100 every time. Over the 28 years' study period we had to make *some* changes because of unforeseen developments but we were careful lest comparability should be compromised. **Results:** The study period coincided with a period of unprecedented increase in the incidence of breastfeeding in Norway. This was accompanied or paralleled by considerable changes in feeding routines. Some examples:

- In 1973 58% of the wards let mothers and babies spent less than five hours together daily while 3% let them spend 24 hrs. together. In 2000 the picture had changed: 83% spent 24 hrs together, and only three maternity units restricted the time together to less than sixteen hours daily.
- In 1973 92% of the wards practices scheduled feeding times, and one third *never* allowed any deviation from the schedule.
- In 1993 the picture was totally changed; from this time, mothers were allowed to breastfeed whenever they so decided.

The use of supplements has changed considerably over time. Most notably, the use of sucrose or glucose water, which in 1982 was used by 90% of the wards, was only used by 7% in 2000. Homemade cow's milk mixtures were used by 41% of the units in1973 but had gone totally out of use in 1991. Infant formula is the most frequently used substitute "when needed".
Reflections: Data available showing trends in breastfeeding from 1858 up to today indicate that from the end of the 1920s, when the feeding of human babies with human milk could be substituted with that of another species, the cow, a large-scale shift in feeding pattern occurred. In Norway as in most other countries in the world the change happened gradually. The mothers did not wish for this change away from breastfeeding, but feeding advice and routines inevitably resulted in inability to keep up the lactation process. Changes back to "normalcy" began to occur in Norway when maternity ward routines changed, at the same time as mothers made known their wishes and views concerning the lactation process. **Conclusions:** The changes in breastfeeding rates in Norway were accompanied by radical changes in maternity ward routines over the same period, as documented in this study. It also reveals willingness on the part of responsible health workers to make changes in the ward routines as requested by maternal reasoning and global recommendations alike. **Supported by** the Norwegian Board of Health, Oslo, Norway.

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A19. RATES OF EXCLUSIVE BREASTFEEDING SINCE BIRTH IN UGANDA

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Introduction: Exclusive breastfeeding is recommended as the best feeding alternative for infants up to six months and its benefits have been widely demonstrated. Demographic Health Survey (DHS) for Uganda 2000-2001 based on 24-hour recall only, stated that breastfeeding in Uganda is universal, and that two in three children younger than six months of age are exclusively breastfed. We studied infant feeding in Uganda and looked at breastfeeding rates based on food history since birth. **Method:** A cross-sectional survey on infant feeding practices was performed in Mbale District, Eastern Uganda 2003. We interviewed 763 randomly selected care-takers - infant pairs, and 727 mothers were analysed on feeding patterns. A questionnaire containing 24-hour recall and retrospective questions on selected food items was used. Data analysis was done with SPSS. Three categories were made based on WHO's definitions: 1) Exclusive breastfeeding 2) Predominant breastfeeding and 3) Complementary feeding. A life-table analysis was made for each category. Termination was introduction of a feed breaking up exclusive breastfeeding into predominantly or complementary fed infants. For the exclusively breastfed no feeds had been introduced. **Results:** Prelacteal feeding was given to 57% of the infants; 52% got water-based drinks and 5% got milk or semi-solid food. The exclusive breastfeeding rate was as follows; three days after birth it was 0.44, by one month 0.20, by two months 0.12, by three months 0.07, by four months 0.03, and less than 0.005 by six months. Complementary feeding: The cumulative proportion not introducing any feeds by three days is 0.94, by one month is 0.68, by two months 0.46, by three months 0.30, by four months 0.14 and 0.03 by six months. **Discussion and conclusions:** The discrepancy in our numbers compared to the DHS-data might partly be due to us not allowing infants who have received prelacteals into the exclusive breastfed group and partly because our retrospective questions in addition to the 24-hour recall gives less optimistic estimates.

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A20. DETERMINANTS OF EARLY WEANING AMONG INFANTS HOSPITALISED IN THE FEDERAL DISTRICT, BRAZIL

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Background In consideration of the high association of premature weaning with infectious diseases, we chose to investigate possible determinants of early weaning among infants less than one year of age. **Methods** From April 2002 to February 2003, a cross-sectional study that examined infants admitted to public hospitals in the Federal District of Brazil was performed. Analysis of a proportional and representative sample from all eleven hospitals was conducted. Its basis was the number of babies admitted (≤ 360 d) in every hospital during the year 2000. This information was made available by the Federal District Health Bureau. Information on socio-economic factors and behavioural, breastfeeding, and weaning practices was compiled using a questionnaire containing open and closed questions. A total of 303 mothers were interviewed. Chi-square test was used to compare proportions. Logistic regression was used to determine the risk factors involved in early weaning. **Results** Of the hospitalised infants, 55% were boys and 45% were girls. When divided by weaning age (under 180 days), a higher proportion of boys (59%) compared with girls was seen (p=0.06). Factors associated with high risk of premature weaning were: caesarean delivery, smoking, and mother's perception of breast-milk insufficiency. **Conclusions** We have shown an unexpected tendency of high proportion of hospitalised male babies with early weaning which may be linked to lack of access to human milk protecting factors. Strategies for breastfeeding counselling should focus on mothers of baby boys and their breast-milk demands as well as to the reduction of the risk factors associated with premature weaning in the Federal District, Brazil.

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A21. IMPACT OF A LESS INTENSIVE PEER COUNSELLING METHOD ON EXCLUSIVE BREASTFEEDING PRACTICES IN RURAL BANGLADESH

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Background: Breastfeeding until two years of age is almost universal in many south Asian countries, but the rate of exclusive breastfeeding (EBF) remains low. In our previous study in urban Dhaka with individual peer counselling significantly increased the rate of exclusive breastfeeding, but was labour intensive. We therefore tested, whether a less intensive group counselling would be equally effective. **Methods:** The study was done in Anowara, a rural thana in Chittagong district, southeast part of Bangladesh during the years 2000 to 2001. Of 12 unions, three were randomised to either a) individual counselling at home, b) group counselling or c) no counselling (control). Volunteer peer counsellors were recruited in each area and trained for 40- hour utilising WHO/UNICEF Breastfeeding Counselling Module. Counselling occurred twice during the last trimester of pregnancy, three times in the first fortnight after delivery and monthly for five months. Trained research assistants collected data on infant's feeding status, disease morbidity, anthropometry every months. Data were entered and analysed utilising SPSS PC statistical package. **Results:** 329 mother/infant pairs were enrolled of which 114 were in individual, 109 in groups and 106 in the control. At 6 months, the rate of EBF in these three groups were 89%, 81%, and 11% respectively for individual, group and control group ($p<0.001$). Mothers in the intervention groups initiated breastfeeding earlier than control mothers (12.1±15.4 vs. 6.2± 12.0 h, $p<0.01$) and more likely to fed colostrum as first food (48%, 29% and 12% respectively for Individual, Group and Control respectively $p=0.001$). There were fewer episodes of diarrhoea, ARI and ear infections in the intervention groups compared to control (($p<0.02$). Peer counselling methods can be scaled up and be incorporated in national programs to improve child survival.

Travel award: I Kabir was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A22. AN INTRODUCTION TO THE SECOND INFANT FEEDING PRACTICES STUDY

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Background: In 1993-1994, the Food and Drug Administration (FDA) conducted the first Infant Feeding Practices Study (IFPS), a longitudinal study of US mother-infant pairs about infant feeding behaviours. Many infant feeding practices have changed over the past decade, including the availability of new infant formula, increased breastfeeding rates, and possible increased intake of dietary supplements by infants and mothers; thus there is a strong need to understand these and other changes and to examine the determinants of current infant feeding practice in the context of this new environment. Therefore, the FDA in collaboration with Centers for Disease Control and Prevention (CDC), the National Institutes of Health, and Office of Women's Health will conduct a second IFPS (IFPS-II) to provide new data to describe infant feeding practices as well as to evaluate the National Breastfeeding Awareness Campaign (NBAC). **Methods:** Similar to the first IFPS, the IFPS-II will draw its sample from a consumer mail panel of 500,000 households throughout the US. In contrast to the first IFPS, the IFPS-II will over-sample lower educated, African American, and Hispanic women as well as those living in the NBAC's Community Demonstration Project areas. After being identified as eligible for the study, mother-infant pairs will be followed with a total of 11 mailed questionnaires from late pregnancy through the infant's first year. The prenatal questionnaire collects information on the mother's health, employment situation, and infant feeding plan; whereas the neonatal questionnaire collects data on maternity care practices as well as the infant's birth and infant's feeding The remaining questionnaires, administrated monthly from 2-7 months then at 9, 10.5 and 12 months after the birth of the child, collect data related to feeding, health status, and information sources. These postnatal questionnaires were composed of nine different modules and will be administered in various combinations. **Results and Conclusion:** Subject recruitment will start about January 2005 and continue for about 6 months until a sample size of approximately 3000 pregnant women is obtained Because each mother will be in the sample for about 15 months, data collection for the entire study is expected to be completed by September 2006. This study will provide detailed information about foods fed to infants, factors that may contribute to infant feeding choices and to breastfeeding success and other issues of interest to FDA, including infant food allergy, and experiences with breast pump. It will also measure dietary intake of pregnant women and new mothers.

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A23. CONDUCTING A RANDOMISED CLINICAL TRIAL (RCT) WITH AN UNDERSERVED POPULATION OF MOTHERS WITH VERY LOW BIRTHWEIGHT (VLBW; <1500G) INFANTS

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Background: In the US, women who deliver VLBW infants are disproportionately African American and low-income (LI; WIC-eligible), subgroups that are significantly less likely to initiate lactation than the general population. As a result, these mothers have not been included in RCTs of lactation interventions, limiting generalisability of findings. The purpose of this study was to describe the process and outcome of conducting a RCT in which our sample demographics reflect women who *deliver* VLBW infants rather than the subgroup that *typically chooses* to provide milk. **Methods**: This RCT compared the comfort and effectiveness of 3 milk expression patterns using the Symphony breast pump (Medela Inc., McHenry, IL). The labour-intensive protocol required that mothers: maintain detailed milk volume records separately for the right and left breasts, collect 1-ml milk samples separately for the right and left breasts before and after each pumping for a 24-hour period, and use the Symphony breast pump in the NICU at the same time for 6 out of 9 consecutive days while the investigator measured outcome variables. Mothers completed a Likert-type questionnaire rating their perceptions of research burdens and incentives. **Results:** Of the 35 women completing the study, 46% did not plan to provide milk at the time of birth, 40% were African American, and 48.6% were LI. There were no missing data for the trial, and all mothers completed all 6 randomisations. Whereas all mothers reported that their primary incentive for participation was to "help my baby", LI women rated economic incentives more highly than non-LI women. Overall perception of subject burdens was low for both groups. **Conclusion:** We conclude that underserved mothers of VLBW infants will complete a RCT of lactation interventions with the proper balance of incentives and burdens. However, funding agencies must recognise that additional investigator time and expertise must be budgeted to recruit and retain these participants. **Acknowledgement:** Partially funded by Medela Inc., McHenry, IL.

Importance of study incentives (1=not important, 5=very important	LI	Not LI
Help baby (4.60±0.79)	4.61	4.60
Help other mothers (4.35±0.98)	4.50	4.27
Symphony at home (4.29±0.81)	4.56	4.13
Free pump in style (4.17±1.13)*	4.56	3.93
Learn about breastfeeding (4.04±1.27)*	4.56	3.73
Free parking (2.83±1.66)*	3.56	2.40
Free bras (2.79±1.35)	2.89	2.73
Free taxi service (2.38±1.76)**	3.44	1.73
Magnitude of study burdens (1=very hard, 5= very easy)	LI	Not LI
24-hour drops (3.40±1.23)	3.78	3.17
6 days to SCN (3.46±1.38)	3.33	3.53
Drops with nurse (3.50±1.29)	3.78	3.33
Pumping with nurse (3.96±1.04)	3.89	4.00
Milk volume log (4.15±0.95)	4.33	4.03

**p<0.05; *p approached significance

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A24. TRAINING OF PEER COUNSELLORS FOR EXCLUSIVE BREASTFEEDING IN RURAL UGANDA

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Background: Exclusive breastfeeding for the first six months of life reduces infant mortality by 13 %. Despite many women initiating breastfeeding, exclusive breastfeeding rates remain low in Uganda. We conducted a pilot study in Iganga district, rural Eastern Uganda to assess the feasibility for training peer counsellors to support exclusive breastfeeding. **Methods:** From March to July 2004, a descriptive cross sectional study was designed in Iganga using qualitative methodologies. In conjunction with the office of the District Director Health Services, community leaders were sensitised about the study and they selected the mothers for training as peer counsellors using the agreed on criteria. The La Leche League training curriculum for peer counsellors was conducted by two lactation consultants. Focus group discussions were held to determine cultural beliefs and practices that affect exclusive breastfeeding. Monthly follow up discussions were held with PCs, recruited mothers and fathers. **Results:** Despite providing community leaders with an agreed selection criteria, two selected PCs did not fulfil all criteria. All proposed PCs turned up for the training and discussed many cultural beliefs and practices some of which don't support exclusive breastfeeding. Some beliefs scare mothers like: "if you express milk to the ground, the baby dies". The PCs realised some of the beliefs they held before were baseless after receiving the basic facts about breastfeeding. On follow-up, the PCs recruited an average 15 mothers each, whom they offered breastfeeding support. The common problems identified by PCs were: inadequate breast-milk, cracked nipples, breast engorgement, mastitis and breast abscesses. Helping mothers to position their babies correctly reduced these problems. A 28year old PC said, "Mothers are happy to realise that they actually have enough breast-milk". The mothers in the Community were happy to have someone to help them with their breastfeeding problems. **Conclusion:** Training rural Ugandan women in basic breastfeeding counselling and support to peer is feasible. The PCs were able to find time to support their breastfeeding peers in addition to their busy daily schedules. The PCs were readily accepted by their communities.

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A25. HEALTH PROFESSIONALS' KNOWLEDGE, ATTITUDES AND PRACTICES IN LACTATION MANAGEMENT AT GOVERNMENT HOSPITALS, KISUMU, KENYA

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Background: Diarrhoea is the second major cause of child morbidity and it causes 20% of under five deaths in Kenya. Breast-milk is the gold standard in early childhood nutrition. Studies indicate that the prevalence of diarrhoeal diseases in infants has remained high. The statement problem was to evaluate information on lactation management by health professionals. The objective of the study was to help determine the level of health professionals' knowledge, attitudes and practice in the management of lactation. **Methods:** A survey interview schedule, observation and focus group checklists were designed for the study. A multi-stage sampling was used. Hospitals in the district were selected purposively while the target population of doctors; nurses' nutritionists and clinical officers were conveniently sampled because they are the primary health information providers Sample size was 113. Quantitative data was analysed using statistical package for social sciences (SPSS). Qualitative data collected was organized, coded and analysed. Chi-square tests were used to test the relationships between the categorical variables at a significant level of 0.05. **Results:** Findings revealed that health professionals' level of knowledge was moderate with a mean of 16.1 (53.7%) out of 30 scores. There were few areas in which gaps in knowledge were demonstrated. 43 (38.1%) were not knowledgeable of initiation time of breast-milk for caesarean babies and reported between one to two days while 76 (67.3%) reported inappropriate complementary age. Only 21 (18.6%) said that they would advice mothers with breastfeeding children to continue breastfeeding. Only 55 (48.7%) were aware of the period of exclusive breastfeeding. Attitude towards lactation management was positive for most 95 (84.1%) respondents. Actual hospital practices were ascertained through observations and focus group discussions. From observation initiation time for caesarean babies took more than 8hrs. **Conclusions:** It is recommended that training curriculum include both technical and communication techniques along with hands on experience In-service training in lactation management be accessible to all health professionals.

Travel award: R Olwambula was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A26. INFANT FEEDING OF HIV-POSITIVE WOMEN: ATTITUDES AND PERCEPTIONS OF COMMUNITY MEMBERS IN BOBO-DIOULASSO, BURKINA FASO

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Background: Infant feeding by HIV-positive women remains a dilemma for developing countries: risk of MTCT linked with exclusive breastfeeding, or sanitary risk associated with replacement feeding. Before implementation of the Kesho-Bora project (study on utilisation of HAART in reducing mother to child transmission (MTCT of HIV) in situations of predominant breastfeeding and for improving maternal health), we wanted to know the attitudes and apprehensions of the populations in question. **Methods:** a qualitative investigation combining focus group discussions (FGD) and individual in-depth interviews were conducted with communities from March to May 2004 in Bobo-Dioulasso city. The interviewers were chosen according to the criteria of homogeneity, age and gender. **Results:** A total of 254 participants took part in the 22 FGD organised with the men, women, adolescents (men and women), elderly women and PLWHA. Sixteen in-depth interviews were carried out with the key informant communities. The most alternative feasible between exclusive breastfeeding and replacement feeding had recorded divergent opinions within the different groups. At every group level, the majority preferred replacement feeding because of the lack of postnatal MTCT risk. The advocates for exclusive breastfeeding cited the nutritive, immunological and psychological value for the children and the mothers, and the difficulties of access to replacement feeding. **Conclusion**: the majority of the participants were in favour of replacement feeding of infants of HIV-positive women in spite of the financial costs associated with its implementation, because of the greater likelihood of eliminating risk of HIV MTCT by exclusive breastfeeding.

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A27. NEWBORNS OF HIV-POSITIVE MOTHERS: EXPERIENCES OF THE CENTRE OASIS FROM 1999-2002, A COMMUNITY-BASED HIV/AIDS CARE ORGANISATION IN OUAGADOUGOU, BURKINA FASO

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Background: Since the emergence of HIV/AIDS in Burkina Faso, community-based organisations have come to play a central and essential role in the struggle against the pandemic. After several years of caring for, and providing social support to seropositive women, community-based organisations were confronted with the fact that a significant number of these women desired to have children. It is estimated that 7% of pregnancies in Burkina Faso involve seropositive women. This occurs in a context where there is limited accessibility to antiretrovirals (ARVs), known to significantly decrease mother-to-child-transmission. Furthermore, there is no national programme on the prevention of mother-to-child-transmission (PMTCT). **Objectives:** Evaluate the prevalence of HIV amongst the newborn children of seropositive mothers, and reduce mother-to-child-transmission by the only means available at the time (interventions centred on breastfeeding). **Methods:** A network was created through which community-based organisations directed seropositive mothers and their newborns to the Centre Oasis. Bi-monthly prenatal medical consultations were organised. A collective support group was created. Educational sessions were held to teach mothers about the benefits of breastfeeding and the difficulties of being seropositive, and about PMTCT. Each mother received personal counselling on choosing between breastfeeding and formula milk. Finally, consent was taken from parents to test newborns for the presence of HIV at the age of 18 months. **Results:** The cumulative results during this four-year period (1999-2002) were satisfactory. 11 newborns received formula milk, 7 newborns received exclusively breast-milk until the age of 6 months, and 3 newborns received both formula and breast-milk due to social pressure. Consent was received to have every child tested for HIV. 5 newborns tested positive for HIV, and 11 tested negative. 3 deliveries benefited from nevirapine for PMTCT. Additional benefits included: parents were educated about HIV/AIDS, bi-weekly community meals were organised, a successful discussion and support group was created, every HIV-positive child is being followed at the Centre Oasis, and 3 grandparents also decided to be screened for HIV. **Conclusion:** The prevalence of HIV amongst infants born to seropositive mothers was elevated, but the management of HIV-infected children remains a difficult task due to: lack of access to ARVs, difficulties with adherence to treatment, the lack of pediatric formulations for certain ARVs. The dilemma of whether or not seropositive mothers should breastfeed persists. This is particularly pronounced in primiparous women who feel a personal and social pressure to share their maternal love and affection via breastfeeding.

Travel award: F Sawadogo was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A28. BREAST AUGMENTATION AND BREASTFEEDING: KNOWLEDGE AND PRACTICES OF LAS VEGAS SURGEONS

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Background: Any positive health benefit from breastfeeding (BF) can only occur if lactation is successful. Although BF rates of initiation in Las Vegas (LV) are at or above the national average, early cessation is common. Breast augmentation (BA) is one factor that can interfere with success. Part of the mystique of LV, renowned for its entertainment industry, is young, large-breasted women – making BA commonplace in women of childbearing ages. A survey of board certified plastic surgeons (BCPS) was conducted to determine their training, knowledge, practices and educational needs in terms of BA and BF. **Methods**: A four-page questionnaire was mailed to all 36 board certified plastic surgeons (BCPS) in LV. Responses were analysed using SPSS and comments were summarised. **Results:** Nineteen responses were received with two BCPS noting they do not perform BA, resulting in a 50% response rate (n=17). Sixteen surgeons were male. Ages ranged from 36-72 y. Years in practice ranged from 5-37 (mean=18y). Number of augmentations done monthly ranged from 4 to 60, with a total of 304/month. All surgeons used round saline implants. Almost half (48%) inserted implants via the periareolar area, none via navel insertion, 35% through inframammary incisions and 18% through axillary incisions. Primary source of lactation knowledge was obtained during residency training (75%). Relationships of utilisation of periareolar incisions and age, number of years in practice, knowledge of risks of surgery to lactation and discussion of risks with women were not statistically significant**. Conclusion:** Almost half the BCPS reported inserting implants in the periareolar area – a procedure known to interfere with BF; yet 71% of these surgeons erroneously believe their method of insertion minimises potential problems. Furthermore, 50% of these BCPS reported not discussing alternative surgery if a woman were interested in future lactation. Hence, there is a need to educate board certified plastic surgeons performing BA in the LV area. Over 50% of all BCPS responded that they were interested in receiving such continuing medical education for several BF-related topics, including anatomy and physiology of lactation, and discussing surgical implications of implants.

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A29. THE BABY FRIENDLY HOSPITAL INITIATIVE IN A NEONATAL UNIT IN A TEACHING HOSPITAL IN SOUTH BRAZIL

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Background: The Baby Friendly Hospital Initiative was launched in early 90´s aiming to provide guidelines for better hospital practices to support breastfeeding; the objectives of BFHI are to implement the "Ten Steps to successful breastfeeding" and do not accept donation of breast-milk substitutes. When a neonate is born premature, low-birth weight or sick, they are often separated in neonate intensive care units (NICU), which do not normally follow the general rules of the BFHI. However, it can be observed that changes in hospital practices and in attitudes of health workers when a hospital gets the title of BFHI, might "contaminate" other units. The aim of this study was to assess the breastfeeding practices among newborns admitted to a neonatal unit immediately after birth and with 6 months of life in a hospital before and after BFHI. **Methods:** The medical records of all newborns admitted to a NICU in 1994 (285), before the hospital be a BFH, and in 1998 (368), after the hospital become a BFH were reviewed to get information on infant feeding practices in two moments: during hospitalisation postpartum and with 6 months. The duration of breastfeeding and exclusive breastfeeding, and the differences between the two years were assessed using Kaplan Meyer and the Log-Rank tests. Logistic regression and Cox analysis were performed. **Results:** There was an important change in the proportion of infants given breast-milk exclusively (1.9% in 1994 and 41.7% in 1998) during hospitalisation, as well as feeding with exclusive infant formula, observed in 17.7% of neonates in 1994 and no longer noted in 1998. The median duration of exclusive breastfeeding increased from 12 days in 1994 to 45 days in 1998. **Conclusion:** The implementation of the BFHI in the studied hospital contributed towards an increase in the exclusive breastfeeding of newborn babies during neonatal care and during the first six months of life. We discuss the appropriateness of implementing Steps n.1 (having a breastfeeding policy) and n.2 (training of all health workers in its implementation), which might have contributed to better infant feeding practices in the NICU. The impact of having been fed the premature and low-birth weight babies with human milk might be resulted in less morbidity and mortality, as shown in other studies.

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A30. INFANT AND YOUNG CHILD FEEDING IN WESTERN UGANDA: CURRENT KNOWLEDGE, PRACTICES AND SOCIO-ECONOMIC INFLUENCES

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Background: Breast- and complementary feeding, if adequately promoted and practiced, can prevent up to 19 % of all childhood deaths in low-income countries. However, the extent of implementation of the current feeding recommendations is still unknown for many local settings in low-income countries. This study assesses knowledge and practices related to infant and young child feeding as reported by mothers/carers. The influence of household socio-economic status on feeding knowledge and practice is also examined. **Methods:** A cross-sectional study that used a two-stage probability proportional to size cluster design to sample 720 children aged below 2 years in the rural district of Hoima, Uganda. Age specific feeding patterns were assessed and analysed with logistic regression. **Results:** Despite universal initiation of breastfeeding, pre-lacteal use was high (43%), with educated mothers more prone to the practice. The median duration of exclusive breastfeeding in the last 24 hours and any breastfeeding was 3.5 and 21.3 months, respectively. In the 24 hours preceding the survey, 10% of infants were fed using a bottle or a cup with spout; 93% of 0-5.9 months breastfed 6 or more times; 21% of 2-3.9 months and 81% of 6-8.9 months had had a complementary food. Over 50% of carers did not know that adding oil could improve complementary food. Among children above six months, 52% were complemented twice or less, 36% received dairy milk and 55% had specially prepared food 24 hours before the survey. The least poor were more likely to use milk compared to the poorest; and educated mothers were more likely to prepare special complementary foods compared to the un-educated. **Conclusions:** Knowledge and practice for feeding infants and young children varied with socio-economic status. Nutrition promotion in Uganda should counteract the likely deterioration of breastfeeding especially use of pre-lacteals as mothers get more educated.

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A31. USE OF INFANT FORMULA IN SHIHEZI CITY, PEOPLE'S REPUBLIC OF CHINA

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Background: With rapid economic development in China, more and more infants are being given infant formula and other substitutes. This trend is sometimes reinforced by traditional perceptions about breastfeeding. A cross-sectional survey in Shihezi, China, in 1996, found that the full breastfeeding rate was only 38%, mixed feeding 56% and bottle feeding 6% at one month. The rate were 9%, 68% and 23% at six months, rate that are well below Chinese and international targets. A more detailed study of breastfeeding is needed to provide the data necessary to implement a comprehensive health promotion program. **Methods:** A longitudinal study of infant feeding practices is being undertaken in Xinjiang Province in the far west of China, along the border with Kazakhstan. Mothers (n=1163) who delivered babies in Shihezi hospitals from January 2003 to April 2004 are interviewed in hospital and are visited or contacted by telephone at regular intervals for 12 months obtain details of infant feeding practices. The data were obtained at 15 days postpartum. **Results:** 59% of mothers were from Shihezi city, 18% Xinjiang Province and 23% other provinces. 34% mothers were farmers and workers, 32% housewife, 26% salespeople and 8% professionals. The rate of bottlefeeding was 11%, mixed feeding 16% and full breastfeeding 72%. The most common reasons for bottle feeding were the mother's hepatitis B virus status (37%) and perceived insufficient breast-milk(42%). Almost all hepatitis B positive mothers (96%) were not breastfeeding. Among the sample of 158 babies who were not fully breastfed, 86.2% were fed formula, 7.6% fresh milk, 5.7% cereals and 1.9% general milk powder (some babies are included in two categories). Babies born after 37 weeks were more likely to be breastfed. The full breastfeeding, mixed feeding and bottle feeding rates by gestational age were 65%,18% and 18% (<=37 weeks), 77%, 16% and 6% (38-40 weeks) and 80%, 17% and 3% (>40 weeks) respectively (X^2=11.36, $p<0.05$). Breastfeeding was significantly more likely when the mother made an early decision to breastfeed. Female babies were more likely to be bottle fed. The bottle feeding rate for males was 9% and females 14% (X^2=6.89, $p<0.05$). **Conclusion:** The bottle and mixed feeding rate in Shihezi was high (27%) two weeks after discharge. The most common reasons for bottle feeding were the mother's hepatitis B virus status and perceived insufficient breast-milk.

Travel award: F. Xu was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A32. INDEPENDENT OF BODY ADIPOSITY, BREASTFEEDING HAS A PROTECTIVE EFFECT ON GLUCOSE METABOLISM IN YOUNG ADULT WOMEN

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Background The objective of this study is to determine if any association between reproductive experience and anthropometric or sub-clinical metabolic alterations of glucose metabolism exist. **Methods** Sixty-seven women were recruited from the University of Brasilia Hospital and were evaluated at 12-18 months post-partum. Demographic, socio-economic, physical activity, anthropometric, and health history data (biochemical, reproductive) were obtained. After 12-h overnight fasting, a 2-h oral glucose tolerance test was performed. Blood samples were collected at several points: basal time, after intake of 75% D-glucose solution (100ml), and every 30 minutes thereafter. Blood glucose and lipids were measured by enzymatic assays. Blood insulin was measured by radioimmunoassay. **Results** In multiple regression analysis, dependent logarithmically transformed (logt) variables (Increased Area Under Glucose Curve - IAUGC, Increased Area Under Insulin Curve – IAUIC, Insulin Peak – IP, Homeostasis model of Assessment - HOMA) were adjusted for parity, age, lactation index (LI), body mass index (BMI), percentage body fat (PBF), waist circumference (WC), superior skinfold sum (SSS) to inferior skinfold sum (ISS) ratio (SSS/ISS), and oral contraceptive use. PBF was positively associated with logt-IAUIC (p = 0.004) and IP (p = 0.006). However, lactation index was negatively associated with logt-IAUIC (p = 0.02). IAUGC and HOMA did not present significant associations. **Conclusions** We conclude that during the postnatal period, independent of parity, body adiposity accumulation is associated with initial alterations in insulin secretion. Furthermore, independent of body adiposity, breastfeeding has a long-lasting protective effect on insulin response.

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A33. PATTERN OF LACTATIONAL AMMENORRHEA AMONGST NURSING MOTHERS IN ILE-IFE OSUN STATE, NIGERIA

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Background: Lactational Ammenorrhea Method (LAM) is used as a temporary method of family planning. In 1988, a consensus meeting brought to the attention of family planning providers and demographers, the health and fertility benefits of breastfeeding (Kennedy et al 1989). Breastfeeding is the primary cause of postpartum infertility that resulted in lactational ammenorrhea. (Bougrants and Potter 1983) .In societies like Nigeria where breastfeeding is prolonged and universal contraception is rare, duration of breastfeeding determines length of birth spacing. (Van Giemata 1978). In many of the rural and urban poor countries infants suckle frequently as often as 12 to 14 hours. As soon as complementary foods are added duration and frequency of suckle reduces especially in affluent societies prolactin levels decline and ovarian activity resumes resulting in menstruation (WHO 1981) A study in Guatemala showed a small significant effect of the caloric intake in pregnancy and lactation, evidenced by the mother weight in reducing or increasing duration of ammenorrhea. This study seeks to examine the pattern of lactational ammenorrhea amongst nursing mothers with the view of assessing when they resume menstruation, duration of breastfeeding, introduction of complementary feeds and the weight of the mother. **Methods:** Two hundred and fifteen mothers were recruited for the study using a prevalence rate 20-50% of mothers resuming menstruation, mothers attending the three primary health care facilities were selected serially as they come to the clinic information was collected using pretested structured interviewer questionnaire. Results were analysed using frequencies and statistical test for association where applicable. **Results**: Only 33 % of mothers used the LAM method, 58.3% resumed menses as early as 6 months postpartum, 37%of them breast-fed their babies had an average duration of ammenorrhea of 3-5 months, of this 85% breast-fed exclusively had an average duration of 9-12months. Mothers less than 50kg had a prolonged duration of 9-12 months and introduction of feeds earlier than 6 months had an average duration of 3-5 months ($P<0.05$). **Conclusion:** Few mothers use LAM as method of family planning, average duration of postpartum ammenorrhea is 5 months, weight of mother and introduction of complementary of feeds reduced the duration of post partum ammenorrhea.

Travel award: O Esimai was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A34. HEALTH, MOOD, STRESS, AND IMMUNE BENEFITS OF LACTATION

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Background: It is known from animal studies that lactation confers stress resistance. This might potentially be accompanied by immunologic protection during stress states. Clinical benefits of lactating may therefore include a health-protecting effect for the mother. The specific aims of the research were to analyse health, stress, mood and selected biological markers of stress and immune function in 200 postpartum mothers. Mothers were studied at 4-6 weeks postpartum and exclusive breastfeeders were compared to formula feeders and non postpartum controls. **Methods**: Mothers were visited in their homes and blood samples were obtained along with the completed instruments. Flow cytometry, mitogen stimulated T cell proliferation, stimulated whole blood assays for Th1 and Th2 cytokines, and serum ELISAs for a panel of endocrine and immune variables performed on serum samples. **Results:** The overall frequency of individual maternal symptoms of infection since birth of the baby was lower in lactators ($p=.06$). Infections symptom severity was highly correlated to perceived stress, postpartum stress, life events stress, and negative moods in the entire sample. Lactators had the same reports of life events, but significantly lower perceived stress, depression, anger and tension scores, and higher fatigue scores compared to formula feeders ($p<.01$). Lactators also showed a higher ratio of Interferon-gamma:Interleukin-10, suggesting upregulation of Th1 cellular immune function. A decreased ratio was associated with depression, tension, and anger moods. Serum cortisol and ACTH levels were correlated with several infection symptoms, and cortisol was also associated with increased EBV capsid antigen IgG, suggesting that stress may decrease Th1 function, and allow latent viruses to escape immune surveillance. Breastfeeders had lower EBV titers. Postpartum mothers generally appeared to have health, stress, and immune benefits compared to non postpartum controls. **Conclusions:** The data support a protective stress, mood, health, and immune benefit to breastfeeding mothers, and thus provide new scientific evidence for the benefits of breastfeeding to maternal health. **Supported by:** NIH RO-1-NR05000.

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A35. IMPACT OF VERY PRETERM DELIVERY ON THE TIMING OF LACTOGENESIS II IN WOMEN

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Background: The nutritional and immunological benefits of human milk are critically important for very preterm (VP) infants. Mothers of VP infants have to express their milk, but successful progression to full lactation is often difficult presumably because of preterm delivery and associated medical intervention such as antenatal steroid treatment. Lower volumes of milk and greater variation in biochemical markers of lactation have been recorded compared with term mothers. We investigated the timing of lactogenesis II by quantifying milk volume and biochemical markers in milk expressed over the first 10 days postpartum by mothers of VP infants. **Methods:** Women presenting with anticipated preterm delivery were recruited to study the effects of antenatal steroids on lactogenesis II. Those who delivered before 34 weeks' gestation and whose infants had not commenced breastfeeding were included (N=56). Milk volume (mL/24h) was measured and milk samples (1mL) were collected daily for measurement of lactose and citrate. Maternal, obstetric and neonatal data were collected. ANOVA for repeated measures was used to investigate predictors of volume, lactose and citrate. **Results:** Gestational age at delivery was 24-28 weeks' (N=18), and 29-33 weeks' (N=38). A total of 362 expression days were recorded with a median volume of 273 mL/24h. Smaller milk volumes were obtained when mothers expressed <6 times per day ($P<0.001$). Decreasing gestation predicted lower lactose concentration ($P<0.001$) and approached significance with citrate ($P=0.08$) and daily milk volume ($P=0.09$). Significant increases in volume were observed by day 3 in 29-33 week mothers, but not until day 4 in <29-week mothers. Antenatal steroid treatment together with gestation was related to milk volume ($P=0.022$). In 29-33 week mothers, steroid treatment 3 – 7 days before birth resulted in lower volumes than those treated <3 or >7 days before birth ($P=0.030$). No other maternal, obstetric or neonatal factors were associated with milk volume or lactose and citrate concentration. **Conclusions:** Lactogenesis II was delayed in mothers of VP infants with the greatest delay occurring at earlier gestations. Antenatal steroid treatment between 3 – 7 days before birth was associated with lower milk production.

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A36. CHANGES IN AXIAL BONE MINERAL CONTENT OF GAMBIAN WOMEN DURING LACTATION

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Background: Physiological changes occur in human lactation such that Ca is released from the skeleton in early lactation and replenished in later lactation or after breast-feeding stops (1). Recovery at vulnerable skeletal sites may be compromised if maternal Ca intakes are low. Women in rural areas of The Gambia breast-feed on demand for about 2y while consuming a diet that is very low in Ca (300-400mg/d). A supplementation study in lactating Gambian women found no significant effects of Ca intake on bone mineral content (BMC) of the radius (2). The aim of this study was to measure BMC of whole body, spine and hip longitudinally during 12 months of lactation in Gambian women. **Methods:** 45 women from the rural village of Keneba, The Gambia took part (mean±SD: age 28±8y; parity 4.5±2.9 range 1-10). BMC and bone area (BA) of the whole body (WB), lumbar spine L1-4 (LS) and hip (total hip TH, including femoral neck FN) were measured in each individual at 2, 13 and 52 wk lactation by DXA (Lunar MD). **Results:** Significant decreases in BMC ($p<0.001$ unless stated) were observed at 13 wk using hierarchical repeat measures ANOVA with BA adjustment (ΔBMC_{13-2}±SE: WB = -0.8±0.3% ($p=0.01$); LS = -3.5±0.6%; TH = -3.0±0.6%, of which FN = -4.4±0.7%). These decreases were sustained or were of greater magnitude at 52wk (ΔBMC_{52-2}±SE: WB = -2.0±0.3%, LS = -2.7 ±0.6, TH = -5.7±0.6%, of which FN = -6.8±0.7%). **Conclusion:** Like lactating women in other countries, Gambian women experience substantial decreases in BMC at the axial skeleton during early lactation, but unlike women in Western populations, there is no evidence of recovery during later lactation. A follow-up study is in progress to determine whether BMC is recovered from axial sites after lactation ceases, or whether these results indicate long-lasting mineral depletion of the maternal skeleton in Gambian women.

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A37. THE INFLUENCE OF PREGNANCY AND LACTATION ON α- AND γ-TOCOPHEROL LEVELS IN PLASMA AND IN ERYTHROCYTES OF ADOLESCENTS

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Background: Tocopherols (TOC) are regarded as protective against chronic diseases in later life. αTOC is the main TOC in plasma and tissues, but there is increasing evidence that γTOC also plays an important role. Furthermore, plasma γTOC and γTOC:αTOC ratio have been proposed as possible markers for nutrition-related risk, including poor dietary choices, which are common during adolescence. Pregnancy and lactation during this period may increase the risk for nutrient deficiencies. The aim of this study was to investigate the influence of pregnancy and lactation on αTOC and γTOC levels and their ratio in plasma and in erythrocytes of adolescents. **Methods:** Fasting blood samples were obtained from three groups of adolescents, with similar age, years post menarche and vitamin E intake: non-pregnant and non-lactating (NPNL, n=26), pregnant (P, n=39; 32.7±3.9 weeks gestation) and lactating (L, n=64; 8.7±5.1 weeks post-partum). Tocopherols were extracted from plasma and erythrocytes and determined by reverse-phase HPLC. ANOVA was used for comparison between groups. Variables are shown as means with standard deviations. **Results:** Plasma levels of α- and γTOC (μmol/mmol cholesterol) were, respectively: NPNL, 4.2±1.5 and 0.9±0.5; P, 3.1±1.6 and 0.9±0.4; L, 2.5±1.3 and 0.6±0.4. Erythrocyte levels of α- and γTOC (μmol/L of packed red cells) were, respectively: NPNL, 2.8±1.2 and 1.0±0.5; P, 2.1±0.9 and 0.3±0.2; L, 3.3±1.8 and 0.6±0.3. L adolescents presented lower ($p<0.01$) levels of both TOC in plasma and of γTOC in erythrocytes than NPNL, whereas P adolescents presented lower ($p<0.01$) levels of both TOC in erythrocytes and α-TOC in plasma than NPNL. Plasma and erythrocyte levels of both TOC in L were different ($p<0.01$) from those in P. Plasma γ:α TOC ratio was higher ($p<0.01$) in P than in L and NPNL. Erythrocyte γ:α TOC ratio was lower ($p<0.01$) in L and P than in NPNL. Plasma γTOC correlated with BMI in NPNL ($r=0.40$) and in L ($r=0.54$). **Conclusion:** In general, pregnancy and lactation affected αTOC and γTOC levels and their ratio in plasma and in erythrocyte membrane of adolescents. These changes may reflect increased utilization of TOC for erythrocyte synthesis and placental transfer during pregnancy and for milk secretion during lactation. **Financial support**: CNPq (Brazil).

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A38. DETERMINANTS OF DELAYED ONSET OF LACTATION

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Background: Lactogenesis II (LGII) has been defined as the onset of copious milk secretion associated with parturition and usually occurs between 36 and 96 hours postpartum. The sudden feeling of breast fullness is perceived by women as the 'coming in' of their milk. The onset of LGII can be adversely affected by a variety of factors including maternal and fetal stress. Recent studies suggest that a high prepregnancy BMI is associated with later onset of LGII. **Methods:** Subjects were 450 women participating in the second Perth Infant Feeding Study (PIFSII) in Australia, who were either exclusively or partially breastfeeding at discharge from hospital. Subjects completed a self-administered baseline questionnaire in the early postpartum period and were asked to indicate on what day their milk had 'come in'. Perceived timing of the onset of LGII was categorised as either early or delayed (<72 h pp vs. ≥72 h pp). Multiple logistic regression was used to evaluate the relation between the delayed onset of LGII and a number of factors known or suspected to be associated with LGII. Those factors entered into the model included method of delivery, gestational age, parity, mother's pre-pregnancy BMI, maternal infant feeding attitude, whether the mother smoked prior to becoming pregnant and whether the infant had been admitted to the Special Care Nursery. **Results:** In total 54 women (11.8%) had delayed onset of LGII. Mean prepregnancy BMI was 24.3 (±5.1). Eighteen per cent of women were overweight (BMI 25 – 29.99 kg/m^2) and 14% were obese (BMI>30 kg/m^2). The only variables to be independently associated with the delayed onset of LGII were delivery method and parity. Women who had undergone a Caesarean section were more likely (OR=2.70 95% CI 1.46-5.01), and multiparous women were less likely, to have experienced delayed onset of LGII (OR=0.40 95% CI 0.22-0.75). **Conclusion:** This study failed to show an independent association between maternal obesity and delayed LGII, which has been reported in other studies.

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A39. HIGH RISK OF VITAMIN B-12 DEFICIENCY IN PREDOMINANTLY BREASTFED GUATEMALAN INFANTS IS REDUCED BY THEIR CONSUMPTION OF DAIRY PRODUCTS

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Background: Vitamin B-12 deficiency is highly prevalent in many countries including Guatemala, where ≈50% in women at 3 mo of lactation had low plasma B-12 and 31% had low milk B-12. Here the prevalence and predictors of low serum B-12 were investigated in Guatemalan infants. **Methods:** A census identified infants <5 mo in nine low income communities in Guatemala City. Exclusion criteria were length/age <-3Z or weight/age <-2Z. At age 6.7±0.6 mo, data collected (n=127) included maternal size, education and SES; infant anthropometry and morbidity; breast-milk intake (2 d test weighing); weighed infant food intake during day and recall intake at night (3 d); and serum B-12 and folate. **Results:** Mean infant weight/age was –1.1Z, length/age, -1.6Z, and weight/length, 0.3Z. Almost all (86%) were breastfeeding; 57% of the total energy intake of 580±143 kcal/d was from breast-milk, and 22% from "dairy" products (75% dry milk and 25% powdered infant formula), with low amounts of other vitamin B-12 sources. Energy intake from dairy products substituted for breast-milk (r=-0.81, P0.0001). B-12 deficiency was found in 24% of infants (serum B-12 <200 pg/mL) and 37% had marginal status (200-300 pg/mL). Only 5% had low serum folate (<3 ng/mL). Serum B-12 correlated with weight/height (r=-0.24, P0.02) but not with other size measures or morbidity. The main predictors of infant serum B-12 were intake of breast-milk (negatively, r=-0.33, P<0.0001) and animal source foods (ASF) (0.33, P<0.0001) especially dairy (r=0.40, P<0.0001). In a subsample of 40 infants retested 6 mo later, prevalence of deficient serum B-12 had increased from 35% to 48%, and serum B-12 remained correlated with values 6 mo earlier (r=0.49, P<0.001) and with recent breast-milk (r=-0.37, P<0.01) and dairy (r=0.23, P<0.05) intake. **Conclusion:** Vitamin B-12 deficiency is highly prevalent in these young infants. Maternal deficiency may cause infant B-12 depletion at birth followed by low B-12 in breast-milk. Higher consumption of cow's milk or infant formulas improves infant status because they contain ≥10X and 3X more B-12/kcal respectively than breast-milk. Functional consequences of low serum B-12 in these infants is under investigation, but there is a clear need for maternal B-12 supplementation and/or food fortification.

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A40. BREASTFEEDING AND ITS INFLUENCE ON INFANT ADIPOSITY BEFORE AND AFTER WEANING: PRELIMINARY RESULTS

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Background: Breast-milk may have a beneficial effect on the growth pattern and body composition of infants, which may result in a lower incidence of obesity and its attendant complications later on in life. **Methods:** We compared anthropometry, skin-fold measurements and fat mass in breastfed (BF, n=63) versus formula-fed (FF, n=37) term infants before and after weaning. The BF group was exclusively breastfed for at least 2 months of age, while the FF group was predominantly or exclusively formula-fed. Anthropometry, skin-fold measurements at 4 different sites and percentage fat mass as determined by the deuterium oxide dilution technique for measuring total body water were performed at 3 months of age before the babies were weaned, and again after weaning at 12 months of age. **Results:** All 100 babies were term at birth [(BF) 39.3 ± 0.9 weeks; (FF) 38.8 ± 1.0 weeks]. Maternal characteristics such as ethnicity, age and body mass index, and the infants' sex ratio, weight, length, head circumference, body mass index and mid-arm circumference at birth were similar between the 2 groups. Solids were first introduced at 5.2 ± 1.1 (BF) vs 4.7 ± 0.9 (FF) months (p=0.026). There were no significant differences between the two groups with regards to anthropometry, mid-arm circumference (MAC), skin-fold measurements, body mass index (BMI) or percentage fat mass (% Fat) before weaning at 3 months of age or after weaning at 12 months (table). **Conclusion:** Breastfed infants were weaned later than formula-fed infants. There was a trend towards breastfed infants having less body fat when compared to formula-fed infants both before and after solids were introduced although the difference was not statistically significant. We hope to show a significant difference when the number of infants in our cohort is increased.

Anthropometry and body fat in breastfed (BF) and formula-fed (FF) infants at 3 months and 12 months of age (mean±SD)

Age (mo)	BF or FF	MAC (cm)	Triceps (mm)	Biceps (mm)	S'scap (mm)	S'iliac (mm)	BMI	% Fat
3	BF	14.9±1.9	9.1±1.8	6.4 ±1.3	8.4±1.4	8.4±2.1	17.0±1.7	22.0±6.5
	FF	14.2±0.9	9.1±1.7	6.9±1.9	8.2±1.5	8.1±1.9	16.7±1.9	22.7±5.8
12	BF	15.0±0.9	8.6±1.6	5.8±0.9	8.4±1.7	6.5±1.2	16.5±1.5	18.4±2.7
	FFa	15.0±1.1	8.1±1.3	5.8±1.9	7.8±1.9	6.8±2.0	16.5±1.1	19.1±2.9

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A41. NEONATAL ZINC DEFICIENCY IN ARTIFICIALLY REARED RATS – DEVELOPMENT OF AN EXPERIMENTAL MODEL

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Background: Prematurely born infants are frequently growth retarded and nutritional zinc deficiency has been suggested as a possible cause of this growth retardation. Although infant formulas are supplemented with zinc, little is still known about the role of zinc during the early postnatal period. **Objective:** The aim of this study was to develop an experimental model of neonatal zinc deficiency and evaluate the effect of moderate zinc deficiency on the growth, organogenesis, and glucose metabolism of neonatal suckling rats. **Methods:** Four-day old suckling rats underwent intragastric cannulation and then were artificially fed either a normal zinc formula (**NZF**; 10 mg Zn/L) or with low Zn content formula (**LZF**, 5 mg Zn/L) for 8 days. Artificially reared rats were compared with dam-fed littermates (**DF**; Zn content of rat milk is 10-14 mg/L). Body weight gain was recorded daily. Organ weights, tissue and serum zinc, glucose and insulin levels were measured. **Results:** Body weight gain was not different between groups. Organ weight of spleen was significantly reduced in the LZF group compared to the NZF or DF groups ($p < 0.05$). Growth of other "zinc-sensitive" organs, such as brain, intestine, pancreas, liver and kidney was not affected. Serum Zn level and femur Zn content was significantly decreased in the LZF group compared to the NZF and DF groups. Serum glucose level was significantly decreased in the RMS group compared to BF controls ($p < 0.05$), whereas serum insulin levels were not affected by diet. **Conclusions:** The ability to maintain zinc homeostasis during the early postnatal period is highly dependent on dietary zinc intake. Reduced serum Zn levels in the RMS and RMS-Zn groups indicate suboptimal Zn status as compared to BF controls. However, reduced serum zinc levels did not affect linear growth and the development of most organs in neonatal rats. The only change resulting from nutritional zinc deficiency was in the spleen indicating the possible importance of Zn for the maturation of the neonatal immune system. **Supported by**: NIH grant HD-39657 (to BD).

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A42. EFFECT OF COMPLEMENTARY FEEDING WITH COWS' MILK ON SLEEPING METABOLIC RATE IN BREAST-FED INFANTS

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Background: Higher values for growth and energy utilisation have been found in formula-fed as compared to breast-fed infants. The mechanism may be through a higher protein content of formula. The effect of complementary feeding with cows' milk on components of energy metabolism in breast-fed infants is unknown. **Methods:** Components of energy metabolism, in particular sleeping metabolic rate (SMR) and minimal observable energy expenditure (MOEE) were compared between two sub-groups of breast-fed infants aged 8.7 months of age: (1) BM group (n=37), i.e. receiving breast-milk as the only source of milk; (2) BCM group (n=25), i.e. receiving cows' milk in addition to breast-milk. Total energy expenditure (TEE) was measured using doubly labelled water (DLW); sleeping metabolic rate (SMR) and minimal observable energy expenditure (MOEE) were measured using respiration calorimetry; breast-milk intake was measured using the ^2H_2O turnover method; complementary food intake was measured by 1-day food weighing; body composition was calculated from the isotope data; anthropometric indices were assessed. **Results:** MOEE was 399 (SD 42) kcal/d in the BM group compared to 444 (SD 50) kcal/d in the BCM group ($p<0.001$). Mass-specific MOEE was 48.0 (SD 5.9) kcal/kg/d in BM versus 51.6 (SD 7.6) kcal/kg/d in BCM infants ($p=0.041$). Comparisons between feeding groups based on SMR gave similar findings. MOEE (kcal/d) was mediated by protein intake and infant weight (R^2=43.1%). Fat mass index (kg/m^2) and weight gained from birth (kg) tended to be higher in BCM as compared to BM infants ($p=0.150$ and $p=0.188$ resp). **Conclusion:** Complementary feeding with cows' milk alters sleeping metabolic rate in breast-fed infants. The effect is mediated by protein intake and infant weight. The tendency towards an increased growth rate and higher fat mass index suggest that the increased metabolic rates of BCM infants may be of concern in relation to the development of obesity later in life.

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A43. PROLONGED BREAST-FEEDING AND MORTALITY IN GUINEA-BISSAU, WEST AFRICA

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Background: Prolonged breast-feeding in developing countries is suspected to be associated with malnutrition, morbidity and mortality. In Guinea Bissau, West Africa median length of breastfeeding is 22 months. We have described a protective effect of breast-feeding for infants and that mother´s decision of weaning is associated with length of breast-feeding and subsequent mortality. The purpose of the present study was prospective description of mortality among prolonged breastfed children. **Method:** A cohort of children born between 1st of September 1998 and 31st August 1999 was followed from 18 months to 36 months. To be included the child should be alive, living in the area and have a known breast-feeding status 18 months. Mortality risk (MR) was estimated as hazard ratio. Medians were compared using Kruskall-Wallis two sample test (KW). **Results:** Of 1,189 children 76.5% (910/1189) were breastfed at 18 months. At 24 months 31.2% (346/1108) and at 30 months 4.0% (41/1025) were breastfed. 44 children died during follow-up of which 40 were breastfed at 18 months; 15 died while they were still breastfed. 21 were weaned before death and 4 had unknown breastfeeding status. Overall mortality was highest between 18-24 months 1.9% (22/1189) compared with 1.1% (between 24-30 months and 0.98% (10/1025) between 30-36 months. Mortality risk between 18-36 months was elevated for children breastfed at 18 months compared with weaned children (MR 2.29 (0.81-6.52). Mortality risk was highest between 18-24 months (MR 2.94 (0.68-12.62) declining at 24-30 months (MR 0.87 (0.24-3.11). Between 30-36 months none of the breastfed children died. 527 of the study children had weight and height measured in the period 18-24 months (391 breastfed and 136 weaned at 18 months). There was no difference in median weight or height comparing breastfed and weaned children. Children who subsequently died had significant lower weight and height compared with children who survived (breastfed and weaned) (Median weight 9.95 kg versus 10.5 kg (KW p=0.02) (Median height 78 cm versus 80 cm. KW p=0.007). **Conclusion.** Breastfed children at 18 months experiences an elevated mortality compared with weaned children which is seen mainly between 18-24 months where most of the children are weaned. The mechanism is unclear, but it could be selection bias as both height and weight of children who subsequently died was lower at 18 months.

Travel award: MS Jakobsen was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A44. INFANT SLEEP PATTERNS IN THE FIRST TWO POSTNATAL DAYS ASSOCIATE WITH TEMPERAMENT CHARACTERISTICS AT 6 MONTHS: EVIDENCE FOR DEVELOPMENTAL CONTINUITY

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Background: There is a need to define instruments that are sensitive to infant functional development, particularly as a response to feeding strategies. Along this line, infant sleep patterns and temperament have been investigated in the current study. **Methods:** We examined the relationship between quiet sleep (QS), active sleep (AS), wakefulness (W), AS to QS transition (AS-QST), arousals in AS (Ar/As), AS:QS (AS/QS), sleep-wake transition (S-WT), quiet sleep bout length (QSBL), AS bout length (ASBL), mean sleep period (MSP) and length of sleep period (LSP) in the first 48 postnatal hours using the non-intrusive Motility Monitoring System. Temperament was assessed (6 months) using the Infant Characteristics Questionnaire (ICQ; Bates et al, 1979).

Temperament	Day 1 (n = 12)	Day 2 (n = 14)
Dull	QS (r = -0.67, P = 0.05) W (r = 0.69, P = 0.05)	QS (r = -0.56, P = 0.05) S-WT (r = 0.64, P = 0.01) QSBL (r = -0.55, P = 0.05) ASBL (r = -0.58, P = 0.05) MSP (r = -0.56, P = 0.05)
Unadaptable	AS-QS (r = -0.62, P = 0.05) Ar/As (r = 0.68, P = 0.05) AS/QS (r = 0.63, P = 0.05) LSP (r = 0.59, P= 0.05)	
Fussy		S-WT (r = -0.72, P = 0.01) MSP (r = 0.56, P = 0.05)

Conclusions: An increased percentage of QS is indicative of a more mature CNS. A low score on the ICQ is generally indicative of more favourable temperament attributes. The significant negative correlations on both days between QS and "dull" point to enhanced neurodevelopment. Conversely, less desirable AS:QS and Ar/AS had positive correlations with a more problematic temperament score. The results clearly demonstrate a relationship between infant sleep characteristics in the first 48 postnatal hours and temperament at 6 months. The findings add a developmental link from our previous study indicating a relationship between prenatal nutrition and sleep patterns of the newborn. **Funded:** in part by USDA IFAFS and the ADA Fdn.

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A45. BREAST-MILK IL-7 MAY INFLUENCE THYMIC T-CELL PRODUCTION IN YOUNG GAMBIAN INFANTS

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Background: In rural Gambia there is evidence that being born during the annual hungry season may impair immune function in young adulthood. We are investigating possible mechanisms for this programming. Ultrasonographic measurements showed that thymic size in infants was lower in the hungry season. The biggest difference was at 8 wks of age, a time when babies were exclusively breast-fed, had good growth and appeared healthy. We have also previously shown that breast-milk immune factors are depressed in the hungry season. We therefore hypothesised that breast-milk factors may influence thymic function and drive immunological changes. The current study tested temporal associations between IL-7, a thymotrophic cytokine, and a measure of thymic T-cell output. **Materials and Methods:** We conducted a prospective cohort study of 138 exclusively breast-fed babies studied in both hungry and harvest seasons. Blood samples at 8wk post-partum were analysed for lymphocyte subsets and signal joint TCR rearrangement excision circles (sjTRECs), a marker for thymic function. Whole breast-milk was collected from the mothers at mid-morning by maternal extraction. IL-7 levels were determined from the milk samples at Wk1 and Wk8 postpartum by ELISA. Student's T-test was used to compare means between the two seasons. **Results:** Breast-milk from mothers of hungry season babies had significantly lower IL-7 than the harvest season at wk1 (79pg/ml vs 103pg/ml, P=0.02) with a similar trend at wk 8. By 8wks of age, those born in the hungry season (n=46) had significantly lower sjTRECs than those born in the harvest season (n=53) (0.92 versus 2.12 sjTRECs/100 T-cells, P=0.006). **Conclusion:** The higher sjTREC levels in the babies born during the harvest season accompanied by higher breast-milk IL-7 levels of the mothers, suggested a possible role for breast-milk IL-7 as a mediator of thymic function in early life.

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A46. BREASTFEEDING, COMPLEMENTARY FEEDING, AND NUTRITIONAL STATUS OF YOUNG CHILDREN IN A RURAL DISTRICT OF BURKINA FASO

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Introduction: In a recent paper on child survival, promotion of exclusive breastfeeding (EBF) and improved complementary feeding (CF) were respectively ranked first and third, among the most effective preventive actions for reducing under-five mortality in developing countries. The promotion of breastfeeding (BF) should be considered in combination with an improvement in CF practices, both being one process. This survey aimed at analysing feeding practices and nutritional status of young children in a rural district of Burkina Faso. **Methods**: A cross sectional study has been implemented in January and February 2004 at Kongoussi. A random sample of 376 children aged 6-23 months has been constituted, using the "probability proportionate to size" cluster sampling. Anthropometrics and socio-economic data were collected. A blood sample was taken for haemoglobin (HB) and zinc measurements. Children anthropometrics measures were transformed into Z scores with the NCHS reference population, under Epinut software. The chi square test was used to compare proportions. **Results**: Among the children 4,5% were exclusively breastfed when aged 0-4 months. The remaining 95,5% in addition to BF were also fed with water (94,7%), others liquids (57%), infant formula (5,1%), solids foods (1,3 %). Prelacteal feeding concerned 41,0% of children. Continued BF at 12-15 months was 98,5%, and 87,2% at 20-23 months. The proportion of CF was 82,7%. Timely introduction of CF was 50,7%. The first food given to children was unfortified cereals (59,3%) or fortified cereals (23,4%). The users of fortified cereals proportion was 35,7%, 20,7%, and 20,2% for high, medium and low socio-economic index ($p = 0,011$). The type of cereal was not statistically associated with maternal education status, age or children rank and gender. Among the children aged 6-11 months wasting was 13,3%, 24,6% and 39,4% respectively for fortified cereals, unfortified cereals, and lack of CF ($p = 0,017$). Stunting, anaemia and zinc deficiency were not associated with CF mode. **Conclusions**: These results reveal a high prevalence of BF, but a low prevalence of EBF. Babies are usually given water or other liquids particularly traditional beverages based on plant decoction. Delayed introduction of CF and use of unfortified cereals exist, and are causes of wasting in youngest children. Studies that aim to highlight the foundation of such practices are needed.

Travel award: HZ Ouedraogo was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A47. A NEW METHOD FOR ESTIMATION OF POSTNATAL GROWTH VELOCITY IN EXTREMELY LOW BIRTH WEIGHT INFANTS (ELBW)

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Background: Growth velocity (GV; g/kg/d) is an important dependent variable in infant nutrition studies. However, no uniform method for calculating GV in ELBW infants has been reported. Because the calculation of actual daily GV is labor-intensive, investigators have estimated GV using varying mathematical methods, making comparisons across studies difficult. The purpose of this study was to assess the accuracy of three commonly used methods of estimating GV and of a new method of estimating GV, an exponential model, which should be appropriate to estimate growth in biological systems. **Methods**: Actual GVs were calculated from daily weights for 83 ELBW infants admitted to the NICU and compared to estimated GVs from each of the four models for these same infants. Actual GV was calculated as $[(W_{n+1}-W_n)x1000]/[(W_n+W_{n+1})/2]$ in daily increments until discharge, where W_n= weight in grams on day "n" and W_{n+1}= weight in grams on the following day. Methods of estimating GV included: the 2-Point Birth Weight model (net weight gain divided by time interval and birth weight); Linear model (linear regression of weight vs. time divided by birth weight); 2-Point Average Weight model (net weight gain divided by time interval and average weight); and the Exponential model ($GV = [1000 \times \ln (W_n/W_1)] / (D_n-D_1)$, where D = day. **Results**: The 2-Point Birth Weight and Linear models were inaccurate with mean absolute errors of 50.6%-96.4%. The 2-Point Average Weight model was fairly accurate, with mean absolute errors of 0.1%-7.5%. The Exponential model was extremely accurate, with mean absolute errors of 0.01%-0.06%. Further analysis showed that the accuracy of the other three models was significantly affected by birth weight, length of stay and chronic lung disease, whereas the Exponential model was unaffected by these factors. **Conclusions:** The accuracy of GV estimates varies widely among currently used methods of calculating GV. The Exponential model provided accurate estimates of GV and provides the accuracy and ease of use that is lacking in current infant growth research.

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A48. EFFECT OF HUMAN MILK INTAKE ON LENGTH OF STAY FOR VERY-LOW-BIRTH-WEIGHT INFANTS

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Background: There is conflicting evidence that human milk (HM) feeding can reduce length of stay (LOS) in very-low-birthweight (VLBW) infants. The objective of this study is to determine the threshold volume of HM/kg/day needed to decrease infant LOS. As a result of active promotion of breastfeeding, lactation support, and follow-up we have a larger sample receiving HM (73% received $\geq$ 10ml/kg/day, 15 % received < 10ml/kg/day and 12%, none) and wider variation in milk consumption (0-144 ml/kg/day) than in studies previously published. This large sample may provide adequate power to determine the minimum amount of HM needed for a significant decrease in LOS. The hypothesis of this research was that VLBW infants who receive the most HM would have the shortest LOS. **Methods**: Data from VLBW infants (n=202), born at Forsyth Medical Center, Winston-Salem, NC whose mothers were enrolled in a previously reported study on maternal feeding decision, lactation counselling, and anxiety were analysed. Infant HM/kg/day intake and LOS was measured during hospitalisation. Group assignment was based on the amount of HM received during the first 28 days. HM/kg/day at 28 days was divided into the lower 50th percentile, <55 ml/kg/day (LH28), and the higher 50th percentile, >55 ml/kg/day (HH28). Data were further analysed and HM/kg/day for the total hospitalisation was divided into the lower 50th percentile, <67 ml/kg/day (LHT) and higher 50th percentile, > 67 ml/kg/day (HHT). **Results:** There was no difference in gestational age (GA) (HH28: 28.9 ± 0.22 wks; LH28: 28.4 ± 0.25 wks) between the two groups, however birthweight (BW) was greater in the HH28 group (HH28: 1188 ± 20.4 gm; LH28: 1091 ± 20.4 gm). Total LOS was significantly lower in HH28 (45 ± 2.3 days) compared to the LH28 (54 ± 2.3 days). However, LOS was not different when comparing LHT and HHT for total hm/kg/day intake, despite controlling for GA, BW and days on supplemental oxygen. **Conclusions:** These results suggest that HM in the first 28 days of life is critical to the health of VLBW infants and a threshold amount of 55 ml/kg/day appears to be needed to result in a shorter length of hospitalisation.

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A49. GROWTH RETARDATION AND INADEQUATE WEANING IN INFANTS ATTENDING DAY CARE CENTRES OF THE SOCIAL SECURITY SYSTEM IN GUADALAJARA, MEXICO

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Purpose. To identify associated factors to growth retardation of infants between 3 to 12 months of age attending to day care centres of the social security system of Guadalajara, Jalisco, Mexico. **Methods.** Analytic and cross sectional design. 123 infants of 3 to 12 months of age, normal birth weight, no pathology, attending five day care centres of the social security system of the metropolitan area of Guadalajara were included. Growth retardation was considered when deficit of length/age was greater than –1 SD (Z score). Data of demographic, socioeconomic, dietetic characteristics and morbidity were obtained. Chi square test and Odds Ratio for the statistical and epidemiological meaning were estimated. **Results.** *Risk factors:* duration of weaning after four months [OR 6.71 (1.92-24.4), p = < 0.025]; mother income less than three minimum salaries [OR 3.95 (1.38-11.4), p = 0.003]; older vs. younger than six months of age [OR 3.43 (1.11-6.97), p < 0.02], non justified cause of weaning [OR 2.54 (1.20-5.36), p = 0.025], cow's milk intake [OR 2.97 (1.33-6.65), p = 0.04). Conclusion. The duration between initial and the end of weaning affected the weight for age index as well as the length for age index which seems to indicate that when mothers insisted on prolonging human lactation for few months longer once of infants had entered to a day care centre, the risk of deficit was increased maybe because the production of human milk was significantly decreased due to a lesser frequency of breastfeeding and/or because mothers and day care centre provided an inadequate and insufficient complementary feeding including in some cases the use of cows milk after six months of age.

Factors associated with deficit in the index length / age (> - 1 Z score) in infants attending day care centers of social security system in Mexico (n = 123)

Variable	Deficit in exposed n/N (%)	Deficit in non exposed n/N (%)	OR (CI 95%)[1]	P
Age of ending weaning (> vs ≤ 4 months)	12/24 (50)	7/54 (12.9)	6.71 (1.92 - 24.4)	< 0.001
Mother's income (< vs ≥ 3 minimum salaries)	12/33 (36.4)	11/87 (12.6)	3.95 (1.38 -11.37)	0.003
Age group (> vs ≤ 6 m)	19/71 (26.8)	5/52 (9.6)	3.43 (1.09-11.49)	0.02
Motive for weaning: (non justified vs justified)	6/12 (50)	13/66 (19.7)	2.54 (1.20-5.36)	0.025
Cows milk intake >6 months of age: (yes/no)	4/8 (50)	19/113 (16.8)	2.97 (1.33-6.65)	0.04
Mother's occupation (manual worker or employee/ professional)	22/91 (24.2)	2/30 (6.6)	4.46 (0.92 -29.45)	0.07

[1] OR =Odds ratio (confidence interval)

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A50. ROLE OF HUMAN MILK IN PROTECTING AGAINST BRONCHOPULMONARY DYSPLASIA IN EXTREMELY LOW BIRTH WEIGHT INFANTS

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Background: Infection and inflammation are contributory to BPD in ELBW infants. The anti-infective and anti-inflammatory properties of human milk (HM) may confer protection. The objective of this study was to determine if ELBW infants who receive HM while in the NICU have a lower incidence of BPD or death when compared to similar infants who never receive HM. **Methods:** The study population for this project was drawn from the study population (N=1433) enrolled in the NICHD Neonatal Research Network Parenteral Glutamine Supplementation Trial. Infants who died without ever receiving enteral feeds were excluded. 1220 infants with prospectively collected nutritional and outcome data were analyzed; the total volume of HM ingested over the first 28 days was divided into tertiles (low, middle, high). The primary outcome was BPD or death, with BPD defined as O_2 requirement at 36 weeks corrected age. Regression models evaluated the effect of HM on outcomes after adjusting for gestational age, gender, race, centre, and severity of illness [including severe intraventricular hemorrhage (IVH grades 3-4), necrotising enterocolitis (NEC, Bell's stage 3-4), and late sepsis (onset after 72 hrs of life)]. **Results:** Characteristics of the participants in the Glutamine Trial are shown in the Table; 68% of the study infants received some HM in the NICU. HM intake was more common in infants of married women, who received more frequent prenatal care and had a higher level of education. No difference in overall severity of illness was noted between groups. Although there was also no difference in the overall incidence of BPD, when the volume of HM over the first 28 days was evaluated in tertiles and adjusted for severity of illness, infants receiving the highest tertile of HM had significantly less BPD or death then infants receiving no HM, OR 0.64 (95% CI 0.44 - 0.93), $p < 0.03$. [Similar findings were observed with late sepsis; see Abstract no 56 by J Meinzen-Derr, et al]. **Conclusions:** High volume HM intake was associated with a reduction in the incidence of BPD or death in ELBW infants. Future studies should be directed towards understanding the mechanism(s) by which HM can protect against lung injury in the premature infant.

Enteral feeding groups	No HM (n=457)	HM (n=976)	p-value
Birth weight (g)	787 $\pm$ 138	777 $\pm$ 133	NS
Gestational age (wk)	26.2 $\pm$ 2	26.1 $\pm$ 2	NS
Maternal age (y)	26.8 $\pm$ 7	27 $\pm$ 7	NS
Married (%)	28	49	<0.0001
Prenatal care (%)	83	95	<0.0001
College degree (%)	5	17	<0.0001

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A51. MILK-BORNE EGF REGULATES ILEAL BILE ACID TRANSPORT IN EXPERIMENTAL NECROTIZING ENTEROCOLITIS

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Background: The development of NEC is characterized by pathological changes in the intestinal mucosa of terminal ileum and proximal colon. Milk-borne EGF decreases the incidence and severity of NEC in the neonatal rat model (Dvorak et al., 2002). Cytotoxic bile acids (BA) are associated with damage to the intestinal mucosa. In rodents, active BA transport from the ileum is reported to be absent in the early postnatal period. Apical sodium dependent bile acid transporter (ASBT), first expressed in rat ileum at weanling, is involved in BA absorption. The aim of this study was to evaluate the effect of enteral administration of EGF on ileal BA levels and transport in the neonatal rat NEC model. **Design/Methods:** NEC injury was induced in neonatal rats fed rat milk substitute (RMS) with or without EGF supplement (500ng/mL) and stressed in cold and hypoxia. Dam fed (DF) littermates were subjected to the same stress regimen. After 96 hours, rats were sacrificed. Total BAs were measured in ileal flushes and serum. Expression of ASBT was evaluated using immunohistochemistry and RT real-time PCR. **Results:** Total BAs were significantly decreased in ileal flushes from EGF (204±89μM) and DF (149±71μM) compared to the RMS group (722±350μM). ASBT was not expressed in DF rats or in animals fed EGF. In contrast, ASBT was highly expressed in neonatal rats with NEC, with localization in the brush border of enterocytes. Expression of ASBT mRNA was elevated in the distal ileum of RMS rats compared to healthy controls. EGF treatment significantly lowered mRNA expression of ASBT compared to the RMS rats. Conclusions: We speculate that premature expression of ASBT, induced by formula and stress in the immature intestine, is associated with increased BA levels. Increased levels of BA can damage the intestinal mucosa, possibly via ASBT mediated increases of intracellular BA to cytotoxic levels. Enteral administration of EGF may protect the intestinal mucosa from the cytotoxic effects of BA through regulation of gene expression of BA transporters. **Supported by:** NIH grant HD-39657 (to BD).

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A52. BREAST MILK CYTOKINES ARE ASSOCIATED WITH PARASITIC GASTROINTESTINAL WORM INFECTIONS IN ZANZIBARI INFANTS

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Background: Several of the cytokines described in breast-milk have also been described in association with helminth infections. The aim of this research was to determine the association of cytokines expressed in breast-milk with GI nematode infections in nursing infants. **Methods:** 105 Zanzibari infants, 6 -11 mo old, were studied in a cross-sectional case-control design. Worm-infected cases were age matched to uninfected controls in a 1:4 ratio. Mothers' milk was collected by manual expression from each breast on two days. Concentrations of IL-4, IL-6, IL-12, IL-13, TNF-α, IFN-γ, and TGF-β_2 were measured using whole milk samples with Quantikine® or Pelikine™ ELISA assay kits. Milk samples were coded positive or negative for each cytokine. Infants' stools were collected on two days, and examined for the presence of *Ascaris* (large roundworm), *Trichuris* (whipworm), and hookworm eggs by Kato Katz and gravity sedimentation. Unadjusted odds ratios were calculated to describe the associations of milk cytokines with the infant outcomes of GI nematode infection, severe anaemia (haemoglobin < 7 g/dL), and mid-upper arm circumference (MUAC). **Results:** Proportions of milk samples positive for each cytokine were: IL-4: 3%, IL-6: 21%, IL-12: 9%, IL-13: 9%, TNF-α: 10%, IFN-γ: 15%, and TGF-β_2: 54%. The following cytokines were positively associated with GI nematode infection: IL-4 (Odds Ratio: 8.7; *P=0.08*), IL-12 (6.3; *0.01*), and IL-13 (20.5; *<0.01*). Positive associations were also observed between IL-6 and severe anaemia (OR: 4; *p=0.01*), as well as IL-12 (4.3; *0.06*) and TNF-α (3.15; *0.13*) with a MUAC < 130 mm. **Conclusions:** Breast-milk cytokines were associated with outcomes in breastfed infants. IL-4 and IL-13 are believed to confer host resistance to GI nematode infections, and so the observed positive association of IL-4 and IL-13 with nematode infection is unexpected. Further research with larger numbers of infants needs to be conducted to confirm these results.

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A53. THE 24-H EXCRETION OF LACTOSE IN URINE AS A MEASURE OF INCREASED PERMEABILITY OF THE LACTATING BREAST DURING INFLAMMATION

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Background/hypotheses: 1) The 24-h excretion of lactose in urine will provide a reliable measure of whether any changes observed in milk composition during breast inflammation, are as a result of increased permeability in the breast; 2) Increased severity of either systemic or breast symptoms during mastitis will predict an increased 24-h urinary excretion of lactose. **Methods:** A convenience sample of 26 mothers, at risk for developing mastitis, was followed prospectively from Day 5 post partum to the end of their lactation. Milk from each breast, blood, 24-h urine samples and data on breast and systemic pathologies were collected at baseline intervals, daily during the occurrence of any breast inflammation and at 7 days following resolution of symptoms. Hypothesis testing of all outcome measures was based on analyses of variance with repeated measures. All analyses were adjusted for stage of lactation and co-existing breast and systemic pathologies. **Results:** There were increases in [Na] ($p<0.001$), [Cl] ($p<0.001$) and [serum albumin] ($p<0.02$), and a decrease in [lactose] ($p<0.003$), in the milk from mastitis breasts compared to the contralateral breasts. Despite the extreme changes seen in [Na], [Cl] and [lactose], the [glucose] remained relatively stable. The 24-h excretion of lactose in urine was higher during mastitis ($p<0.001$), peaking at the commencement of the mastitis and decreasing over time until there was no significant difference at the time of follow-up when compared to the mothers with no mastitis ($p<0.25$). Despite a close correlation between [lactose] in blood and the 24-h excretion of lactose in urine ($p<0.01$, $r=0.8$), [lactose] in blood was not found to be a significant predictor for mastitis ($p<0.31$). An increase in severity of breast symptoms was associated with an increase in the 24-h excretion of lactose ($p<0.022$). **Conclusions:** Excretion of lactose in urine provides a reliable marker for changes occurring in breast permeability. Increased severity of breast symptoms experienced during mastitis was a significant predictor for an increased excretion of lactose in urine. The [serum albumin] and [glucose] in milk did not always conform to the increased permeability of the paracellular pathway model during mastitis, questioning the nature of the structure of the tight junctions within the breast.

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A54. AUSTRALIAN BREASTFED INFANTS HAVE LOWER ANTIBODY TITRES TO HIB-PRP

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Background: Breastfeeding is an important factor in the protection of infants against some invasive diseases including *Haemophilus influenzae* type b. There are conflicting reports appearing in the literature[1,2] as to whether this is due in part to direct effects on the immune response to immunisation. **Methods:** Healthy term infants were enrolled from postnatal wards at the Women's and Children's Hospital. Infants were eligible if they were born at ≥ 37 wk gestation and had a birth weight ≥ 2500g. Infants were immunised against *Haemophilus influenzae* type b with Hib (PRP-OMP) (Liquid PedvaxHIB) at 2 and 4 mo of age according to the national immunisation schedule. Blood samples were taken by venepuncture at 7.3 ± 0.5 wk of age (prior to immunisation, n=254) and at 31.6 ± 1.3 wk of age (93 d after the 2nd immunisation for Hib, n=230) for measurement of plasma antibody concentration by ELISA. **Results:** Breastfed infants had gestational ages that were greater than formula fed infants and were heavier and longer at birth [P<0.05]. Breastfeeding mothers were less likely to smoke during pregnancy (14% compared with 33%) and were more socially advantaged than women who chose to formula feed [P<0.05]. Breastfed infants had lower plasma concentrations of IgG antibody to Hib PRP at 7 weeks of age and at 7 months of age (Mann Whitney-U test) and a lower proportion of breastfed infants achieved antibody concentrations to Hib PRP greater than 1.0 μg/mL (Pearson Chi-Square test) (Table). **Conclusions:** Breastfeeding is associated with lower plasma antibody titres both prior to and after immunisation with Hib (PRP-OMP) conjugate vaccine.

[1]Pabst HF, Spady DW. *Lancet* 1990;336:269-70.
[2] Scheifele D *et al*. *Lancet* 1992; 340:1406.

	Breastfed	Formula Fed	P
	Hib PRP IgG (μg/mL)		
	Median (10th, 90th CI) [Number of Subjects]		
7 weeks	0.35 (0.16, 0.82) [87]	0.6 (0.23, 1.55) [153]	0.000
7 months	1.07 (0.18,13.32) [92]	2.14 (0.38, 11.27) [138]	0.002
	Proportion of Infants with Hib > 1.0 μg/mL		
7 months	47/92 (51%)	101/138 (73%)	0.001

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A55. INACTIVATION OF CLINICAL HERPES ISOLATES BY LIPID-PEPTIDE MIXTURES

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Background: Most studies which examine the antimicrobial activity of human milk utilize laboratory adapted strains of viruses and bacteria. However, different clinical isolates of infectious agents often have varying susceptibilities to antimicrobial agents. The present study was undertaken to determine whether mixtures of antiviral lipids and peptides, which synergistically and rapidly inactivate laboratory strains of herpes simplex virus (HSV) were equally effective when tested against panels of clinical isolates of HSV-1 and HSV-2. **Methods:** The ether lipid 1-0-octyl-sn-glycerol (OG) was combined with varying concentrations of the antimicrobial peptide LSA5. Lipid-peptide mixtures were tested against panels of HSV-1 and HSV-2 consisting of one laboratory isolate of HSV-1 and one of HSV-2 as well as five clinical isolates each of HSV-1 and HSV-2. Viral inactivation studies were performed over a 1-hour period. **Results:** Mixtures of 3 mM OG and 9 μM LSA5 reduced viral titers by at least 1,000-fold for all clinical isolates of HSV-1 and HSV-2 in the panels. The time required for complete viral inactivation, however, varied. Some HSV-1 isolates were completely inactivated in $\leq$ 10 minutes whereas the infectivity of other HSV-1 isolates was reduced 10,000-fold in 60 minutes, but some infectivity remained. Inactivation of HSV-2 clinical isolates showed the same time variability as HSV-1. All of the HSV-2 clinical isolates were inactivated in less time than the laboratory strain by the synergistic effect of OG and LSA5. **Conclusion:** These results show that the susceptibility of clinical HSV isolates to inactivation by antimicrobial mechanisms present in human milk varies between isolates and with laboratory adapted strains. Interestingly, clinical isolates were sometimes more sensitive to inactivation by lipid-peptide mixtures. This study indicates that to obtain an accurate picture of the *in vivo* efficacy of protective factors in human milk future studies should use, when possible, panels of clinical isolates of infectious agents.

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A56. FACTORS ASSOCIATED WITH SUBCLINICAL MASTITIS AMONG HIV-INFECTED AND UNINFECTED WOMEN IN LUSAKA, ZAMBIA

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Background: Subclinical mastitis – raised milk sodium/potassium (Na/K) ratio – has been associated with poor infant growth and increased risk of postnatal mother-to-child HIV transmission. Several causes of subclinical mastitis – maternal infection, micronutrient deficiences, poor lactation practice – have been individually documented but their relative importance has not been investigated in the same women. **Methods**: Women (198 HIV-infected, 189 HIV-uninfected) from Lusaka, Zambia were recruited at 34 weeks gestation and followed up to 16 weeks postpartum for collection of information on their and their infant's health, infant growth, and infant feeding practices. Milk samples were collected from each breast at 11 postpartum visits and blood at recruitment and 6 weeks postpartum. Milk Na/K ratio was analysed by flame photometry. An overall geometric mean Na/K ratio was calculated for each woman. **Results**: Both unilateral and bilateral subclinical mastitis (Na/K ratio > 1.0) were common in early lactation; bilateral, but not unilateral, decreased with time. Geometric mean milk Na/K ratio and the proportion of women with subclinical mastitis were higher among HIV-infected than uninfected women. Other factors associated in univariate analyses with higher Na/K ratio were primiparity, high maternal 1-acid glycoprotein (AGP) or low plasma vitamin E at 6 weeks, premature infant, low infant weight or length at 6 and 16 weeks, and non-exclusive breastfeeding or giving the infant solid foods. In a multivariate analysis including all factors significant in univariate analyses, primiparity and raised AGP were strongly associated with raised Na/K ratio and there was a borderline association of Na/K ratio with feeding the infant solids and with low infant length at 6 weeks. **Conclusion**: The main factors associated with subclinical mastitis which are amenable to intervention are a maternal acute phase response, likely to infection, and feeding the infant solids before 16 weeks of age. The association of raised Na/K ratio with poor infant growth has been seen in several studies. Further studies are required to determine the impact of improved postpartum medical follow-up, especially management of maternal infections and especially in primiparous women, on the prevalence of subclinical mastitis and, among HIV-infected women, the associated high milk viral load. **Funding:** The Wellcome Trust.

Travel award: L Kasonka was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A57. DIARRHEOGENIC *E. COLI* AS A CAUSE OF GASTROENTERITIS IN BREAST-FED INFANTS IN JAPAN

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Background: We investigated diarrheogenic serogroups of *E. coli* as a causative factor of infantile gastroenteritis. **Patients and methods**: Eleven infants (age: <4 months) who visited our hospital with complaints of bloody diarrhea were subjected to examination by routine stool culture as well as commercially available enzyme immunoassays and Verotoxin-specific latex agglutination. Twenty-two age-matched infants without any signs or symptoms of digestive tract disorders were examined as controls. **Results**: Ten of the 11 patients (91%) were fed breast-milk and bloody diarrhea continued or was recurrent for more than 1 month in 9 patients. When compared with the rate of breast-feeding (54%) in one-month-old infants visiting our hospital for routine medical checks, the rate among the 11 subjects (91%) was high but not significant. None exhibited evidence of allergic colitis. *E. coli* was isolated in routine stool cultures from 6 patients (6/11: 55%) and all of these were O-antigen positive (O-1, O-25, O-124, O-125) and Verotoxin negative, while *E. coli* was isolated in 18 of 22 controls and 5 of these 18 (5/22: 23%) were O-antigen positive (O-1, O-18, O-125) and Verotoxin negative. No other pathogens were isolated from stool cultures of the subjects. **Conclusion**: The present results indicate that we may consider diarrheogenic serogroups of *E. coli* to be a causative factor of infantile gastroenteritis in Japanese breast-fed infants with continuous or recurrent diarrhea.

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A58. HUMAN MILK AND LATE-ONSET SEPSIS IN INFANTS 401-1000G: A SECONDARY ANALYSIS

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Background: Human milk feeding may decrease the rate of sepsis among very low birth weight infants. Few studies examine the dose effect of human milk on infection risk. The hypothesis of this analysis is that a higher intake of human milk during the first 14 days of life reduces the incidence of late onset sepsis in the first month of life among infants 401-1000g. **Methods:** Analysis of data on 1326 of 1433 infants enrolled in the NICHD Neonatal Network Glutamine Trial at 15 centers between Oct 1999 and Aug 2001; infants were excluded if they had died <72 hours of age (n=36), had missing data for sepsis or age of death (n=4), or not in the hospital for at least 14 days (n=67). Type of feeding, late-onset sepsis (confirmed septicemia after 72 hrs of age), potential confounding variables/risk factors were collected. Human milk (HM) intake was defined as the volume of human milk (cc human milk per Kg infant weight) in the first 14 days of life. Crude associations between sepsis infection, occurring within the first 35 days of life, and feeding were tested using a two-sample t. Association between HM and sepsis, controlling for covariates, was assessed using the Cox proportional hazards model. **Results**: Among all study infants, 33% (n=441) had at least one episode of sepsis by age 35 days accounting for 80% of all infants who had sepsis during the study period. Infants with at least one late-onset sepsis episode had slightly lower mean maternal age (26 vs. 27yrs;p=0.02), birth weight (744 vs. 796g; p<0.0001), gestational age (25 vs. 26 wks; p<0.0001) and were more likely to have Apgar <5 at 5 minutes (17% vs. 11%; p<0.04). In bivariable analysis of feeding, infants with late-onset sepsis were fed a lower cumulative volume of HM (cc/Kg) in the first 14 days (95 vs. 176; p<0.0001), had a longer duration in days to their first enteral feed (8.8 vs. 6.8; p<0.0001), and had a longer duration in days to their first human milk feed (11.1 vs. 8.8; p=0.0004). Proportional hazards model results indicate a protective effect of HM, after adjusting for gestational age, number weeks on parenteral nutrition, and Center. For each 100 cc/kg increase in HM intake during DOL 1-14, the hazard ratio decreased by a factor of 0.93 (95% CI=0.88-0.98; p=0.02). **Conclusion:** Increasing volume of human milk intake in the first 14 days of life is associated with a decreasing risk or hazard of late onset sepsis within the first 35 days in extremely low birth weight infants.

Travel award: J Meinzen-Derr was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A59. INCIDENCE OF DIARRHEA AND ACUTE RESPIRATORY INFECTION (ARI) IN BREASTFED INFANTS DURING 18 MONTHS OF LIFE

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Background: There is increasing evidence of high risk morbidity in non breastfed infants in underdeveloped countries. The purpose of the study was to compare the incidence of diarrhea and ARI in breastfed infants vs. non breastfed infants. **Methods:** A cohort study: An original sample of 493 dyad mother-infant were included from the obstetric service at the West General Hospital. Each dyad localized in the beds 2 and 4 from each one out of six obstetric wards was recruited and information about socio-demographic and mother-infant characteristics were obtained. Information about type of feeding (including exclusive breastfeeding before four months and the use of complementary foods after that age), and the occurrence of diarrhea and ARI's (cough and fever) were also obtained in each visit at 1,2,3,4,6,9,12 and 18 months after discharge. Similar information was obtained from non breastfed infants. Non parametric tests and Relative Risk (RR) were estimated for the association between type of feeding and episodes of diarrhea and ARI's. **Results:** 63.5% of mothers were younger than 25 y, 91% were housewives, 88.6% were married or living as a couple and 51.9% had 6 or less education years. In 63.9% of cases the yearly family income was lower than $1500 US dollars. 493, 469, 362, 303,216, 151, 115 and 89 dyads mother-infant were followed up during the seven clinical visits respectively. 85 to 90% of infants evaluated were receiving breast-milk at each visit. A total number of 1636 visits and 97,929 observation days were computed. There were 46 episodes of diarrhea in the whole sample with an incidence density (ID) in breastfed infants of 139 cases/1000 person-years vs. 277 cases/person-years in non breastfed infants [RR = 0.50 (0.28, 0.90)]. 113 episodes or ARI's were also identified with an ID in breastfed infants of 223 cases/1000 person-years vs. 965 cases/1000 person-years in non breastfed infants [RR = 0.23 (0.16, 0.34)]. **Conclusion**: There was a strong protective effect of breastfeeding against infection. The absence of breastfeeding might have a negative impact on the physical growth, nutritional status and morbidity of these children.

Travel award MI Santos-Torres was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A60. FAECAL CALPROTECTIN LEVELS IN BREAST-FED INFANTS

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Background: Faecal calprotectin has been proposed as a sensible marker for gastrointestinal inflammation in children and adults. Increased levels have been reported in healthy infants during the first months of life, but data about the effects of the kind of feeding on calprotectin concentration in feaces are scanty and controversial (1,2). The aim of our study was to evaluate faecal calprotectin levels in healthy infants fed exclusively with breast-milk or a standard infant formula. **Methods:** Between September 2003 and June 2004 stool samples of 62 healthy infants (35 exclusively breast-fed and 27 exclusively formula-fed; mean age 51.54±22.11days, range: 12-90 days) were collected at our Department. Exclusion criteria were infections and intake of anti-inflammatory drugs. Stool samples were stored at –20°C until they were analysed. Faecal calprotectin levels were detected using a quantitative ELISA (Calprest, Eurospital SpA, Trieste, Italy). The study protocol was approved by the Hospital's ethical committee and parents gave written consent to the inclusion of their infants in the study. Statistical analysis was performed using Student's t-test. A value of $p<0.05$ was considered to be statistically significant. **Results:** The mean faecal calprotectin concentration among healthy infants was significantly higher in breast-fed than in formula-fed ones (565.81±381.08 mg/kg *vs* 342.80±240.27 mg/kg; $p=0.045$). **Conclusion:** It is well known that faecal calprotectin concentration is higher in young infants than in healthy adults and show a wide interindividual and age-dependent variation. In our healthy infants, we observed higher levels in breast-fed than in formula-fed ones. This finding could be due to cytokines and other immunostimulating and growth factors (such as leptin and G-CSF) present only in human milk (3), which contribute to the development of infants gastrointestinal immune system.

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A61. ARE CULTURES OF MECHANICALLY-EXPRESSED HUMAN MILK PREDICTIVE OF THE PATTERN OF INFECTION IN EXTREMELY PREMATURE INFANTS IN THE NICU?

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Background: Mother's milk has been mentioned as a source of infection in extremely premature infants and some clinicians recommend bacterial cultures of milk prior to feeding. We tested the hypothesis that bacterial flora in expressed mother's milk is related to the pattern of infection in the extremely premature infants. **Methods:** Aliquots of mechanically-expressed human milk were collected weekly (1 to 11 weeks) from 187 mothers of infants born less than 30 weeks gestation (n=236). Milk was diluted serially. Samples and controls (sterile water and sodium chloride) were inoculated onto MacConkey and Columbia CNA agar plates and incubated for 48 hours at 35 ^{o}C to determine "milk flora.". Pathogens isolated from the same infants blood, cerebrospinal fluid, and urine prospectively throughout hospitalization were tabulated. **Results:** 2,336 organisms were isolated as milk flora (66% were Gram-stain positive) from 91% of mothers. The percentage distribution of milk flora and pathogen isolates differed [Table]. Of all milk samples, those with the same organism isolated as pathogen and as milk flora were tabulated as [Concordance] = [# Milk Flora and Pathogen Isolate]/[#Milk Flora].

Organism Group	Milk Flora	Pathogen Isolates	[Concordance]
Staphylococcus epidermidis	43%	14%	2.4%
Coagulase negative Staph	5%	46%	26%
Staphylococcus aureas	6%	11%	4.7%
Enterobacter	6%	3%	1.5%
Klebsiella	3%	5%	2.8%
Pseudomonas	4%	1%	0 %
Serratia	1%	2%	6.9%
Citrobacter	0.6%	0.5%	7.7%
Acinetobacter	9%	0	-
Stenotrophomonas	4%	0	-

Conclusion: The data do not suggest that random milk cultures are predictive of infection in the infant. However, certain organisms rarely present in milk should be reconsidered as potential problems in extremely premature infants. Microbiological surveillance of human milk in the NICU might be more cost-effective if techniques to detect only selected unusual pathogens are employed.

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A62. INFLUENCE OF BREASTFEEDING ON THE ANTIBODY RESPONSE TO HIB AND PNEUMOCOCCAL CONJUGATE VACCINES GIVEN AT 3, 5 AND 12 MONTHS OF AGE

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Background: PncCRM (Prevenar®) is a heptavalent pneumococcal conjugate vaccine against serotype 4, 6B, 9V, 14, 18C, 19F and 23F. Sweden has a routine vaccination schedule of three doses, i.e. at 3, 5, and 12 months. Recent findings indicate a stimulatory effect on the immune response by breastfeeding in invasive Hib disease. This study was performed in order to relate the duration of breastfeeding to the antibody concentrations to each of the pneumococcal serotypes PncCRM vaccination, after the 2nd dose (6 months), before (12 months) and after the 3rd dose (13 months) as well as to Hib at 6 and 13 months. **Methods:** This was an open non-randomised multi-centre study enrolling 101 healthy Swedish infants. PncCRM was administered concomitantly, but at separate site, with DTaP/IPV/Hib at 3, 5, and 12 months. Antibody concentrations were determined at KTL by EIA for anti-Pneumococcal IgG from serum samples taken at 3, 6, 12 and 13 months, and for anti-Hib IgG from serum samples at 6 and 13 months of age. Breastfeeding data was calculated for days of almost exclusive as well as total (any form of) breastfeeding. **Results:** At three months of age, before the immunization, the children breastfed 30 days or more showed lower antibody concentrations against Pn6B, 19F and 23F than those children breastfed shorter than 1 months. At 13 months of age 7 out of 95 children do not reach the protective level 0.2 mgram/mL against Pn 6B, 5 of these were breastfed <90 days (Fischers Exact test, P=0.026); 4 children did not reach 1 microgram/ml against Hib and all those were breastfed 1 month or less (P=0.007). **Conclusion:** Children breastfed exclusively 90 days or more had a higher proportion above a protective antibody level against Pn6B and Hib compared to those less breastfed.

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A63. BREASTFEEDING PROTECTS AGAINST MORE SEVERE MANIFESTATIONS OF CLINICAL MEASLES INFECTION

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Background: It is well known that ongoing breastfeeding is protective against many infectious diseases. There are recent indications that breastfeeding may have long-lasting protective effects against infectious diseases beyond the period of breastfeeding itself. **Method**: We analysed data on 10 2307 individuals from the 1970 British Cohort Study. Data was registered regularly since birth, with breastfeeding data collected at 5 years of age while information on clinical measles infection, as well as socio-economic variables was collected at the age of 10 years. Breastfeeding was categorised as follows with counts in brackets; breast-fed yes < 1month (1611), bf yes 1-3 mo (1016) , bf yes 3mo or more (1108), bf yes but not known how long (21) , and never breast-fed (6451). **Results:** Breastfeeding three months or more showed to be protective against more severe manifestations of clinical measles infection after controlling for crowding, social class, measles vaccination and sex with an OR 0.75 (0.6-0.9). Measles vaccination was highly protective OR 0.14 (0.13-16), a well as high social class, OR 0.45 (0.3-0.6), while there was an increased risk with crowding with an OR of 1.5 (1.2-1.7) in the most crowded group. No difference between the sexes was found. Mean age for the disease was 4.5 years with no difference between the breastfeeding groups. **Conclusion**: Our findings support a long-lasting protective effect by breastfeeding against more severe manifestations of clinical measles infection up to the age of 10 years. The mechanism behind this finding needs to be further studied.

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A64. REDUCING THE CMV INFECTIVITY OF MOTHER'S OWN BREAST MILK FOR EXTREMELY PRETERM INFANTS

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Background: Infants can acquire primary postnatal CMV infection from the breast-milk of their CMV infected mothers. CMV transmission has been shown in 37% of preterm infants of CMV infected mothers, with symptomatic infection in half of these infants. The infants most at risk of symptomatic infection are extremely low birth weight infants and those who acquire CMV early. An increased risk of adverse neurodevelopmental outcome in low-birth weight infants who acquired CMV early has been suggested. Preventing CMV transmission in extremely preterm infants from maternal milk is a clinical problem. Heating breast-milk removes CMV but also removes beneficial properties of breast-milk. Freezing is not as harmful to the protective effects of breast-milk. Previous small studies of freezing (to -20°C) breast-milk with naturally acquired CMV showed a reduction in CMV titres, or elimination of CMV. These studies used a culture method with a low sensitivity. Aim: A pilot study to determine the length of freezing at -20°C required to eliminate CMV from breast-milk, using a more sensitive culture method. Methods: Breast-milk was collected from CMV seropositive women. The breast-milk was frozen in 1 ml aliquots at -20°C in a quality-controlled freezer. CMV culture was performed at day 0,1,3,5,7,10 and 14 after freezing. CMV was cultured in human embryonic fibroblasts in tube cultures and monitored for the characteristic CMV cytopathic effect. Immunofluorescence with CMV-specific monoclonal antibodies gave a sensitive measure of the presence of CMV. CMV culture was chosen as the endpoint as it is the marker of CMV infectivity PCR while more sensitive does not necessarily indicate infectivity. Results: Breast-milk was collected from 19 women, PCR detected CMV in 12, CMV was cultured in 5 samples on the day of collection. After 7 days of freezing at -20°C, CMV could not be cultured in any of the samples. **Conclusions:** We recommend freezing breast-milk to -20°C for 7 days as a relatively simple method to substantially reduce the CMV infectivity of breast-milk for extremely preterm infants.

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A65. DOCOSAHEXAENOIC ACID REDUCES THE CATABOLIC EFFECT OF SEPSIS ON NUTRITIONAL STATUS OF CRITICALLY ILL NEONATES

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Background: Human milk provides protection to the recipient infant not only against infectious diseases, but also against the severity and anorectic response of infections. Such protection is provided through a number of factors present in milk. Special focus has been paid to n-3 fatty acids' derivatives such as docosahexaenoic acid (DHA) because it seems to modulate the inflammatory response by attenuating cytokines production and secondarily their catabolic effect. Recently, a study that compared leukocytes' cytokines production of breast-fed (BF) and formula-fed (FF) infants did not find differences in the *ex-vivo* production of IL-1β and TNF-α. Another study conducted in our lab demonstrated that increases of IL-1β and TNF-α after an immunologic challenge were not different between BF and FF infants. One limitation of these studies is that the fatty acids pattern of young infants depends not only on dietary sources, but also on maternal origin. Therefore, we evaluated the effect of the acute administration of DHA to infected neonates on their nutritional status, under the hypothesis that if DHA attenuates leukocytes' cytokines production, their nutritional status would not be affected. Methods: A randomised clinical trial was conducted. Post-surgery neonates who acquired sepsis in the hospital were selected. They were randomly assigned to receive DHA (100 mg/day) or placebo for 14 days. Anthropometric measurements were obtained at sepsis diagnosis (baseline) and at the day 14 of treatment (day 14). Differences of nutritional status indicators between baseline and day 14 were obtained and transformed to percentage of change. Comparisons were made with paired t test, Student's t test or Mann-Whitney-U test as adequate. **Results**: Thirty-nine neonates were followed; 20 received DHA and 19 placebo. The nutritional status mean of neonates was not different, at baseline or at day 14, between groups. However, the percentage of change demonstrates higher deterioration in the nutritional status of children in the placebo group (table). **Conclusion:** These results suggest that acute DHA supplementation preserve the nutritional reserves of critically ill neonates during an episode of infection.

	DHA (n= 20)	Placebo (n=19)
Weight	4.7 ± 2.0	0.6 ± 1.7
Length	1.7 ± 0.4	0.7 ± 0.2[b]
Head circumference	1.2 ± 0.4	0.2 ± 0.4
Arm circumference	2.2 ± 1.2	-3.7 ± 1.3[ab]
Biceps	7.8 ± 6.1	-11.8 ± 4.6[ab]
Triceps	4.1 ± 4.7	1.0 ± 5.6
Suprailiac	4.3 ± 6.2	-11.3 ± 6.5[a]
Subscapular	4.4 ± 4.9	-0.2 ± 4.4
Thigh	4.4 ± 3.8	-6.2 ± 5.7
Skinfolds sum	2.9 ± 2.8	-6.8 ± 4.1
Fat mass	9.3 ± 4.0	-7.6 ± 4.4[ab]
Fat free mass	4.5 ± 2.1	-0.6 ± 2.0[b]

Mean ± SE, [a] One tail paired t test, $P \leq 0.05$, [b] Student's t test or Mann-Whitney U test, $P \leq 0.05$.

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A66. THE EFFECTS OF MATERNAL DIET AND ACUTE EXERCISE ON LONG-CHAIN POLYUNSATURATED FATTY ACID CONCENTRATIONS IN BREAST MILK

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All authors were affiliated with the UNCG at the time this research study was conducted

Background: Long-chain polyunsaturated fatty acids (LCPUFA) are essential for infant growth and visual and cognitive development. [LCPUFA] in breast-milk are dependent on maternal diet and body fat stores. Since exercise increases mobilisation and utilisation of fatty acids, maternal activity may also influence the [LCPUFA] in breast-milk. The objective of this study was to determine the effects of exercise on breast-milk α-linolenic acid (LNA), linoleic acid (LA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) concentrations. A second objective was to determine if lactating women consume adequate amounts of LCPUFA to compensate for those used for energy during exercise. **Methods:** [LCPUFA] in milk were measured at 12 wk postpartum in women who participated in an exercise (ES) and a rest session (RS). The ES consisted of brisk walking or jogging for 30 min. Breast-milk was expressed before exercise (time 0) and 10 and 60 min after exercise. During the RS, milk was collected at the same time, with women resting for the 30-min test period. Body composition was assessed by underwater weighing. Dietary intake was recorded for 3 d. **Results:** [LA] and [LNA] increased from time 0 to 60 during ES, but decreased from time 0 to 60 in RS. There were no significant changes in DHA, EPA or AA. Percent body fat was significantly correlated with [LA] ($r= 0.62$, $p= 0.02$) and with [LNA] ($r= 0.77$, $p= 0.001$) at time 60 after the ES. There were no significant correlations between % body fat and milk [LCPUFA] during the RS. Fatty acid intake (LA: 11.3 ± 6.0 and LNA:1.0 ± 0.6 g/d) was close to the Adequate Intakes of LA (13 g/d) and LNA (1.3 g/d) for lactation. **Conclusions:** The significant relationship between % body fat and [LA and LNA] 60 min after the ES suggests a greater mobilisation of LA and LNA during exercise in women with a higher % body fat. The trend towards an increase in [LA and LNA] after ES indicates that LA and LNA may actually increase in breast-milk after exercise. These results suggest that women consuming adequate amounts of LCPUFA can exercise moderately without decreasing [LCPUFA] in their breast-milk.

Travel award: M Bopp was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A67. DIET AND SUCKLING MODIFY PRIMATE MILK FATTY ACID COMPOSITION. EXOGENOUS MUFA MAY ACT IN THE CONTROL OF MILK FATTY ACIDS

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Background We studied *Callithrix penicillata* milk to determine total lipid and fatty acid content due to normal captive diet and due to a docosahexaenoic acid (DHA) rich diet. Also examined was local mammary adaptation of fatty acid composition upon the presence or absence of suckling. **Methods** Animals were studied during mid-lactation (14-16d post partum). For three days, a control diet (CD) group (n=3) was fed their normal diet and an experimental diet (ED) group (n=4) received salmon as a source of DHA. Animals had one of their teats sealed for 3 hours prior to sampling. Milk samples from each teat were manually expressed from dams under light anesthesia. Total lipid content was determined by crematocrit, while fatty acids were analysed by gas chromatography. Data were compared by Student t-test and bivariate correlation. **Results** Detectable amounts of EPA and DHA were measured in milk from only the ED group. In unsealed teats, there was decreased MCFA and increased MUFAs, while PUFA remained unchanged. Correlation analysis revealed a significant negative correlation between MCFA and MUFA ($r = -0.74$, $p = 0.05$) and between C8:0 and C16:1 ($r = -0.71$, $p = 0.014$). **Conclusions** Diet and suckling modify milk fatty acid distribution in primates. Also, results indirectly indicate that MUFA may control the activity of mammary lipogenic enzymes in the local synthesis of MCFA.

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A68. MATERNAL DIETARY SUPPLEMENTATION WITH FISH OIL IN PREGNANCY AFFECTS BREAST MILK POLYUNSATURATED FATTY ACID COMPOSITION AT SIX WEEKS POST-PARTUM

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Background: Many studies have shown that the polyunsaturated fatty acid (PUFA) composition of breast-milk is acutely influenced by supplementation of the mothers' diet during lactation but the effects of supplementation during pregnancy have not been investigated. We hypothesised that maternal dietary supplementation with fish oil during pregnancy would affect breast-milk PUFA composition during lactation. **Methods:** In a randomised controlled trial 83 women received 4g fish oil capsules (containing 3.7g n-3 PUFA) (n=40) or 4g olive oil capsules (n=43) daily from 20 weeks gestation until delivery. Breast-milk was collected at day 3 and week 6 postpartum and fatty acids were analysed by gas liquid chromatography. General linear regression models with repeated measures were used to compare the outcomes between the fish and olive oil supplemented groups, and between measurement times. **Results:** Key PUFA differed between, and from day 3 to week 6 within, supplementation groups. The percentage composition of 20:4n6 (AA) was lower in the fish oil supplemented group at day 3 but no difference between groups was noted by week 6. Greater levels of 20:5n3 (EPA) and 22:6n3 (DHA) were found in the fish oil group, at both day 3 and week 6. Differences were also observed within groups at each time point. For both the fish and olive oil groups, 18:3n3 increased with time whereas 20:4n6 and 22:6n3 decreased. 18:2n6 increased over time in only the olive oil group and 20:5n3 increased over time for the olive oil group but decreased over time for the fish oil group. **Conclusions:** Supplementation of maternal diet with fish oil during pregnancy provided remarkably high breast-milk EPA and DHA levels at day 3 and week 6 postpartum. In particular, DHA levels were up to four times greater than that previously reported for Australian women. Furthermore, both n-3 PUFA were significantly greater when compared to those in the olive oil supplemented group at each time point. Even though levels of AA were lower in the fish oil group at day 3, actual levels were within literature values. Furthermore, for AA, the difference between groups was not apparent at week 6. This study suggests that maternal diet during pregnancy can affect breastmilk PUFA composition during lactation.

Changes in fatty acid levels (wt %) Values are mean ± SE; NS: Not significant

FA	Olive oil group		Fish oil group		Diff olive-fish (P)		Diff day 3-week 6 (P)	
	Day 3	Week 6	Day 3	Week6	Day 3	Week6	Olive	Fish
18:2n6	8.73± 0.29	10.7± 0.53	8.84± 0.37	9.86± 0.44	NS	NS	↑0.0002	NS
18:3n3	0.72± 0.04	1.02± 0.7	0.76± 0.04	0.91± 0.05	NS	NS	↑0.0001	↑0.0277
20:4n6	0.61± 0.02	0.38± 0.01	0.55± 0.02	0.35± 0.01	0.0154	NS	↓0.0001	↓0.0001
20:5n3	0.05± 0.004	0.07± 0.005	0.16± 0.01	0.1± 0.008	0.0001	0.022	↑0.0104	↓0.0001
22:6n3	0.5± 0.03	0.26± 0.02	1.14± 0.08	0.42± 0.04	0.0001	0.0359	↓0.0001	↓0.0001

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A69. LONG CHAIN-POLYUNSATURATED FATTY ACID (LCPUFA) STATUS AT BIRTH IS SIMILAR IN PRETERM AND TERM BRAZILIAN INFANTS

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Background: LCPUFA are indispensable functional and structural components of all cellular membranes, and the availability of LCPUFA during early life appears to affect the quality of growth and the development of visual and cognitive functions. Several studies have shown that later LCPUFA status of infants is determined both by the status at birth and post-natal diet. The aim of this study was to investigate the PUFA status at birth in preterm and term infants using the red cell membrane fatty acid composition as a marker. **Methods:** Cord blood samples from preterm (26 to 36 weeks of gestation; $n = 30$) and term (37 to 42 weeks of gestation; $n = 30$) infants were taken at birth. Simultaneously, maternal blood samples were also taken. Fatty acid composition of the red cell membrane was determined by capillary gas chromatography after direct transesterification. **Results:** The PUFA contents (weight%) in red cell membrane of preterm and term infants were (mean ± SD), respectively: 18:2n-6: 3.3 ± 0.2 and 3.5 ± 0.1; 20:3n-6: 2.1 ± 0.1 and 2.3 ± 0.1; 20:4n-6: 15.6 ± 1.1 and 13.7 ± 0.7; 22:4n-6: 2.7 ± 0.2 and 3.1 ± 0.2; 22:5n-6: 1.1 ± 0.1 and 1.4 ± 0.1; 22:5n-3: 0.6 ± 0.1 and 0.5 ± 0.1; 22:6n-3 (DHA): 4.5 ± 0.1 and 4.0 ± 0.1. There were no differences (t test) between preterm and term infants in PUFA contents, except for DHA ($P<0.01$), or in Δ6 and Δ5 desaturase activity indices and DHA deficiency index. DHA sufficiency index was higher ($P<0.001$) in preterm (3.95 ± 0.97) than in term (2.85 ± 0.84) infants. The differences found between preterm and term infants were not present in their mothers. No association (multiple regression analysis) between PUFA status at birth and increase in weight and length at 6 months post-partum was found for preterm and term infants. **Conclusion:** Our study showed that, the preterm infants may not be in disadvantage in comparison with term infants regarding LCPUFA status at birth, when a functional marker such as cell membrane composition is evaluated, in contrast to studies that used the composition of plasma phospholipids as a marker of status. **Financial support:** CNPq (Brazil).

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A70. TRANSCRIPTIONAL REGULATION OF Δ6D AND Δ5D DESATURASES BY DIETARY LINOLEIC ACID CONTENT IN LACTATING MAMMARY GLAND

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Background: Dietary essential fatty acids; linolec acid (LA) and linolenic acid (ALNA) are converted to the important long chain polyunsaturated fatty acids (LC-PUFAs) arachidonic acid (AA) and docosahexaenoic acid (DHA) respectively. Newborns need LC-PUFAs for cerebral and cognitive development and they obtain them from milk. Despite the low intake of these LC-PUFAs, milk of mothers with marginal nutrition in Mexico has adequate levels of AA that could be synthesised in the maternal body. Also, has been demonstrated that expression of mRNA 6D and 5D desaturases (required to synthesise LC-PUFAs) is positively regulated by some transcriptional factors like SREBP1 in liver. The synthesis of LC-PUFAs by lactating mammary gland has not been evaluated. The objective of this research was to know if rat lactating mammary gland expresses mRNA for Δ6D and Δ5D desaturases, and if this expression is regulated by dietary LA content. **Methods:** Rats were fed during gestation and lactation with 2, 5 and 10% of corn oil (contains 60% LA). The animals were killed 12th day of lactation to measure the desaturases (Δ5D and Δ6D) mRNA in mammary gland and liver. **Results.** Δ6D and Δ5D are expressed in the mammary gland and this expression is regulated by dietary LA. The lower corn oil content the higher the expression of both desaturases. Also, SREBP1 transcription factor is more expressed in mammary gland of rats fed with low corn oil diet than in rats fed high corn oil diet. **Conclusions:** This is first study that suggests lactating mammary gland has capacity to synthesise LC-PUFAs, and the Δ6D and Δ5D expression is regulated by dietary LA content. Besides, increase in SREPB1 expression is a compensatory mechanism to increase desaturases transcription in case that the intakes of PUFAs by the mother is low. Also, this research add to knowledge in that organs other than liver contribute to LC-PUFAs synthesis of milk necessary for newborn brain development mainly when intakes of the mother of polyunsaturates is low. This work was supported by CONACYT-México Grant No. 39804.

Travel award: M Rodriguez was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A71. CYCLES OF GESTATION-LACTATION AFFECT POLYUNSATURATED FATTY ACID METABOLISM IN LACTATING WOMEN

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Background: Gestation-lactation (GL) cycles seem to affect polyunsaturated fatty acid (PUFA) metabolism, which may have consequences to later maternal health, but the possible effects of previous GL cycles on PUFA status/metabolism during lactation have not been determined. The aim of the present work was to investigate the effect of parity on the composition of plasma nonesterified (NEFA) and erythrocyte membrane (EM) fatty acids and on their associations in lactating women. **Methods:** Fasting blood samples were obtained from primiparous (n= 14) and multiparous (n= 8) lactating women (26.0±1.4 years; 56.3±5.6 days of lactation; mean±SE) attending a public day-care clinic in Rio de Janeiro, Brazil, in a cross-sectional design. They were exclusively or predominantly breastfeeding and had similar dietary habits. Fatty acids were analysed by capillary gas-chromatography with split (EM) or splitless (NEFA) injection. **Results:** Eighteen fatty acids were identified in the plasma samples, and 24 fatty acids in the EM samples. There were significant differences between primiparous and multiparous lactating women (ANOVA) in both plasma and EM samples and no interactions with age. Contents (wt%) of 20:4n-6, 20:5n-3, and total saturated NEFA were higher ($P< 0.05$) in multiparae than in primiparae (mean±SE): 2.97±0.27 vs 2.00±0.18; 0.58±0.07 vs 0.37±0.05; 52.1±1.76 vs 47.1±1.15, respectively. In contrast, the content of 18:2n-6 was lower ($P< 0.005$) in the EM of multiparae (8.02±0.42) than in primiparae (10.4±0.30). The content of 18:2n-6 in plasma was correlated with EM 18:2n–6 (r= 0.52; $P= 0.059$) in primiparae, and with EM 20:4n-6 (r= 0.94; $P= 0.006$) in multiparae. **Conclusions:** Erythrocyte membrane 18:2n-6 levels in primiparae seem to be more dependent on 18:2n-6 plasma NEFA levels than in multiparae. The correlation between plasma 18:2n-6 and EM 20:4n-6 for multiparae is indirect and probably dependent on PUFA metabolism in the liver. Long-term effects of GL cycles on PUFA metabolism result in changes in NEFA and EM composition and their interactions during lactation. Financial Support: CNPq.

Travel award: AG Torres was awarded a travel grant to attend the 12th ISRHML meeting in Cambridge.

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A72. BREASTMILK ALPHA-DEFENSINS AND TRANSMISSION OF HIV-1 FROM MOTHER TO CHILD

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Introduction: It is not known whether neutrophils present in breastmilk produce α-defensins 1-3, which are known to have anti-HIV activity. We set out to quantify α-defensins in breastmilk of HIV-1 infected women, define correlates of α-defensins production and role in postnatal transmission of HIV-1 from mother to child. **Methods:** α-defensins were measured in the breastmilk specimens from HIV-1 infected women in Nairobi. HIV-1 viral load was calculated in maternal plasma and breastmilk at 1 month postpartum and infant infection status determined at birth and months 1,3,6,9, and 12 by HIV-1 DNA PCR and HIV-1 RNA. **Results:** Among 330 breastfeeding women, 280 (85%) provided breastmilk specimens and 60 (21%) of their infants became HIV-1 infected during follow up, 40 (67%) of whom became infected during or after birth. We detected α-defensins in 40% of the women. Alpha-defensins were positively correlated with breastmilk viral load, parity, and presence of clinical mastitis ($p<0.05$ for all comparisons). Independent of breastmilk viral load, plasma viral load was inversely associated with α-defensins levels in breastmilk. Women with detectable defensins had higher levels of MIP-1α, MIP-1β, RANTES, and SDF-1 in breastmilk ($p\leq0.01$). Controlling for plasma viral load, the odds of infection increased 3-fold for every log increase in breastmilk virus among women with undetectable defensins (OR 3.6; 95% CI 2.1-6.1; $p<0.001$), compared with a 2-fold increased risk among women with detectable defensins (OR 1.7; 95% CI 1.0-2.8; $p=0.05$). **Conclusion:** These results suggest a complex interplay between virus levels, chemokines and defensins, a better understanding of these relationships may provide insights into developing novel strategies to reduce HIV-1 transmission from mother to child.

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A73. THE CELL CONTENT OF HUMAN BREASTMILK: FACTORS INFLUENCING THE VARIATION OF CELL NUMBER, VIABILITY AND CULTURING POTENTIAL

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Background: Breastmilk contains mammary secretory epithelial cells (lactocytes) and immune cells. Cell numbers decline from $3.0x10^6$ to $2x10^3$ cells/mL from week 1 to 26 post-partum (pp) although lactocytes increase from 2% to 90% of total cell number. However the effect of method of collection of milk on variability in lactocyte number and viability has not been studied. **Methods**: Mothers (n=12) expressed milk for 10min using a Medela electric breast pump and samples collected every 30s and pooled according to degree of breast fullness (DoF) calculated from variation in the fat content of milk over 24h. Cells were washed from milk, counted on a haemocytometer and lactocytes identified using morphological characteristics. Cell death was determined using propidium iodide exclusion and visualised using acridine orange/ethidium bromide staining. Apoptosis was determined using DNA laddering. Statistical significance is $p<0.05$. Cell numbers are $x10^4$ cells/mL. **Results**: Between 1-6mo pp the mean cell count (±SD) for all women was 19±2 (range; 0.3-99). In relation to a DoF of 1.0-0.81, 0.8-0.61, 0.6-0.41, 0.4-0.21 and 0.2-0 respectively, the mean±SE total cell number (7±3, 9±5 , 20±10, 26±8.6, 43±20), total cell viability (%; 47±11, 47±8 65±5 64± 4 69±11), lactocyte number (1±0.5, 2±1, 5±1, 5±1, 9±3), and lactocyte viability (%; 28±6, 26±5, 46±5, 47±4, 58±7) increased significantly with milk removal. Apoptosis was the predominant method of cell death. There was a significant difference in mean±SE cell viability (%; 63±6 versus 74±6) between the Medela Classic and an experimental Symphony Breastpump. **Conclusions:** These data show significant differences between lactocyte number and viability dependent upon the DoF of the breast, the stage of lactation, and breast pumps. Primary cell cultures can be grown from low cell number and viability, and variation in cell numbers and viability could influence the interpretation of results. Therefore when deriving primary cultures these variables should be considered to minimise variability in the experimental protocol.

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A74. INDICATORS FOR RAISED SODIUM AND ASSOCIATED CHANGES IN BREASTMILK DURING THE FIRST THREE MONTHS POST PARTUM

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Background: The term sub-clinical mastitis has been applied in situations where lactating women present with increased [Na] in milk after Day 3 post partum and in the absence of weaning. However this definition may obscure other potential causative factors. This study investigates factors, other than mastitis, that may be associated with changes in milk [Na] over the first 3 months post partum. **Methods:** Reference samples of milk from both breasts, in addition to blood and 24-h urine samples, were collected from 26 mothers at days 5, 14, 30, 60 and 90 post partum. During this time data on any breast or systemic pathologies being experienced were also collected. Data obtained were used to identify biochemical changes occurring as a result of breast or systemic events. Hypothesis testing of all outcome measures was based on analyses of variance with repeated measures. All analyses were adjusted for stage of lactation and co-existing breast and systemic pathologies. **Results**: Variation from normal milk composition was observed in both asymptomatic and symptomatic women. There was a marked increase in [Na] and [Cl], and a decrease in [lactose] in milk in asymptomatic women, at Days 5 and 14. These changes were associated with birth at, or before, 38 weeks gestation, caesarean birth and increased [C-reactive protein] in blood above the normal mean for that time post partum. Of the non-inflammatory breast pathologies identified: low supply, perceived oversupply and nipple trauma all showed an association with an increase in milk [Na] ($p<0.001$; $p<0.001$, and $p<0.004$, respectively) and a decrease in milk [lactose] ($p<0.05$; $p<0.015$ and $p<0.001$, respectively). Changes were also observed in the concentrations of chloride, glucose, lactoferrin, sIgA in milk and the 24-h excretion of lactose in urine. **Conclusions:** These findings demonstrate that a mother's health status, and in particular breast health, must be taken into consideration when establishing a normal range for breastmilk composition. A better understanding of how various breast and systemic pathologies affect milk composition and breast physiology will be gained if a possible causative association for the increased [Na] in breastmilk, and associated breastmilk changes, are investigated and defined.

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A75. IDENTIFICATION OF ANTIBODY TO COW MILK PROTEINS IN SERA OF TYPE 1 DIABETES MELLITUS POSITIVE CHILDREN

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Background: Studies are equivocal about relationship of enfant early ingestion of cow protein and onset of type 1 diabetes mellitus. **Methods:** Western blots of two-dimensional electrophoretic separations of cow Milk-Fat Globule Membrane (MFGM) proteins were assayed with 10 coded serum samples from the Joslin Diabetic Center (Boston,MA). **Results:** Serum samples, uncoded, were from 5 type 1 diabetes positive patients, ages 7-14 yrs; 5 samples were from controls, ages 21-30yrs. All sera had antibody to some proteins; 4 of 5 in both groups had antibody to butyrophilin. A doublet at approximately 16kD, pI 5.1, 5.3 identified as a genetic variant of the monomeric form of β lactoglobulin was the only area positive in all diabetes samples and absent in all controls. Significant homology between β lactoglobulin and glycodelin (PP14) was discovered Glycodelin is known to directly modulate T cells. **Conclusions:** The possible significance of the relationship of antibody to β lactoglobulin to an immune system modulator needs to be discussed.

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A76. THE FUNCTIONAL SIGNIFICANCE OF MILK-DERIVED IL-12

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Background: We have described the presence of interleukin 12 (IL-12) in human milk samples collected from day 3 postpartum to 8 weeks of lactation[1]. The function of milk-borne IL-12 is not known, however its biological activities in other systems suggest the potential for a role in addressing the balance between Th2-like responses known to predominate *in utero* and Th1-type cytokine driven cell mediated responses that are required after birth. For example, reduced mononuclear cell IL-12 production in the neonatal period has been associated with stronger neonatal Th2 responses and increased risk of subsequent atopic disease[2]. **Objective:** Using the IL-12-deficient (p40-/-) mouse model, the aim of this study was to determine the ability of milk containing IL-12 to restore the capacity of p40-/- mice to produce IFNγ, a Th1-type cytokine. **Methods:** IL-12 p40-deficient C57BL/6 mice and control C57BL/6 mice (wild type, WT) were bred under specific pathogen-free conditions. Cross foster experiments were carried out using litters of p40-/- and WT pups born within 24 h and commenced when pups were 1-2 days old. At day 16, spleens were collected from mouse pups and splenocytes ($2x10^6$/mL) cultured *in vitro* with concanavalin A (5μg/mL) for 24h, 72h or 5 days. Culture supernatants were harvested for analysis of IFNγ concentration ELISA. **Results**: see Table.

IFNγ pg/mL, mean ± SEM (number of mice)			
	Wild Type	IL-12 p40-/-	Cross Fostered p40-/-
24 h	[a]1644 ± 262 (10)	[b]426 ± 66 (11)	[b]663 ± 105 (16)
72 h	[a]3201± 295 (10)	[b]1821 ± 340 (11)	[ab]2525 ± 284 (14)
5 days	[a]2974 ± 374 (8)	[a]2106 ± 420 (11)	[a]2642 ±394 (10)

[a,b] Different superscript letters indicate significant differences between groups, $P < 0.05$, ANOVA

Conclusions: Spleen cells isolated from suckling IL-12 p40-/- mice produced less IFNγ in response to *in vitro* stimulation with Con A than spleen cells from WT pups at the same age. Cross fostering IL-12 p40-/- pups onto WT dams with IL-12 in their milk increased the capacity of pups lacking IL-12 to produce IFNγ. These data support the hypothesis that the provision of cytokines and growth factors via breast-milk may assist in countering defects in the infant's own cytokine network in the first few months of life.

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A77. BREAST MILK CALCIUM CONCENTRATION IS ASSOCIATED WITH THE *VAN91*I RESTRICTION LENGTH POLYMORPHISM OF THE PARATHYROID HORMONE RECEPTOR GENE

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Background: There are marked differences in breast-milk Ca concentration between women and between populations that are independent of maternal Ca intake (1). Parathyroid-hormone related peptide (PTHrP), acting through the PTH/PTHrP receptor (PTHR1), plays a key role in Ca regulation during human lactation. The aim of this study was to determine whether breast-milk Ca concentration is influenced by polymorphisms in the PTHR1 gene. **Methods:** Eighty one women from Cambridge, UK were studied at 2, 7 and 13 weeks of lactation. Breast-milk samples were analysed for Ca (2). *Van 91*I restriction fragment length polymorphisms of PTHR1 were determined using the method of Heishi et al (3). Repeat measures ANOVA with Sheffé post-hoc tests was used to determine the influence of PTHR1 genotype on breast-milk calcium. **Results:** The women were: age=32.2±4.1 y, parity=1-4; height=1.66±0.06 m; weight at 2 wks=70.6±10.9 kg, calcium intake at 2 wks=1406±533 mg/d (mean±SD). Breast-milk Ca concentration (mg/l) averaged 288±42, 301±50 and 288±42 at 2, 7, and 13 weeks respectively, and was consistent within individuals over time ($P \leq 0.0001$). The observed frequencies of PTHR1 genotypes were in Hardy Weinberg equilibrium: VV=13.6%, Vv=55.6%, vv=30.8%. There were significant differences between women in breast-milk Ca concentration depending on their PTHR1 RFLP (p=0.0016): vv-VV=21.9±5.9%, p=0.002; Vv-VV=15.4±5.5%, p=0.024; vv-Vv=6.4±3.8%, p=0.25 (difference±SE%, p value of Scheffé post-hoc test). **Conclusion:** Breast-milk Ca concentration is associated with maternal PTHR1 genotype. The magnitude of effect is similar to the variations seen between women and between populations. Differences in PTHR1 genotype frequency may underlie ethnic differences in breast-milk Ca concentration.

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A78 DETECTION OF ADIPONECTIN IN HUMAN MILK: POTENTIAL IMPLICATIONS FOR EARLY METABOLIC REGULATION

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Background: Breastfeeding appears to be associated with lower risk of obesity, diabetes, and dyslipidemia in later life. The mechanisms by which human milk may provide protection are unclear, but research suggests that bioactive factors in human milk influence infant metabolic development. Adipose tissue is a substantial component of human breast tissue, and proteins secreted by adipocytes (adipokines) influence metabolic control. Adiponectin, a major adipokine, influences insulin sensitivity, fatty acid metabolism, and the risk of obesity, type 2 diabetes, and dyslipidemia. Although adiponectin has not been isolated in human milk, its presence provides a plausible mechanism by which human milk feeding could impact metabolism. **Methods:** We assayed adiponectin and leptin (another adipokine, previously characterised in human milk) in 30 skim milk samples from the Cincinnati Children's Research Human Milk Bank. All donors delivered term infants. Mothers gave consent to have their banked human milk utilised for any research study approved by the Cincinnati Children's Hospital Institutional Review Board; this study was approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center. **Results:** Concentrations in human milk ranged from 6.9-80.4 ng/mL for adiponectin and 0.3-2.2 ng/mL for leptin. Adiponectin levels were negatively correlated with day of lactation (range 1-401 days) and positively correlated with levels of leptin. **Conclusions:** This is the first report of adiponectin in human milk, a necessary initial step in characterising the impact of adiponectin on metabolic development. Further exploration of the relationship between adiponectin and metabolic programming is warranted to understand the relationship between early nutrition and adult disease.

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A79. THE CREAMATOCRIT PLUS™: A NEW CENTRIFUGE FOR MEASURING CREAMATOCRITS WITH MOTHERS' MILK

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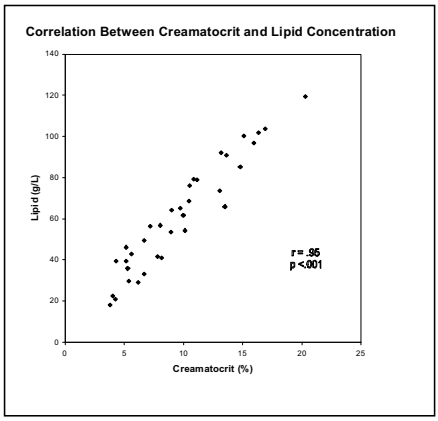
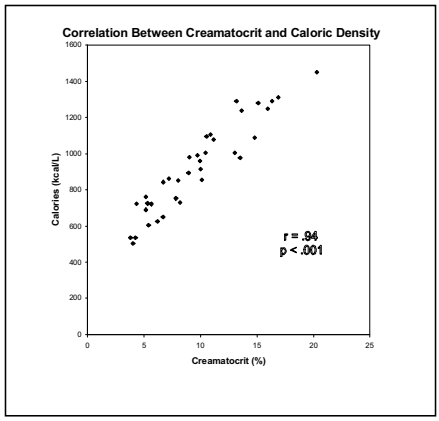
Background: Although the creamatocrit (CRCT) has been used extensively in published research, it has only recently been incorporated into the clinical management of lactation and feeding problems. However, the standard equipment for measuring CRCTs is not conducive to use in the clinical setting. The purpose of this study was to compare the intra- and inter-rater reliability, absolute CRCT values, and predictive accuracy of the Creamatocrit Plus (Separation Technology, Altamonte Springs, FL) with two standard techniques for performing CRCTs: the laboratory centrifuge with a hematocrit reader and with digital calipers. **Methods**: Twelve women each provided foremilk, hindmilk, and composite milk (total = 36 specimens) ≥8 days post-birth. For each specimen, two investigators performed 6 CRCTs: two each using the Creamatocrit Plus, the standard centrifuge with hematocrit reader, and the standard centrifuge with digital calipers. Investigators were blinded to each other's measures. Laboratory measures of lipid and caloric content were performed by biochemists that were blinded to CRCT values. **Results**: Intra- and interrater reliability and absolute CRCT measures were statistically similar for the three CRCT techniques. CRCTs obtained using the Creamatocrit Plus were highly correlated with laboratory measures of lipid (r = .95) and calories (r = .94). (See figures). The resulting regression equations accurately estimated lipid and calories, with small mean differences between actual and estimated lipid (0.003 g/l) and calories (0.004 kCal/l). **Conclusions**: We conclude that the Creamatocrit Plus is comparable to standard CRCT techniques with respect to intra- and interrater reliability, equivalence of CRCT measures, and predictive accuracy. Its 1-pound weight, noiseless operation, and automatic calculation of the CRCT from an imbedded digital reader make it especially attractive for use in the clinical setting. **Acknowledgement**: Funded by Separation Technology, Inc., Altamonte Springs, FL.





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A80. FREQUENCY, VOLUME AND MILK FAT CONTENT OF BREASTFEEDS OF EXCLUSIVELY BREASTFED BABIES

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Background: There is little information on the normal variation in the feeding behaviour of the demand-fed infant. We aimed to provide information on the frequency of breastfeeds and the amount, and fat content, of milk consumed by babies who are exclusively breast-fed. **Methods**: Seventy-one mothers of babies between 1- and 6-months old who were exclusively breastfeeding on demand measured their 24-h milk production from each breast by test weighing their babies before and after each breastfeed. In addition, small milk samples were collected from each breast immediately before and after each breastfeed. The measurement of the fat content of these samples allowed calculation of the degree of fullness of the breast, and the amount of milk available in the breast when the sample was taken. **Results**: The 24-h milk production ranged from 478 to 1298 g, with a mean of 798 g (SD 169). The right breast of 72 % of the mothers produced significantly more milk than the left breast. Babies had 11 breastfeeds per day (range 6–18), and took 76 g (SD 24) at each feed (range 0–240 g). Only 14 % of the babies always fed from both breasts with less than 30 min between breasts, while 27 % of the babies always fed from only one breast. Two-thirds of the babies fed at night (10 pm to 4 am). During this interval these babies had their biggest feed from the more productive breast, and consumed 20 % of their total intake. Babies who did not feed at night had their biggest feed in the morning (4 am to 10 am). The number of feeds was evenly distributed between morning, day (10 am to 2 pm) and evening (2 pm to 10 pm) with fewer feeds at night. The babies took 67 % (SD 11) of the available milk at each feed and rarely emptied the breast. The fat content of the milk was 41 g/L (SD 8) and ranged from 22 to 62 g/L. A high fat feed did not necessarily result in a long between-feed interval. The 24-h fat intake of babies was 32 g (SD 8) and was not affected by the frequency of feeding. **Conclusion:** There is a three-fold variation in total milk production, frequency of feeds and fat intake of babies. Night feeds are common and comprise a significant proportion of the daily intake of most babies. These data suggest that in established lactation the breast does not need to be emptied at each feed to maintain milk production. Mothers should be encouraged to respond to their babies' cues rather than strive to be 'average'.

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A81. INDUCTION OF DRUG METABOLISING ENZYMES BY INFANT FORMULAS AND HUMAN MILK

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Background: Elimination of caffeine used to treat neonatal apnea is 3-fold slower in breast-fed infants than in formula-fed infants. Caffeine is metabolised by cytochrome P450 (CYP)1A and partly by CYP3A4. Paradoxically, human milk reportedly contains higher levels of environmental pollutants, potent inducers of CYP1A, while little is known about presence of inducers of CYP3A4 in mammalian milk. CYP1A and CYP3A4 expressions are regulated by the aryl hydrocarbon receptor (AhR) and the Pregnane X Receptor (hPXR), respectively, which are ligand-activated transcription factors. Also, AhR-mediated CYP1A induction is implicated in carcinogenesis. To gain mechanistic insight into enhanced caffeine elimination in formula-fed infants, we tested the hypothesis that infant formula induces CYP1A and/or CYP3A4, but not human milk, through their respective main regulation pathways. Methods: Mature human milk obtained from 39 lactating women with no medication, and infant formulas (cow milk-based, and soy-based) were tested on HepG2 cells. Rifampicin and dexamethasone were used as a classical PXR activator, and dibenz[a,h]anthracene (DBA) as an AhR ligand. AhR and PXR activation were tested using reporter constructs with respective response elements. Results: Formula but not human milk induced CYP1A mRNA and protein, and activated AhR. The AhR activation was abolished by the co-treatment of 3,4-dimethoxyflavone, an AhR antagonist. Human milk and infant formula also had differential effects on AhR activation mediated by DBA, a potent AhR ligand. On the other hand, both infant formulas and human milk showed mild induction of CYP3A4 mRNA. As expected, formulas and human milk mildly activated hPXR. Importantly, co-transfection of hPXR dramatically increased the activation levels by rifampicin (>70-fold over control), and dexamethasone (>10-fold). However, formula and human milk did not show substantial increase in activation, suggesting that their CYP3A4 induction is mediated by non-PXR pathway. **Conclusion:** Infant formulas contain AhR agonist(s) that increases CYP1A expression. Human milk does not elicit the same effect. In contrast, both infant formulas and human milk induce CYP3A4 through non PXR-mediated pathway.

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