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The Window of Opportunity: Pre-Pregnancy to 24 Months of Age

David J.P. Barker
Renate L. Bergmann
Pearay L. Ogra

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Nestlé Nutrition INSTITUTE

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Preface

Extensive scientific information acquired over the past 5 decades has clearly demonstrated that human fetal development, pre- and postnatal growth, and subsequent expression of physiologic (normal) or pathologic (disease) states is largely a function of the inherited genetic constitution of the individual. But growing evidence suggests that developmental programming of fundamental life processes by early genetic imprinting on the expression of genes occurs by exposure to a diverse spectrum of nutrients, macromolecules, microbial agents and other cellular or soluble components present in the external environment.

The concept of the developmental origins of adult diseases emphasizes the importance of intrauterine and early life events in the etiology of human diseases, and offers explanations for the increasing prevalence of diseases such as obesity, hypertension, diabetes mellitus, atopic dermatitis, asthma, food allergies, systemic lupus, rheumatoid arthritis, and others. Although the precise mechanisms underlying the empirical findings of early programming of such disease states have only partly been elucidated, there is evidence to suggest that a critical 'window of opportunity' may exist in the human infant before and during pregnancy, and up to 24 months of age. Altered exposure to different environmental agents during this critical period may determine the nature of responses in the perinatal period, and the expression of specific diseases states in later life.

This workshop was organized to explore in some detail the nature and the eventual outcome of these complex interactions between the individual and the external environment, relative to this window of opportunity in the fetal and early neonatal period. The participants included many outstanding scientists who have proposed the concepts of such programming and other scholars in basic biology, epidemiology, microbiology and immunology who have made seminal contributions to this field.

Most presentations made at the workshop focused on the impact of perinatal growth, nutrition, environmental microflora, and host immune responses, on the outcome of health and disease in later life. The opening session provided a forum to discuss the basic biology of human growth and its relationship to cardiovascular and renal growth and disease, development of bone matrix, and genetic aspects of disease in later life. The second session concentrated on maternal nutrition before and during pregnancy, including the

impact of under-nutrition and growth restrictions, maternal overweight and diabetes, neonatal overweight perinatal nutritional programming, nutrition in the first 6 months of life and their impact on growth and development in late infancy. The final session of the workshop considered in some detail the patterns of growth and host–pathogen interactions in tropical Africa, induction of antigen-specific immunity in the human neonate and infant, impact of fetal and perinatal viral and parasitic infections on later development of disease, effects of early exposure to environmental microbial flora on the immunologic homeostasis, subsequent immune responses and eventual disease outcome.

This publication includes the full text of the authors' presentations and the ensuing discussions. A comprehensive summary of the presentations and broad conclusions drawn during the deliberations is included under Concluding Remarks.

David J.P. Barker
Renate L. Bergmann
Pearay L. Ogra

Foreword

The 61st Nestlé Nutrition Workshop – Pediatric Program, entitled 'The Window of Opportunity: Pre-Pregnancy to 24 Months of Age', was held in Bali, Indonesia, in early April 2007. The importance of proper nutrition during pregnancy, lactation, and infancy for later health has been addressed from a number of different angles right from the origins of this workshop series. Indeed the very first Nestlé Nutrition Workshop in 1980 'Maternal Nutrition in Pregnancy – Eating for Two', chaired by *John Dobbing*, focussed on the role of nutrition of the mother-to-be in the determination of fetal growth. Some years later, the 36th Nestlé Nutrition Workshop 'Long-Term Consequences of Early Feeding', chaired by *John Boulton, Zvi Laron* and *Jean Rey*, dealt mainly with the impact of nutrition during early infancy on health outcomes in later life, including mental development, obesity, cardiovascular disease and immune response. One presentation by *David Barker* addressed the long-term consequences of fetal growth and also raised the question of the potential importance of nutrition during pre-pregnancy for fetal development.

In 2004–2005, three Nestlé Nutrition Workshops covered various aspects of longer term health effects of nutrition during pregnancy, infancy and childhood:

No. 55: The Impact of Maternal Nutrition on the Offspring
Chairpersons: *Gerard Hornstra, Ricardo Uauy* and *Xiaoguang Yang*

No. 56: Feeding during Late Infancy and Early Childhood: Impact on Health
Chairpersons: *Olle Hernell* and *Jacques Schmitz*

No. 57: Primary Prevention by Nutrition Intervention in Infancy and Childhood
Chairpersons: *Alan Lucas* and *Hugh Sampson*

In 2006, the United Nations Standing Committee on Nutrition, considering the double burden of undernutrition and obesity in many countries, proposed the intensification of efforts to improve nutrition at the local, national and global levels, with major 'focus on the window of opportunity from pre-conception to around 24 months of age, the critical period when the foundation for life long health is set'. Thus with the observed alarming increases in nutrition-related conditions such as obesity, cardiovascular disease, diabetes, osteoporosis, allergy and other disorders of immune function, we felt it was timely to review current knowledge on the influence of nutrition during the critical period of pre-pregnancy through infancy on such conditions. Against this background, the chairpersons of this workshop, *David Barker, Renate Bergmann* and *Pearay Ogra*, designed a workshop program focussing on this window of opportunity, looking not only at

conditions such as heart disease, obesity and diabetes, but also addressing the effects of early nutrition and growth on the development of immune function. We warmly acknowledge the excellent scientific program conceived by the chairpersons. We are also indebted to all the renowned speakers and experts who came from across the globe to review and debate this important topic.

Finally, we wish to thank and congratulate Dr. *Leilani Lestarina* and her team from Nestlé Nutrition Institute – Indonesia for their first class logistic support, allowing the workshop to take place under ideal conditions and the participants to experience the wonderful culture and hospitality of the Balinese.

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61st Nestlé Nutrition Workshop
Pediatric Program
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The Biology of Growth

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Abstract

Variability in human growth is not only in the timing of critical periods within the whole pattern of growth but also in the magnitude and rate of change coincident with the period. In addition, for a radical change in, e.g., height to occur there must also be changes in the anatomical parts that make up total height and these changes are themselves variable. Acceleration, for instance in height velocity, may be the result of different changes in the length of the spine, femur, and/or tibia, each of which may contribute differently to the total process. In addition, not only may the process be variable within a single child, it may also be variable between different children of the same or opposite sexes. The mathematical and statistical problems arising from the seemingly simple process of an increase in height are thus complex. In order to review the biology of human growth this contribution will discuss the principles of growth that are fundamental to our ability to interpret the response of the child to factors that might modify the genetically programmed pattern of growth from conception to maturity. In this way the biology of human growth will be described by a set of phenomena that reflect the actions of biological control mechanisms. These mechanisms are subject to genetic and environmental influences and their expression is characterised by variation in timing, magnitude, and duration.

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Introduction

Our knowledge of the biology of human growth and development is based on research conducted almost exclusively during the last 100 years. Although this research has its foundations in the Age of Enlightenment in 18th century France, the expansion of knowledge in the 20th century was due to the initiation and development of a series of longitudinal growth studies in the United States of America [1]. From 1904 to 1948, 17 such studies were started and 11 completed. Their complexity varied from the relatively simple elucidation of the development of height and weight to more complex data yielding correlations

between behavior, personality, social background and physical development. By the 1950s these studies had reached a level of maturity sufficient for the data to be analyzed to demonstrate not simply the pattern of normal growth in a variety of physical dimensions but also the extent of normal variation. During the years after World War II a series of European studies (for example, in England, France, Sweden, Switzerland, and Belgium), coordinated by the Centre Internationale de l'Enfance in Paris, was able, through the formation of multidisciplinary teams, to study human growth with a greater degree of statistical and mathematical sophistication.

Our understanding of human growth was advanced not only by dealing with the core problem of human variability in normal growth but also by applying this knowledge to the diagnosis of abnormal growth and growth disorders.

Variability in human growth is not only in the timing of critical periods within the whole pattern of growth (e.g. puberty) but also in the magnitude and rate of change coincident with the period. In addition, for a radical change in, e.g., height to occur there must also be changes in the anatomical parts that make up total height and these changes are themselves variable. Acceleration, for instance, in height velocity may be the result of different changes in the length of the spine, femur, and/or tibia, each of which may contribute differently to the total process. In addition, not only may the process be variable within a single child, it may also be variable between different children of the same or opposite sexes. The mathematical and statistical problems arising from the seemingly simple process of an increase in height are thus complex and their elucidation was reliant on the close collaboration of clinicians, biologists and statistical mathematicians.

In order to review the biology of human growth this contribution will discuss the principles of growth that are fundamental to our ability to interpret the response of the child to factors that might modify the genetically programmed pattern of growth from conception to maturity. In this way the biology of human growth will be described by a set of phenomena that reflect the actions of biological control mechanisms. These mechanisms are subject to genetic and environmental influences and their expression is characterized by variation in timing, magnitude, and duration.

Growth is Nonlinear and Discontinuous

Our understanding of the biology of growth is dependent on our ability to detect the pattern of growth of the whole body and its various tissues and organ systems, i.e. the magnitude of change in relation to time. The frequency with which we measure the process of growth dictates the observed pattern. Thus if growth is assessed only at birth and 18 years the pattern of growth will appear to be a straight line suggesting a constant amount of growth per unit time from infancy to adult maturity. If additional measurements are made at

Fig. 1. The pattern of growth in height when assessed at yearly and 6-month intervals. (Redrawn from Tanner JM: Growth at Adolescence, ed 2. Oxford, Blackwell Scientific, 1962).

e.g. 5, 10, and 15 years then it becomes apparent that there are two major components to the pattern of postnatal growth; a childhood component from birth to about 10 years and an adolescent component from 10 years to adulthood. If growth is assessed yearly it is characterized by a series of two or perhaps three curves that may be modeled by logistic functions. These curves divide the process into infant, childhood, and adolescent components from approximately birth to 3, 3–10, and 10–18 years, respectively (fig. 1).

Postnatal growth is, of course, a continuation of the pattern begun at conception. Fetal growth is normally assessed from about 10 to 12 postmenstrual weeks of gestation when the embryo becomes 'recognizably human'. Growth in length or weight appears as a smooth curve but, when examined in terms of velocity (rate of change of distance with time), it demonstrates peaks in growth in length between 20 and 30 weeks of gestation and in weight between 30 and 40 weeks of gestation. If the frequency of assessment of postnatal growth is increased to daily or weekly measurements, as has been done by Lampl et al. [2], then growth in length corresponds to a series of aperiodic saltatory episodes separated by periods of stasis in which no growth occurs. In a sample of 31 infants studied by Lampl et al. [2], periods of stasis lasted from 3 to 63 days and saltation occurred during periods as short as 24 h in which the infants grew up to 2.5 cm in length.

The pattern of growth that emerges as the frequency of assessment increases changes our approach to understanding the control mechanism.

A continuous linear model suggests a mechanism that acts continuously perhaps with the constant release of a growth-promoting hormone. A curvilinear model composed of two or three phases (infancy, childhood, and adolescence) suggests modifications of the controlling mechanism at certain important and perhaps critical periods, at the end of infancy and childhood. A model based on saltation and stasis implies a discontinuous aperiodic control mechanism that underlies a more general mechanism occurring in three phases. One such aperiodic mechanism is found in the control of the cell cycle in which the cell leaves the cycle during G1 and becomes quiescent (stage G0).

Growth Occurs at Different Rates in Different Tissues

Different tissues reflect different growth rates, perhaps because of different lengths of the quiescent G0 phase of the cell cycle. In the first 5 years of life, for instance, the nervous system grows rapidly to the extent that the brain reaches 95% of its adult size by about 7 years of age. Conversely tissues of the reproductive system, e.g., breasts and genitalia, do not demonstrate rapid growth until after 10 years of age. The tissues of the lymphatic system, e.g. thymus, grow rapidly in the first 10 years of life to achieve a size approximately 80% greater than they will be in adulthood but then recede during adolescence. These growth rates are in contrast to the curve of general linear growth that is represented by height with its clear childhood and adolescent components. The growth of adipose tissue, represented by subcutaneous fat, demonstrates clear sexual dimorphism. There is greater acquisition of fat in girls and the development of the classic gynoid distribution with fat accumulation in the gluteo-femoral region in contrast to the android abdominal distribution in boys. An index of body size and composition, such as body mass index exhibits yet another pattern of growth with a sharp increase in the first year followed by a reduction until a rebound, the 'adipose rebound' [3] occurs during early to mid-childhood (fig. 2).

Growing Organisms Demonstrate Allometric Growth

Given these different growth patterns it is not surprising that the organism exhibits variation in the proportions of body segments that change with advancing age. These proportional changes in size, caused by tissue-specific growth rates (allometric growth), are most marked during fetal growth but are apparent throughout childhood and adolescence (fig. 3).

At the beginning of the fetal period (about 9 post-fertilization weeks) the head is 50% of total body length and the lower limbs make up approximately

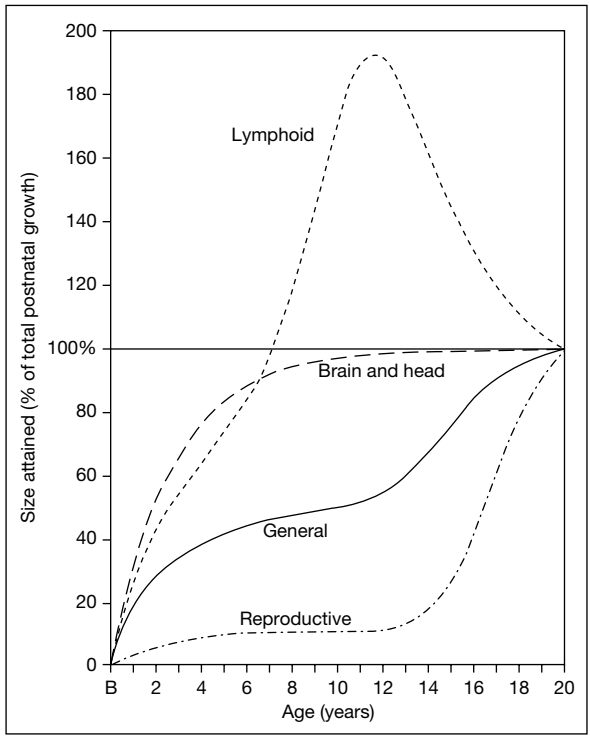

Fig. 2. Growth curves of different parts and tissues of the body, showing the four main types: lymphoid (thymus, lymph nodes, intestinal lymph masses); brain, neural tissue and head (brain and its parts, dura, spinal cord, optic system, cranial dimensions); general tissue (whole body linear dimensions, respiratory and digestive organs, kidneys, aortic and pulmonary trunks, musculature, blood volume), and reproductive tissue (testes, ovary, epididymis, prostate, seminal vesicles, fallopian tubes. From Tanner JM: Growth at Adolescence, ed 2. Oxford, Blackwell Scientific, 1955.)

20% of total length. By mid-gestation (19 post-fertilization weeks) the head has reduced to about 30% of total length and the lower limbs have increased to also form about 30% of total length. These proportional changes, or allometric growth, continue throughout childhood and adolescence achieving adult proportions at the end of growth in length at about 18 years. The controlling mechanisms for allometric growth are far from clear although the pattern of growth of the limbs during prenatal growth may well be affected by the unique nature of the fetal circulation diverting the most oxygenated blood to the head and brain whilst the least oxygenated blood passes through the ductus arteriosus to the descending aorta and lower limbs.

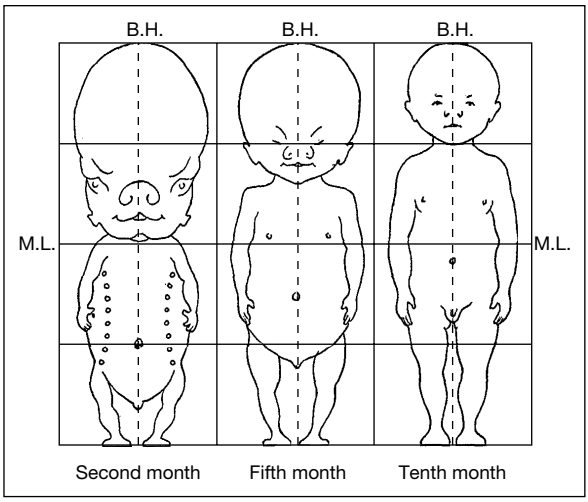

Fig. 3. Proportional changes in fetal growth.

Tissues Exhibiting the Most Rapid Growth Are the Most Sensitive to Insult

When the child comes into contact with factors that are likely to constrain growth it is the tissues that are growing most rapidly that are likely to be more affected. We would not, for instance, expect tissues of the reproductive system (genitalia, breast, etc.) to be influenced by an adverse factor, e.g. malnutrition acting during infancy. On the other hand, malnutrition during infancy is likely to have a long-term effect on the neural tissue which is growing most rapidly at that time. Martorell et al. [4], for instance, recorded the long-term functional deficit attributable to nutritional stunting during the first 3–6 years of life in Guatemalan children. Their analysis of follow-up data on 249 Guatemalan children (120 males) measured at 3 years of age and again as adults showed that the 70% of males and 80% of females who were stunted at 3 years were significantly compromised in terms of both size and functional ability in early adulthood.

Organisms Demonstrate Variability in Their Tempo of Growth

Biological change involves two processes; growth and maturation. The former is structural and the latter functional. Rates of maturation, or tempo of growth, differ considerably within and between the sexes as figure 4 demonstrates.

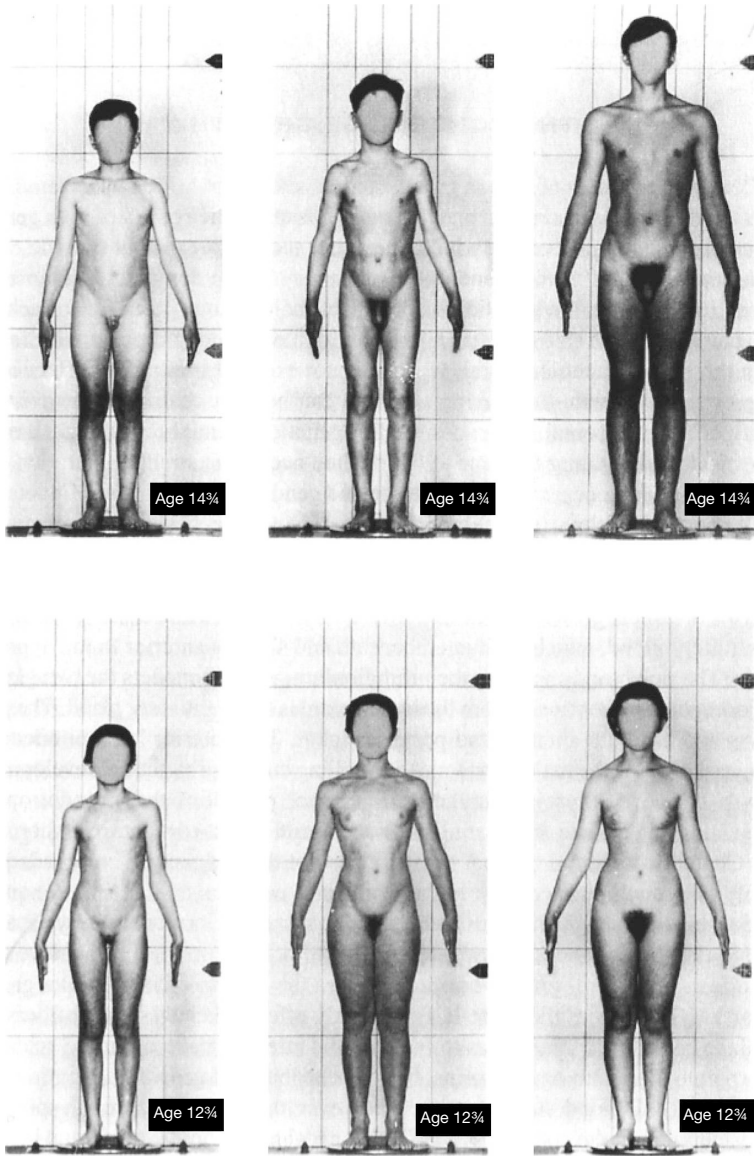

Fig. 4. Three boys and three girls photographed at the same chronological ages within sex; 12.75 years for girls and 14.75 years for boys. From Tanner JM: Growth and endocrinology of the adolescent; in Gardner L (ed): Endocrine and Genetic Diseases of Childhood, ed 2. Philadelphia, Saunders, 1975.)

Figure 4 shows 3 boys and 3 girls who are of the same ages within gender; the boys are exactly 14.75 years of age and the girls 12.75 years of age. The most striking feature of this illustration is that even though they are the same age they demonstrate vastly different degrees of maturity within the limits of normal variation. The average girl will enter the adolescent growth spurt at about 10 years of age, experience peak height velocity at 12 years, and reach adult height (a growth velocity of less than $1\,cm \cdot year^{-1}$) at about 16 years. The average boy will be delayed by 2 years compared to the average girl, entering the growth spurt at 12 years, experiencing peak height velocity at 14 years, and reaching adult height at 18 years. The extra 2 years of pre-adolescent growth is the major reason for the average adult male being taller than the average adult female; 2 years of growth at about $5\,cm \cdot year^{-1}$ result in about 10 cm of extra adult height and a slightly greater peak height velocity accounts for another 2–3 cm. The variability associated with this average pattern can be remarkable as figure 4 demonstrates. It is not surprising that the individual's response to environmental factors affecting the process of growth is dependent on their level of maturity.

Sexual Dimorphism Occurs in Size and Tempo of Growth

In addition to the variability within the sexes, there is clearly variability between the sexes with girls in advance of boys. The 2-year difference in entry to the adolescent growth spurt is the result of a gradually increasing gap in maturity that has it roots in fetal growth evidenced by a 2-week advancement [5]. The differences between the sexes are a mark of sexual dimorphism which becomes more pronounced during adolescence as the functional requirements for successful reproduction become established. However, the male delay in age at entry to pubertal development is not as marked as that for somatic growth. Mean age at entry to puberty, usually marked by a change in breast development in girls and genitalia development in boys differs by approximately 6 months [6, 7]. The comparison of the timing of the adolescent growth spurt with the timing of pubertal development demonstrates that the relationship between somatic growth and maturation differ between the sexes (fig. 5).

Figure 5 illustrates that boys begin their pubertal development prior to the initiation of the adolescent growth spurt, whilst girls have a more synchronized start to both puberty and adolescent growth. The reasons for this degree of sexual dimorphism and the relationship between the different timing of puberty and adolescent growth between the sexes are mostly speculative. Clearly some advantage must result from this differential timing and that advantage must be related to the success of reproduction. As has been suggested by Bogin [5], it may be that the early sexual maturity of late adolescent boys that is not accompanied by the adult size and body composition of men allows them to experiment sexually without representing a physical challenge to adults. Similarly the

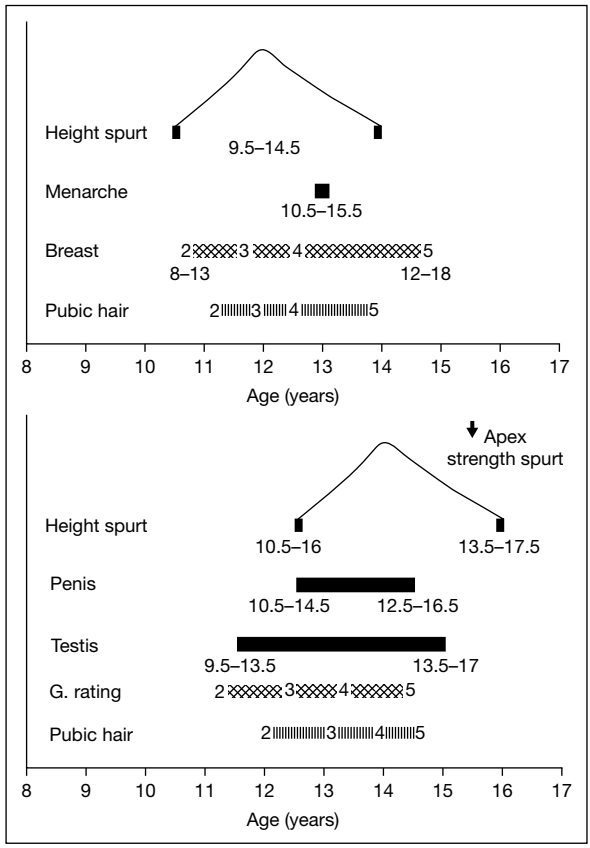

Fig. 5. The differential timing of adolescent growth and pubertal development between boys and girls. Breast, genitalia (G), and pubic hair development are denoted by Tanner scales of 2–5. Numbers represent the normal range for early and late developers exhibiting peak height velocity, menarche, penis and testicular development. From Marshall and Tanner [7].

pubertal timing of adolescent girls results in simultaneous physical and sexual maturity and an increased attractiveness to slightly older, and more capable, male partners than relatively immature boys of similar age.

In the Absence of Insult Organisms Grow to Their 'Genetic Potential'

In order to assess the normality or otherwise of the growth of children we use 'growth reference charts'. These charts depict both the average height to be

expected throughout the growing years (typically from birth to 18 years), and the range of normal heights, in the form of percentile or 'centile' distributions.

Children who do not have constraints upon their growth exhibit patterns of growth that fall steadily and continuously parallel to the centile lines prior to adolescence. However, as the adolescent growth spurt takes place they will depart from this parallel pattern and all adolescents will demonstrate 'centile crossing'. If they are 'early developers' their height-for-age curve will rise through the centiles before their peers and level off early as they achieve their adult stature. 'Late developers', on the other hand, will initially appear to fall away from their peers, as the latter enter their growth spurts, and then accelerate into adolescence rising through the centile lines when their peers have ceased, or nearly ceased growing. Even the child who enters their growth spurt at the average age for the population will cross centile lines. This is because the source data for these reference charts were collected in cross-sectional studies; studies in which children of different ages were measured on a single occasion. They thus reflect the average heights, weights, etc., of the population rather than the growth of an individual child. If one were able to undertake a growth study of the same children over many years (a longitudinal study), one could theoretically adjust the data so that they illustrated the adolescent growth spurt of the average child, i.e. the child experiencing the adolescent growth spurt at the average age. In such a hypothetical situation the growth curve of the average child would fall exactly on the 50th centile line. But that is not the case with growth reference charts based on cross-sectional data; the average child will initially fall away from the 50th centile line as he/she enters the growth spurt and then cross it at the time of maximum velocity (peak velocity) before settling back onto the 50th centile as he/she reaches adult height.

Figure 6 illustrates the typical growth patterns exhibited by early, average, and late developers plotted on a growth reference chart. The early developing girl (E) accelerates into adolescence at about 8 years of age, some 2 years prior to the average, and rapidly crosses centile lines to move from just above the 50th to the 90th centile. However, her growth slows at about 13 years and her height centile status falls back to the 50th centile. Conversely the late developer (L) is almost 13 years old before she starts to accelerate and that delay causes her height centile status to fall from the 50th to below the 10th centile before rising to the 50th centile as she approaches adulthood. Finally the average girl (A) initially falls away from the 50th centile but then accelerates through it at the average age of peak velocity before following the 50th centile as adulthood is reached.

Figure 6 demonstrates more than simply the crossing of centiles by early and late developers. It also tells us something about the control of human growth. These are not hypothetical curves. They are the growth curves of real children who were measured on a 6- or 3-month basis throughout childhood and adolescence [8]. Note that during childhood they are growing on or near

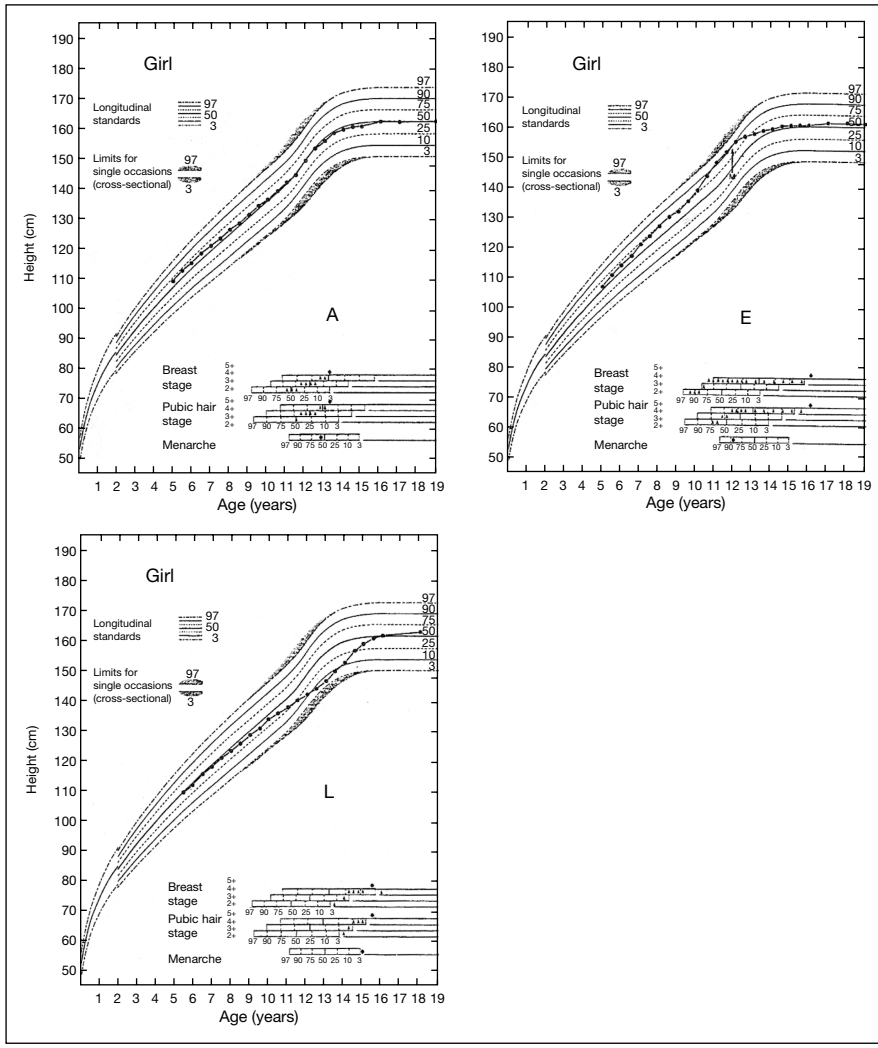

Fig. 6. Growth curves of average (A), early (E), and late (L) developers. Data from subjects 35, 38 and 45 in Tanner and Whitehouse [8].

to the 50th centile and after the deviations brought about by their adolescent growth spurts they return to that same centile position in adulthood. Such adherence to particular centile positions is found time and again when one studies the growth of children. Indeed it is true to say that all children, when in an environment that does not constrain their growth, will exhibit a pattern of growth that is more or less parallel to a particular centile or within some imaginary 'canal'. This phenomenon was described by a British geneticist,

C.H. Waddington, in 1957 and has been termed 'canalization' or 'homeorrhesis' [9]. It is most likely that this pattern is genetically determined and that growth is target seeking in that we have a genetic potential for adult stature and the process of growth, in an unconstrained environment, takes us inexorably towards that target.

In Response to Insult the Organism Demonstrates Growth Faltering

However, it is a truism to say that none of us have lived or been brought up in a completely unconstrained environment. Towards the end of our intrauterine life our growth was constrained by the size of the uterus. During infancy and childhood we succumbed to a variety of childhood diseases that caused us to lose our appetite and at those times our growth would have reflected the insult by appearing to slow down or, in more severe cases, to actually cease.

Waddington [9] likens growth to the movement of a ball rolling down a valley floor. The sides of the valley keep the ball rolling steadily down the central course. If an insult occurs it tends to push the ball out of its groove or canal and force it up the side of the valley. The amount of deviation from the predetermined pathway will depend on the severity and duration of the insult. However any insult will cause a loss of position and a reduction in growth velocity as the ball is confronted by the more severe slope of the valley wall. The magnitude of the loss of velocity will also depend on the severity and duration of the insult. Thus a small insult of short duration will cause a slight shift onto the valley sides which will entail a minor change in velocity. The alleviation of the insult will result in a rapid return to the valley floor at an increased velocity. Having reached the floor normal growth velocity is resumed.

In Response to the Alleviation of Insult the Organism Demonstrates Catch-Up Growth

This analogy may be seen to apply appropriately to the process of human growth. Figure 7 shows the growth chart of a girl who has suffered from celiac syndrome. In this condition there is an abnormality of the lining of the gut and food cannot be absorbed resulting in the child being starved. The result in terms of growth is that the height velocity is gradually reduced as the malnutrition becomes more and more severe.

The reduction in height velocity means that the height distance curve leaves the normal range of centiles and the child becomes abnormally short for her age. So, at the age of almost 12 years she is the average height of a 5-year-old.

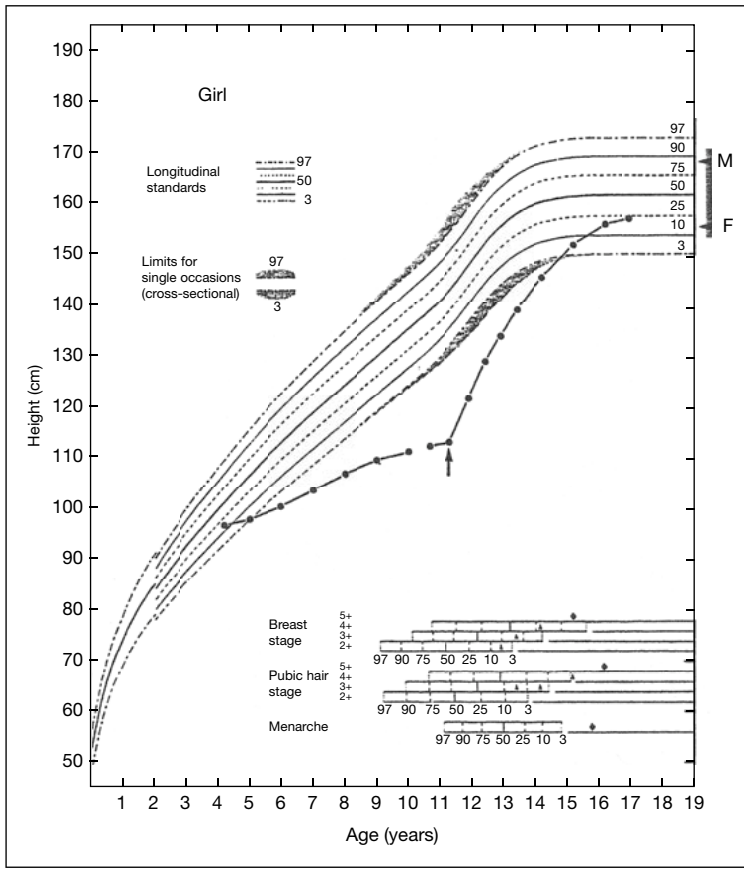

Fig. 7. Catch-up growth exhibited by a child with celiac syndrome. From Tanner and Whitehouse [8].

On diagnosis the child is switched to a gluten-free diet which alleviates the malabsorption. Recovery of height velocity is rapid and jumps from 1 to $14 \cdot \text{cm year}^{-1}$ returning the child to the normal range of centiles within 3 years. Indeed this girl ends up within the range of heights one would expect given the heights of her parents. So she demonstrates 'complete' catch-up growth in that she returns to the centile position from which she most probably started.

Catch-up growth is, however, not always complete and appears to depend on the timing, severity and duration of the insult. This appears to be particularly true in the treatment of hormone deficiencies. Initial diagnosis is often delayed until the child is seen in relation to other children and the deficiency in stature becomes obvious. Usually a hormone deficiency, e.g. growth hormone deficiency, is also accompanied by a delay in maturation. Response to

treatment appears to depend on some pretreatment factors such as chronological age, height, weight and skeletal maturity, i.e. on how long the child has been deficient, how severe the deficiency in height and weight are, and by how much the maturity has been affected.

Growth Is Characterized by 'Critical Periods' of Sensitivity with Long-Term Sequelae

This apparent sensitivity to the timing, intensity and duration of insult has given rise to the recognition of 'critical periods' during which insult appears to have long-term effects in terms of health and wellbeing.

It has long been recognized that there are 'critical periods' during mammalian development when exposure to specific environmental stimuli are required in order to elicit the normal development of particular anatomical structures or their normal functioning. The responses of the organism to these stimuli depend on there being a specific level of anatomical maturation *and* a state of rapid anatomical and/or functional change [10]. So-called 'critical periods' in growth are not confined to the classic definition of a narrow time frame of development during which a particular environmental threshold or limit must exist for normal growth and function to ensue. Using both auxological and epidemiological approaches, Cameron and Demerath [11] suggested a lifespan perspective which encompasses accumulating and interacting risks that are manifest from prenatal life onward. By understanding the process of growth and development, and by scrutinizing the growth process, early variations that lead to later disease can be identified.

The fetus, for instance, appears to respond to insults during the prenatal period through the process of 'programming' which has short-term survival advantage but may have long-term disadvantage in that it is associated with cardiovascular disease, hypertension, type 2 diabetes and later obesity. Low birthweight combined with rapid postnatal growth during infancy also appears to be associated, for instance, with later childhood and adult sequelae in terms of glucose tolerance and obesity. Independent of birthweight, the timing of adiposity rebound during mid-childhood also predicts later obesity. The timing, magnitude, and duration of adolescent growth and maturation are associated with critical body composition changes including the normal acquisition of body fat and bone mineralization. In particular the acquisition of appropriate peak bone mass is critical in determining later risk of osteoporosis.

It may thus appear that having identified growth before birth, during infancy, mid-childhood, and adolescence as being critical then the whole of growth from conception to full maturity exists within a critical milieu. To some extent that is true. The growing organism is continuously sensitive to its environment and is affected by both acute and chronic influences. The question of whether growth variation, per se, programs later health or whether

growth is merely a marker for underlying variation in physiological programming cannot yet be answered with the existing evidence. While the results of epidemiological research implicate growth as a significant factor, overall size or rate of growth is the outcome of multifactorial determinants. Some of these determinants may be related to susceptibility to adult disease, and others may not. Further, there are many genetic and environmental determinants of adult disease that are not specifically related to growth.

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Discussion

Dr. Barker: The existence of catch-up growth says that every child has the ability to accelerate growth. It follows that every child could grow faster than it is actually growing. It therefore follows that no child grows to its genetic potential and it may be an unhelpful concept because the interesting question might be why don't human beings grow faster than they do, knowing that they could under certain circumstances. It may be that the costs of rapid growth are too high. Growth has costs; if you put energy into growth, then you can't do other things, like possibly put repair mechanisms into place. So what is this genetic potential, is it reality or is merely an old it concept?

Dr. Cameron: Quite right, what is this potential? That is why I call it a potential, and yes it does appear that growth is extremely expensive. Some of the work that Lampl et al. [1] showed with their saltation–stasis model was when they assessed 31 infants under the age of 2 and followed them for a period of time. They also took clinical notes on the children while assessing them, and some time later they again looked at these clinical notes and realized that during periods of saltation the parents reported that the children were irritable, hadhigher temperature and colds, whereas

during periods of stasis they were calm, quiet and extensively well. The children tended to get more infection during periods of saltation, which gives some support to the idea that, yes, growth is expensive and it does have a deleterious effect when analyzed in this way. We don't know what this genetic potential is, and this was one of the great discussions in the early 1990s when the WHO was trying to determine how to chart the best of all possible growth and the genetic potential for growth. We don't know what maximum growth is; we believe we can analyze and describe optimal growth if we reduce the factors that we know to constrain growth. Of course, there are unknown factors that will have a constraining effect on growth. There are things we don't know including all the factors that constrain human growth.

Dr. Walker: I want to come back to catch-up growth. You gave us an example of a situation in which a tall father and a mother who was born very small produce low birthweight infants who tend to develop into excessively overweight children with metabolic syndrome. I think that problem exists also in other circumstances in which there is malnutrition and then there is a rebound effect in which children tend to over-expand. Do we know enough about that to make recommendations to curtail catch-up growth, slow it down, and hopefully prevent some of these rebound effects and complications?

Dr. Cameron: Interesting point. The reason why I tend to call catch-up growth rapid growth in infancy is that catch-up growth by definition is growth that returns the child to its pre-insult centile. What we see is again the result of work done to look at the developmental origins hypothesis and in particular the work of Ong and Dunger [2], our colleagues in Cambridge. Children who exhibit rapid growth in the first few years and demonstrate risk factors for type 2 diabetes will start to demonstrate insulin resistance and glucose intolerance at around 7 years according to our data. Children who show more than catch-up growth overshoot, as you quite rightly say, the centile that you would expect them to be on. So as a group they end up with the 75th centile rather than back on the 50th centile. So this is not catch-up growth in our understanding of it from work on growth disorders in the 1970s, 1980s and 1990s; this is unexpectedly rapid growth. If we recognize this, can we start to curtail it so that children don't then demonstrate the adverse risk factors such as centralized fat distribution appearing by the age of 7 or 8 or 9, such as increased fat content and so on? My question back to you will be how do we recognize the child who is going to overshoot as opposed to a child who is going to end up back on the 50th centile?

Dr. Walker: It seems to me that if we recognize that pattern then these are children who need to be carefully followed by the health professional to make sure they don't overshoot. Let them gradually come back to where they should be and do not allow them to overshoot.

Dr. Cameron: Absolutely, but the difficulty is to recognize the child who will overshoot. At the moment one can only really do that by looking at the circumstances that have given rise to growth faltering in the first place or given rise to rapid growth. If one recognizes the child who is growing rapidly, say in the post-weaning period, and looks at a series of circumstances around that, then from research we have determinants of catch-up growth, but unexpectedly rapid growth should be a signal to us that this may be a child who is going to be at risk and therefore we should be watching him. At this moment in time, I think this may be a way to approach this particular situation.

Dr. Ogra: You briefly alluded to the growth of lymphoid tissue and the burst of activity in the early adolescent period. Is there any particular element of lymphoid tissue which is affected by this growth spurt in adolescence? Do you think it may have some important functional implications? Finally, is there any relationship to the sexual dimorphism between the boys and girls, and is it related in any way to the level of immunologic function in adolescence? Have you or anyone else seen any significant functional change related to the increase in lymphoid tissue in adolescents?

Dr. Wilson: As you alluded to, I think there is a potential problem with how the data are ascertained. There is no noninvasive way to do that other than by imaging the thymic mass, which would have to be done by two-dimensional X-rays, with volume estimated by some type of reconstruction.

Dr. Cameron: Some of the early data I am sure is from cadaver material.

Dr. Wilson: The problem with that of course is the nature of the illness that caused the death since lymphoid tissues like the thymic cortex are going to change under stressful situations, so it is not necessarily a very good index. Presumably also there is no good ascertainment of mucosal lymphoid mass, which of course represents the largest lymphoid mass overall in the body, so I would wonder about it. There obviously are some changes in lymphoid function in adolescents. There is this burst of increased risk of tuberculosis disease that is poorly understood and a few other things that do change.

Dr. Ogra: We know in general that when they are young girls are better off immunologically than boys. For example, very early on in life girls have much higher levels of IgA than boys. It will be interesting to know whether the growth spurt is related to an increased availability of any specific substrates of immune function.

Dr. Maldonado: It is clear, at least functionally, that in the neonate there are some early studies looking at the functional differences in growth and development of the immune system. Remarkably those studies were not done until the last decade or so, and most of those are looking at cytokine production and lymphoproliferation, immunogenicity in the context of vaccine administration, etc. It is very clear that there are some functional correlates with proliferation of immune function or lymphoid function perhaps. Again to reiterate what Dr. Ogra said, in the adolescent there are clear differences in HIV-infected children for instance in adolescence than you would see in younger children or in adults in the same situation. Clearly adolescence does have at least some of these functional correlates and I am not sure whether human studies would be possible. Perhaps in animals it might be easier to quantify those differences.

Dr. Prentice: At this meeting we are talking about the first 24 months, but data demonstrate that the pubertal period is critical in terms of catching up some of the lost ground caused by multiple infections. What is our current knowledge in terms of the early growth correlates of the timing, tempo and duration of the pubertal catch-up spurt?

Dr. Cameron: Good question. One of the areas that we are looking at quite extensively at the moment is early determinants of the initiation of puberty, so early determinants of the maturation of the HGA axis. Whether adolescence demonstrates catch-up growth or is compensatory growth for those early insults is a debatable point. Some people describe adolescence as a period of catch-up growth to allow for early insults. The children who have been insulted at an early age during childhood tend to keep growing until their early 20s. The classic picture of human growth in the developing countries, such as South Africa where I work, is for children to keep growing until their early 20s but demonstrate a relatively normal timing in terms of the initiation of the adolescent growth spurt but grow at slower values for longer. So they show this compensatory as opposed to catch-up growth. But the determinants of the initiation of puberty is a fascinating area and one that we haven't got enough research data on because the longitudinal data available to us to do that sort of research are still fairly minimal or the data that we do have had not measured the things that we now know to be important in early life, the first 2, 3, 4 years. So it is an important area.

Dr. Exl-Preysch: I have a question relating to what we were talking about before. I always wonder why are we getting taller than our parents. Is it only because we have

fewer infections and better protein supply, or are there other determining factors? Is it essentially dangerous to be too tall?

Dr. Cameron: We are getting taller, we demonstrate this thing called a secular trend that each generation is taller than the previous generation and matures slightly earlier. One can see that trend quite dramatically if one looks at data from South East Asia. If you look the data from Japan and China you can see dramatic increases in secular trend. Very interestingly there are increases in leg length, particularly the tibia, the anatomical leg, which seems to increase most rapidly when we are recovering from whatever constraint it was that was affecting our growth. Is it just nutrition, is it just good health, we don't know. All of those things aid the secular trend. On looking into my data in South Africa following the end of Apartheid, one of the areas of research is trying to analyze the secular trend being demonstrated by black children who were shorter under Apartheid and are now beginning to get taller What specific determinants we can put against that secular trend? At the moment the situation seems to be that all those things are important in terms of determining the secular trend and the rates at which we grow. Is it bad to be large; well, if you speak to Al Gore he would probably say you are using too many of the environmental resources if you are too large, you eat too much, and so on, compared to someone who is smaller. So it is a good debating point.

Dr. Björkstén: I have an anecdotal comment to this. The Scandinavian Vikings were actually big when they were pillaging Europe, almost as big as we are today, and with Christianity, in the middle ages, we became as tiny as the rest of the Europeans.

Dr. Cameron: It is an important point that the secular trend is not always a positive trend. The secular trend is cyclic, and if one looks back at the ancient Greeks, who divided life into the series of hepdomads, periods of 7 years, the end of the second heptomad, s14 years, was seen as being the point of sexual maturity in girls, probably linked to the age at menarche. In Romeo and Juliette, William Shakespeare talks about Juliette achieving womanhood at 14 years of age. So perhaps menarche then was around about 14. It was not until the 19th century that we started to see menarcheal ages around about the age of 16, and interestingly enough the data we have from the 1860s are Scandinavian which show that menarcheal age in Sweden, Norway and Finland was around about 16 years of age during the 1860s, and now of course it is down to around 12.5 years of year. But secular trends are a cyclic phenomenon; they go up and down. We see negative secular trends particularly in Sub-Saharan Africa, in the least developed countries, and I am sure at the moment in Zimbabwe there are negative secular trends occurring in terms of the growth of those children.

Dr. Malka: Is there any environment hypothesis regarding catch-up growth, like the hygiene hypothesis, in relation to allergy in rural and urban areas? Is there any relation with catch-up growth?

Dr. Cameron: The way we view catch-up growth is as an individual phenomenon. In other words we observe catch-up growth in individuals rather than in groups. Catch-up growth in a clinical situation is when drug treatment is given and the child catches up. Looking at normal children we would not think in terms of catch-up growth as being a sample characteristic. Certainly in terms of accelerated growth in terms of the secular trend, we see differences between rural and urban areas, and the urban environment tends to be one in which children growth more rapidly, they have earlier puberty, and the age at menarche is decreased. My data in South Africa looking at girls and boys living in rural areas as opposed to the urban area of Soweto, show significant differences in height, significant differences in age at menarche, significant differences in the age of Tanner pubertal scaling because they live in urban areas. I tend to think that the data are characterized by a lack of seasonality, by the fact that particular foods can be bought from local supermarkets on any day of the year and

people are not affected by the seasonal supply of those foods, and in the urban environment there is access to better health care. In the rural environment people may have to walk 10 km to the nearest clinic. In the urban environment there is access to sanitation services, access to welfare services, access to better housing, and so on. All these aspects of the urban environment are associated with secular trends. One of the interesting areas that we are beginning to look at now is how these aspects of urbanization affect the growth of the child. For some years now I have had this idea that the normal socioeconomic factors that we apply to human growth are dynamic, they change over time, and the child's response to those factors depends upon the age and maturity of the child. A fact that we know affects growth in the first 3 or 4 years may be maternal education; how well the mother is educated relates to how well she can understand and absorb factors relating to child health and hygiene, for instance, and so clearly has a major effect upon the growth of the infant. Perhaps by the time the child is 5, 6, 7 years of age and is going to school, how well the child is fed and how good the child's socioeconomic environment is in terms of finance become more important, and so family income becomes the important factor of socioeconomic status. These things are dynamic over time and dynamic also in terms of their effect upon the individual whether they are family factors, community factors, or national factors. Some of the research we are doing with social science colleagues at the moment looking at the situation in South Africa is to analyze both family, community and national factors of socioeconomics in relation to the outcome variable of human growth and development.

Dr. Walker: I would like to come back to the comments of Dr. Ogra and Dr. Prentice. There was an experiment in *Nature* in which children who have chronic conditions, such as chronic infection, inflammatory bowel disease and so forth, turn off pubescence and continue to grow for longer periods of time, even into their 20s, as a way of catching up; it is as if nature has decided they need more time to reach their ultimate height. I think it is an example of how that occurs.

Dr. Cameron: I think it is an exquisite relationship, a very complex relationship to look at these things.

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Human Growth and Cardiovascular Disease

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Abstract

Low birthweight is now known to be associated with increased rates of coronary heart disease (CHD) and the related disorders, stroke, hypertension and type 2 diabetes. Associations between low birthweight and later disease have been extensively replicated in studies in different countries. They extend across the normal range of birthweight and depend on lower birthweights in relation to the duration of gestation rather than the effects of premature birth. The associations are thought to be consequences of developmental plasticity, the phenomenon by which one genotype can give rise to a range of different physiological or morphological states in response to different environmental conditions during development. Recent observations have shown that impaired growth in infancy and rapid childhood weight gain exacerbate the effects of impaired prenatal growth. CHD and the disorders related to it arise through a series of interactions between environmental influences and the pathways of growth and development that precede them.

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There is now clear evidence that the pace and pathway of early growth is a major risk factor for the development of a group of chronic diseases that includes coronary heart disease (CHD) and type 2 diabetes, a disorder which predisposes to cardiovascular disease. This has led to a new 'developmental' model for the disease [1, 2]. The model proposes that nutrition during fetal life, infancy and early childhood changes gene expression and thereby establishes functional capacity, metabolic competence, and responses to the later environment [2, 3].

To explore the developmental origins of chronic disease required studies of a kind that had not hitherto been carried out. It was necessary to identify groups of men and women now in middle or late life whose size at birth had

Table 1. Hazard ratios (95% confidence intervals) for death from coronary heart (CHD) disease according to weight at birth and at age 1 year in 10,636 men in Hertfordshire

Weight, kg	Death from CHD	
	before 65 years	all ages
At birth		
≤2.5	1.50 (0.98–2.31)	1.37 (1.00–1.86)
3.0	1.27 (0.89–1.83)	1.29 (1.01–1.66)
3.5	1.17 (0.84–1.63)	1.14 (0.91–1.44)
4.0	1.07 (0.77–1.49)	1.12 (0.89–1.40)
4.5	0.96 (0.66–1.39)	0.97 (0.75–1.25)
>4.5	1.00	1.00
p for trend	0.001	0.005
At age 1 year		
≤8.0	2.22 (1.33–3.73)	1.89 (1.34–2.66)
9.0	1.80 (1.11–2.93)	1.58 (1.15–2.16)
10.0	1.96 (1.23–3.12)	1.66 (1.23–2.25)
11.0	1.52 (0.95–2.45)	1.36 (1.00–1.85)
12.0	1.36 (0.82–2.26)	1.29 (0.93–1.78)
≥12.5	1.00	1.00
p for trend	<0.001	<0.001

been recorded at the time. Their birthweight could thereby be related to the later occurrence of CHD. In Hertfordshire, UK, from 1911 onwards, when women had their babies they were attended by a midwife, who recorded the birthweight. A health visitor went to the baby's home at intervals throughout infancy, and the weight at 1 year was recorded. Table 1 shows the findings in 10,636 men born between 1911 and 1930 [1, 4]. Hazard ratios for CHD fell with increasing birthweight. There were stronger trends with weight at 1 year. A subsequent study confirmed a similar trend with birthweight among women [4]. Table 2 shows findings for a sample of men who had glucose tolerance tests [5]. The percentage with impaired glucose tolerance or type 2 diabetes fell steeply with increasing birthweight. The association between low birthweight and CHD has now been replicated among men and women in Europe, North America and India [6–12]. Low birthweight has been shown to predict altered glucose tolerance in studies of men and women around the world [13–17]. The associations between low birthweight and later disease depend on slow fetal growth rather than premature birth.

Biological Basis

Like other living creatures, in their early life human beings are 'plastic' and able to adapt to their environment. The development of the sweat glands

Table 2. Percentage of 370 men aged 64 years in Hertfordshire with impaired glucose tolerance or diabetes according to weight at birth

Weight, kg	% of men with 2-hour glucose of ≥7.8 mmol/l	Odds ratio (95% CI)[1]
≤2.5	40	6.6 (1.5–28)
3.0	34	4.8 (1.3–17)
3.5	31	4.6 (1.4–16)
4.0	22	2.6 (0.8–8.9)
4.5	13	1.4 (0.3–5.6)
>4.5	14	1.0
p for trend	<0.001	

[1]Adjusted for current body mass index.

provides a simple example of this. All humans have similar numbers of sweat glands at birth but none of them function. In the first 3 years after birth a proportion of the glands become functional, depending on the temperature to which the child is exposed. The hotter the conditions, the greater the number of sweat glands that are programmed to function. After 3 years the process is complete and the number of sweat glands is fixed. Thereafter, the child who has experienced hot conditions will be better equipped to adapt to similar conditions in later life because people with more functioning sweat glands cool down faster.

This brief description encapsulates the essence of developmental plasticity: a critical period when a system is plastic and sensitive to the environment, followed by loss of plasticity and a fixed functional capacity. For most organs and systems the critical period occurs in utero. There are good reasons why it may be advantageous in evolutionary terms for the body to remain plastic during development. It enables the production of phenotypes that are better matched to their environment than would be possible if the same phenotype was produced in all environments. Developmental plasticity is defined as the phenomenon by which one genotype can give rise to a range of different physiological or morphological states in response to different environmental conditions during development [18]. Plasticity during intrauterine life enables animals and humans to receive a 'weather forecast' from their mothers that prepares them for the type of world in which they will have to live [19]. If the mother is poorly nourished, she signals to her unborn baby that the environment it is about to enter is likely to be harsh. The baby responds to these signals by adaptations, such as reduced body size and altered metabolism, which help it to survive a shortage of food after birth. In this way plasticity gives a species the ability to make short-term adaptations, within one generation, in addition to the long-term genetic adaptations that come from natural selection. Because, as Mellanby [20] noted long ago, the ability of a human mother

to nourish her baby is partly determined when she herself is in utero, and by her childhood growth, the human fetus is receiving a weather forecast based not only on conditions at the time of the pregnancy but on conditions a number of decades before [3]. This may be advantageous in populations that experience periodic food shortages.

Until recently we have overlooked a growing body of evidence that systems of the body that are closely related to adult disease, such as the regulation of blood pressure, are also plastic during early development. In animals it is surprisingly easy to produce lifelong changes in the blood pressure and metabolism of a fetus by minor modifications to the diet of the mother before and during pregnancy [21, 22].

The different size of newborn human babies exemplifies plasticity. The growth of babies has to be constrained by the size of the mother, otherwise normal birth could not occur. Small women have small babies: in pregnancies after ovum donation they have small babies even if the woman donating the egg is large [23]. Babies may be small because their growth is constrained in this way or because they lack the nutrients for growth. As McCance [24] wrote, 'The size attained in utero depends on the services which the mother is able to supply. These are mainly food and accommodation.' Research into the developmental origins of disease has focused on the nutrient supply to the baby, while recognizing that other influences, such as hypoxia, stress and maternal size, also influence fetal growth. This focus on fetal nutrition was endorsed in a recent review [25]. The availability of nutrients to the fetus is influenced by the mother's nutrient stores and metabolism, as well as by her diet during pregnancy. In developing countries many babies are undernourished because their mothers are chronically malnourished. Despite current levels of nutrition in Western countries, the nutrition of many fetuses and infants remains suboptimal because the nutrients available are unbalanced or because their delivery is constrained by maternal metabolism. Globally, size at birth in relation to gestational age is a marker of fetal nutrition [25].

A striking feature of the associations between birthweight and later disease is that they are graded, extending across the entire range of birthweights. This implies that what were regarded as normal variations in the delivery of nutrients to the human fetus have profound long-term effects on the health of the next generation [3].

Developmental Origins Hypothesis

The developmental origins hypothesis proposes that CHD, stroke, hypertension and type 2 diabetes originate in developmental plasticity, in response to undernutrition during fetal life and infancy [2, 26]. Why should fetal responses to undernutrition lead to disease in later life? The general answer is clear: the 'life history theory', which embraces all living things, states that

Table 3. Hazard ratios (95% CI) for coronary heart disease in 3,629 men in Helsinki according to the ponderal index at birth (birthweight/length^3) and household income in adult life

Household income in GBP/year	Ponderal index ≤26.0 kg/m^3 (n = 1,475)	Ponderal index >26.0 kg/m^3 (n = 2,154)
<15,700	1.00	1.19 (0.65–2.19)
15,700	1.54 (0.83–2.87)	1.42 (0.78–2.57)
12,400	1.07 (0.51–2.22)	1.66 (0.90–3.07)
10,700	2.07 (1.13–3.79)	1.44 (0.79–2.62)
≤8,400	2.58 (1.45–4.60)	1.37 (0.75–2.51)
p for trend	<0.001	0.75

during development increased allocation of energy to one trait, such as brain growth, necessarily reduces allocation to one or more other traits, such as tissue repair processes. Smaller babies, who have had a lesser allocation of energy, must incur higher costs and these, it seems, include disease in later life. A more specific answer to the question is that people who were small at birth are vulnerable to later disease through three kinds of process. First, they have less functional capacity in key organs, such as the kidney: one theory holds that hypertension is initiated by the reduced number of glomeruli found in people who were small at birth [27]. A second process is the setting of hormones and metabolism. An undernourished baby may establish a 'thrifty' way of handling food. Insulin resistance, which is associated with low birthweight, may be viewed as persistence of a fetal response by which blood glucose concentrations were maintained for the benefit of the brain but at the expense of glucose transport into the muscles and muscle growth [28].

A third link between low birthweight and later disease is that people who were small at birth are more vulnerable to adverse environmental influences in later life. Observations on animals show that the environment during development permanently changes not only the body's structure and function but also its responses to environmental influences encountered in later life [19]. Table 3 shows the effect of low income in adult life on CHD among men in Helsinki [29]. As expected, men who had a low taxable income had higher rates of the disease. There is no agreed explanation for this but the association between poverty and CHD is a major component of the social inequalities in health in many Western countries. Among the men in Helsinki the association was confined to men who had slow fetal growth and were thin at birth, defined by a ponderal index (birthweight/length^3) of ≤26 kg/m^3 (table 3). Among men who were not thin at birth CHD was not associated with income, which implies that they were resilient to the effects of low income.

One explanation for these findings emphasizes the psychosocial consequences of a low position in the social hierarchy, as indicated by low income

and social class, and suggests that perceptions of low social status and lack of success lead to changes in neuroendocrine pathways and hence to disease [30]. The findings in Helsinki seem consistent with this. People who were small at birth are known to have persisting alterations in responses to stress, including raised serum cortisol concentrations [31]. It is suggested that persisting small elevations of cortisol concentrations for many years may have effects similar to those seen when tumors lead to more sudden large increases in glucocorticoid concentrations. People with Cushing's syndrome, the result of overactivity of the adrenal cortex, are insulin-resistant and have raised blood pressure, both of which predispose to CHD.

Infant and Childhood Growth and Coronary Heart Disease

Figure 1 shows the growth of 357 men who were either admitted to hospital with CHD or died from it [32]. They belong to a cohort of 4,630 men who were born in Helsinki. Their mean height, weight and body mass index (BMI, weight/height^2) at each month from birth to 2 years of age, and at each year from 2 to 11 years of age, are expressed as standard deviations (z scores). The mean z score for the cohort is set at zero and a boy maintaining a steady position as tall or short, or fat or thin, in relation to other boys would follow a horizontal path on figure 1. At birth the mean body size of the boys who later had CHD was approximately 0.2 standard deviations below the average and they were thin. Between birth and 2 years of age, the mean z scores for each measurement fell, so that at 2 years the boys were thin and short. After 2 years of age their z scores for BMI began to increase and continued to do so. In a simultaneous regression, both low BMI at 2 years of age and high BMI at 11 years of age were associated with later coronary events ($p < 0.001$ and $p = 0.05$, respectively). When BMI at birth was added to the model, the measurements of body size at each of the three ages were associated with later coronary events ($p = 0.04$ for low BMI at birth, $p = 0.001$ for low BMI at 2 years of age, and $p = 0.03$ for high BMI at 11 years of age).

As with the boys, the mean body size of the 87 girls who later had coronary events was below average at birth (fig. 1). They tended to be short at birth rather than thin, but their mean z scores for BMI fell progressively after birth so that, like the boys, they were thin at 2 years of age. After 4 years of age the z scores began to increase and continued to do so, reaching the average at approximately 8 years of age. Similar to the boys, in a simultaneous regression, body size at each of the three ages was associated with later coronary events ($p = 0.02$ for short length at birth, $p = 0.002$ for low BMI at 2 years of age, and $p = 0.02$ for high BMI at 11 years of age).

In table 4 the findings for boys and girls have been combined to show the simultaneous effect of birthweight and BMI at 2 years of age, divided into thirds, on hazard ratios for coronary events. The highest hazard ratios were

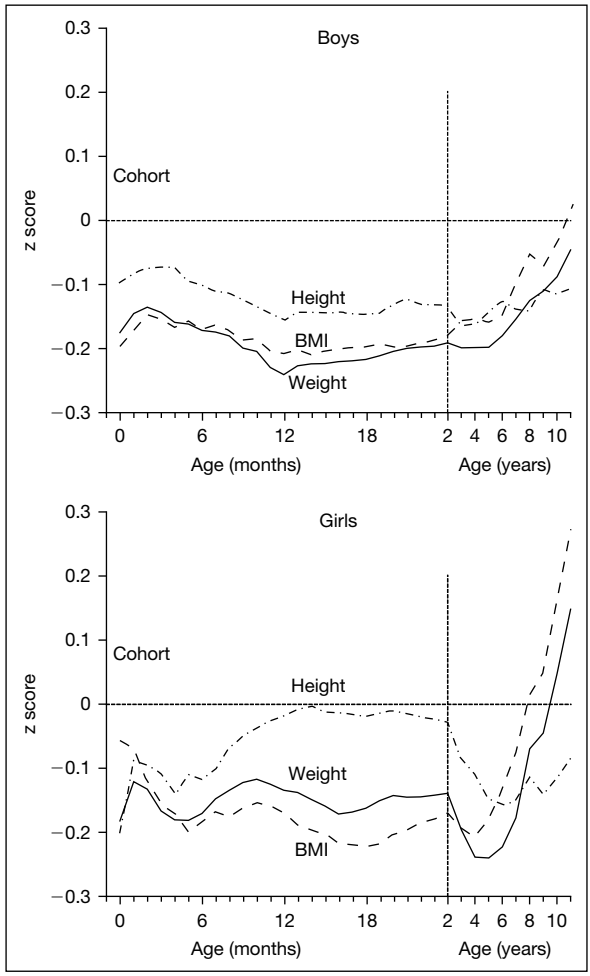

Fig. 1. Mean z scores for height, weight and body mass index (BMI) in the first 11 years after birth among boys and girls who had coronary heart disease as adults. The mean values for all boys and all girls are set at zero, with deviations from the mean expressed as standard deviations (z scores).

among subjects with birthweights below 3.0 kg and BMIs at 2 years of age of 17 or less. Table 5 shows the simultaneous effects of BMI at 2 and 11 years of age. The highest hazard ratios were among people with BMIs below 16 at 2 years of age and above 17.5 at 11 years of age. The hazard ratios in tables 4 and 5 were little changed if they were adjusted for socioeconomic status or income in adult life.

Table 4. Hazard ratios (95% CI) for coronary heart disease according to birthweight and body mass index (BMI) at 2 years of age for boys and girls combined

Birthweight, kg	BMI at age 2		
	<16	16–17	>17
<3.0	1.9 (1.3–2.8)	1.9 (1.2–3.0)	1.3 (0.7–2.2)
3.0–3.5	1.5 (1.0–2.1)	1.6 (1.1–2.2)	1.2 (0.8–1.8)
>3.5	1.7 (1.2–2.5)	1.5 (1.1–2.2)	1.0

Table 5. Hazard ratios (95% CI) for coronary heart disease according to body mass index (BMI) at 2 and 11 years of age for boys and girls combined

BMI at age 2	BMI at age 11		
	<16	16–17.5	>17.5
<16	1.6 (0.8–3.3)	2.4 (1.2–4.9)	3.0 (1.4–6.3)
16–17	1.4 (0.7–3.1)	1.6 (0.8–3.3)	1.9 (0.9–3.9)
>17	1.0	1.3 (0.6–2.7)	1.1 (0.5–2.3)

These observations demonstrate that CHD is independently associated with both prenatal and postnatal growth [33]. One explanation for the associations with small body size at birth and thinness at 2 years of age is that babies who are thin or short at birth and during infancy lack muscle, a deficiency that will persist into childhood as there is little cell replication in muscle after around one year of age [34]. Rapid weight gain in childhood may lead to a disproportionately high fat mass in relation to muscle mass. This could underlie the strong associations between low birthweight, low BMI at 2 and high BMI at 11 and later insulin resistance, which was found when 2,003 subjects in the Helsinki cohort were examined at the age of 62 years [32].

The Helsinki study gives no support to the recent hypothesis that promoting early growth with high intake of nutrients in the first few months after birth will adversely affect cardiovascular health [35]. This hypothesis arose from studies of intermediary markers among young people born prematurely. In the Helsinki cohort at any birthweight and at any period up to 2 years of age, greater weight gain was associated with a lower incidence of CHD in later life.

Type 2 Diabetes and Hypertension

People who were small at birth remain biologically different to people who were larger, and these differences include an increased susceptibility to type

Table 6. Odds ratios (95% CI) for type 2 diabetes and hypertension according to birthweight and body mass index (BMI) at age 11 years among 13,517 men and women in Helsinki

Birthweight, kg	BMI at age 11			
	<15.7	16.6	17.6	>17.6
Type 2 diabetes (n = 698)				
<3.0	1.3 (0.6–2.8)	1.3 (0.6–2.8)	1.5 (0.7–3.4)	2.5 (1.2–5.5)
3.5	1.0 (0.5–2.1)	1.0 (0.5–2.1)	1.5 (0.7–3.2)	1.7 (0.8–3.5)
4.0	1.0 (0.5–2.2)	0.9 (0.4–1.9)	0.9 (0.4–2.0)	1.7 (0.8–3.6)
>4.0	1.0	1.1 (0.4–2.7)	0.7 (0.3–1.7)	1.2 (0.5–2.7)
Hypertension (n = 2,997)				
<3.0	2.0 (1.3–3.2)	1.9 (1.2–3.1)	1.9 (1.2–3.0)	2.3 (1.5–3.8)
3.5	1.7 (1.1–2.6)	1.9 (1.2–2.9)	1.9 (1.2–3.0)	2.2 (1.4–3.4)
4.0	1.7 (1.0–2.6)	1.7 (1.1–2.6)	1.5 (1.0–2.4)	1.9 (1.2–2.9)
>4.0	1.0	1.9 (1.1–3.1)	1.0 (0.6–1.7)	1.7 (1.1–2.8)

2 diabetes and hypertension. Table 6 shows odds ratios for these two disorders according to birthweight and fourths of BMI at age 11 years. The two disorders are associated with the same general pattern of growth as CHD [26]. Risk of disease falls with increasing birthweight and rises with increasing BMI in childhood.

The associations between low birthweight and type 2 diabetes shown in table 2 have been found in other studies [5, 13–17]. The association with hypertension has also been found elsewhere [36]. There is substantial literature showing that birthweight is associated with differences in insulin sensitivity and blood pressure within the normal range [5, 13, 17, 37]. These differences are found in children and adults but they tend to be small. A 1-kg difference in birthweight is associated with an around 3-mm Hg difference in systolic pressure. The contrast between this small effect and the large effect on hypertension (table 6) suggests that lesions that accompany poor fetal growth and that tend to elevate blood pressure, and which may include a reduced number of glomeruli, have a small influence on blood pressure within the normal range because counter-regulatory mechanisms maintain normal blood pressure levels. As the lesions progress, however, possibly through hyperfiltration of the reduced number of glomeruli and consequent glomerulosclerosis, these mechanisms are no longer able to maintain homeostasis. This may initiate a cycle of rise in blood pressure resulting in further progression of the lesions and further rise in blood pressure [27, 38]. A rapid increase in body size after birth may exacerbate glomerular injury because greater body size leads to increased excretory loads and glomerular hyperfiltration [39]. Direct evidence in support of this has come from a study of the kidneys of people killed in road accidents. Those being treated for hypertension had fewer but larger glomeruli [40].

Table 7. Mean fasting insulin concentrations (pmol/l) in elderly people in Helsinki according to PPAR-γ gene polymorphism and birthweight

	Birthweight, kg			
	<3.0	3.0–3.5	>3.5	p for trend
Pro12Pro	84 (n = 56)	71 (n = 161)	65 (n = 107)	0.003
Pro12Ala/Ala12Ala	60 (n = 37)	60 (n = 67)	65 (n = 48)	0.31
p for difference	0.008	0.02	0.99	

The number of subjects is given in parentheses.

Pathways to Disease

New studies, especially the Helsinki studies with their detailed information on child growth and socioeconomic circumstances, increasingly suggest that the pathogenesis of CHD and the disorders related to it depend on a series of interactions occurring at different stages of development. To begin with, the effects of the genes acquired at conception may be conditioned by the early environment. Table 7 is based on a study of 476 elderly people in Helsinki [41]. It shows mean fasting plasma insulin concentrations according to which of two polymorphisms of the peroxisome proliferator-activated receptor (PPAR)-γ gene was present. The Pro12Pro polymorphism is known to be associated with insulin resistance, indicated by elevated fasting plasma insulin concentrations. Table 7 shows, however, that this effect occurs only among men and women who had low birthweight. Conversely, low birthweight has been consistently linked to later insulin resistance [28], but table 7 shows that this effect occurs only among people with the Pro12Pro polymorphism. As birthweight serves as a marker of fetal nutrition [25], this gene–birthweight interaction may reflect a gene–nutrient interaction during development.

The effects of the intrauterine environment on later disease are conditioned not only by events at conception but also by events after birth. Table 6 shows how the effects are conditioned by childhood BMI. Table 3 shows that the effects of a low ponderal index at birth are conditioned by living conditions in adult life. Table 8 shows how the effects of low birthweight on later hypertension are conditioned by living conditions in childhood, indicated by the occupational status of the father [42]. Among all the men and women, low birthweight was associated with an increased incidence of hypertension, as has been shown before [36]. This association, however, was present only among those who were born into families where the father was a laborer or of lower middle class.

It seems that the pathogenesis of cardiovascular disease and type 2 diabetes cannot be understood within a model in which risks associated with

Table 8. Cumulative incidence (%) of hypertension according to birthweight and father's social class in 8,760 men and women in Helsinki

Birthweight, g	Father's social class			
	manual worker	lower middle class	upper middle class	p for trend
<3,000	22.2	20.2	10.5	0.002
3,500	18.8	15.2	10.6	<0.001
4,000	14.5	12.5	10.3	0.04
>4,000	11.1	15.6	15.7	0.11
p for trend	<0.001	0.05	0.79	

adverse influences at different stages of life add to each other. Rather, disease is the product of branching paths of development. The environment triggers the branchings. The pathways determine the vulnerability of each individual to what lies ahead [39, 43].

A clinical study of 2,003 people within the Helsinki birth cohort showed that two different paths of fetal, infant and childhood growth preceded the development of hypertension in adult life [44]. In one, which was associated with more severe hypertension in people who tended to be overweight, small body size at birth and during infancy was followed by rapid weight gain, so that at age 11 years the children's body size was around the average. This is the same path of growth that led to insulin resistance and CHD (fig. 1). In the other path of growth, which was associated with less severe hypertension, slow linear growth in utero and during infancy was followed by persisting small body size so that at age 11 years the children were short and thin. A similar path of growth leads to stroke [45]. One possible process underlying this is that slow growth is associated with impaired development of the cerebral vasculature during a period of rapid brain growth, and also with altered liver metabolism and the development of an atherogenic liver profile. The two different paths of growth may lead to hypertension through different biological mechanisms and may produce two groups of patients who respond differently to medication.

We are beginning to understand the processes through which different paths of development initiate hypertension [39]. The changes occur at different levels and include allocation of stem cells and alteration of gene expression in the embryo, changes in renal growth, and alterations in hemostatic set points that control blood pressure. These changes can make the affected systems more vulnerable to disruptive influences in postnatal life, which include rapid weight gain, oxidative stress, environmental stress and a high salt intake.

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Discussion

Dr. Haschke: One of your slides indicates that low BMI at 1 and 2 years of age is a predictor of poor outcome. If we look at the BMI of breastfed and formula-fed infants we know exactly that breastfed infants grow faster during the first 3 months of life and then they fall back. They clearly have a lower BMI at 1 and 2 years of age than formula-fed infants; the difference being up to 0.3 z scores. The industry is now trying to develop formulas to mimic the growth of the breastfed infants, but is this really the right thing to do to move to a lower BMI with regard to the nutritional factors that influence growth? What is your opinion?

Dr. Barker: Firstly, almost all the children in the Helsinki birth cohort were breastfed because they were born in the 1930s and 1940s, and so the Helsinki data don't permit us to answer your question. But isn't that the kind of thing that the industry should be funding? It would not be difficult to study the age of adiposity rebound of breastfed and formula-fed babies. There is a lot of heterogeneity in the body composition of infants, and paths of growth will be optimal or not according to baby's initial body composition, and no doubt other factors as well.

Dr. Giovannini: To what extent would the quality of a child's physical activity change or reverse an unfavorable programming condition? Is physical activity always beneficial to those born growth-restricted?

Dr. Barker: I can't answer that question. Dr. Prentice is more likely to be able to answer than anybody in the room.

Dr. Prentice: Thank you Dr. Barker, but I don't think I can either. Intuitively, one would say that physical activity is almost always a good thing. One thing we know about physical activity in very young children is that it is driven more internally than externally.

Dr. Barker: We have a colleague in Oregon who studies obesity in monkeys. She measures physical activity using an accelerometer. The monkeys are given a surfeit of food, and obesity is unrelated to the diet they eat. It is all about patterns of activity and there is a wide variation in the amount of activity among different individual monkeys, and individual patterns of activity are stable over time. Some monkeys are very active and others are very inactive.

Dr. Walker: You mentioned and very elegantly described the impact of low birthweight and rapid weight gain on long-term disease. What about the flip side of that, because a major problem in developing countries and in developed countries is the obese woman who becomes pregnant, who then produces a child who is excessively overweight, who then continues to be overweight, and develops the same adult diseases as your prototype.

Dr. Barker: Thank you for pointing that out. It is of course true that women who are overweight may have macrosomic babies who are poor at making insulin and at risk of developing obesity and type 2 diabetes. We are not seeing that this is a path of growth that leads to coronary heart disease or stroke, but it certainly leads to diabetes. A conclusion from that is that there is more than one path of growth leading to a particular disease. In fact there are three known paths of growth leading to diabetes. One begins with small size at birth; the second begins with normal size at birth but slow infant growth; and the third is the one that you have alluded to.

Dr. Cameron: I just wanted to make a comment on the physical activity question earlier. It is always said in research on human growth that a certain minima of physical activity is necessary for normal growth but nobody knows what that certain minima actually is. It appears from research in adults that it is not necessarily the activity one undertakes but how fit one is as a result of that activity that is the risk factor for coronary heart disease and so on. Perhaps that is also true for children; it may not be the activity they do but how their body responds to the activity in terms of fitness.

Dr. Eriksson: I also would like to come back briefly to the question regarding physical activity. The truth is that we don't know how active these children were when they were young, but we know how active they were in adult age. We have one study in which it was shown that those people with the lowest birthweight if they were more active in later life were completely protected against the negative influences of low birthweight, but type 2 diabetes was the outcome [1].

Dr. Vaidya: Dr. Barker's hypothesis worries me for two reasons. The first is that I myself had a low birthweight, I grew a little faster, and my blood pressure is slightly increased. But my main worry is that, in my country where there are so many low birthweight babies, the entire focus of the government, the Indian Academy, pediatricians, mothers and parents is to achieve good growth. There are so many nutritional interventions going on in my country; mothers want their children to grow, pediatricians want the children to grow, and people start to use growth hormone to make IUGR infants grow. Unless this entire process of growth promotion is in some way curtailed, I am very seriously worried about the implications, especially in a country which has such a large low birthweight population.

Dr. Barker: It is well known in India that people become insulin-resistant at levels of fat accumulation which are low by Western standards and it is partly about the tendency of Indian people to accumulate fat centrally. In order to improve the development of babies you have to focus on improving the nutrition of young girl children

because much of a mother's metabolic competence is set in her early life. I entirely agree that governments need to be aware that investments need to be made against longer term outcomes.

Dr. Ogra: That was a very elegant outline of your hypothesis, Dr. Barker. For the past 20 years, more or less, we have been talking about the effects of environmental influences on growth. Have you or anyone else come up with any basic biological markers which can be used more consistently to determine or to predict who may be really at high risk of stroke or heart disease as a function of the nutritional alterations in the fetal or perinatal period, or later in life?

Dr. Barker: That is a central question because that is clearly where we need to go, to develop biological markers of vulnerability which can be measured in children so that individuals can be protected.

Dr. Ogra: We all recognize that every society or country has priorities for its economic resources. In some places priorities with regard to fetal and infant nutrition may not be as high as providing jobs or investing in vaccinations for infections. Therefore, identifying more basic markers and mechanisms of such vulnerability will be very helpful in establishing a high priority for such nutritional approaches.

Dr. Barker: I agree with you and I'd like to add one further point. A group of people who are extremely supportive of this are the economists because early development affects cognitive function and it clearly affects the ability to be a productive part of a work force. Politicians may not care about health but they do care about economics and productivity. So economists are very entranced with ideas about how if money is put into early development it could improve economic productivity, and there is direct evidence of that. In the Helsinki Birth Cohort men who were short at birth earn less, and in a democracy like Finland what you earn is a demonstration of your physiological capacity, of your cognitive capacity.

Dr. K. Bergmann: Looking at statistics on causes of death, one thing that impresses me quite a bit is that the average age at death from cardiovascular diseases is much higher than life expectancy. So dying from cardiovascular diseases may be a sign of being protected from dying from something occurring a lot earlier. Another point is we think that perhaps half or two thirds of all causes of death are from cardiovascular diseases. It is a death of the old people. It may not be necessary to prevent cardiovascular diseases, it may only be necessary to prevent early death from cardiovascular diseases, which also occurs. What does this look like in your studies? Do people die early from cardiovascular disease or do more people die from cardiovascular disease, which makes a big difference?

Dr. Barker: There are a number of points there. Firstly it is a blessing really that what promotes good growth and cognitive development in children also diminishes cardiovascular disease in later life. So there isn't a situation as far as we know that you have to make a decision: are you going to be smart and die young or are you going to be stupid and have a long life, it doesn't work like that. It's a characteristic of places where coronary heart disease is epidemic, and India is an excellent example, that people get it at extraordinarily young ages by Western standards. The same for stroke; there are people in China who have strokes at the age of 25. It's the same with renal failure; In South Carolina in the USA there are large numbers of people on dialysis for chronic renal failure at the age of 25. So the short answer to your question is that poor development is linked to early death; it is also linked to life expectancy in a rather complicated way. Birthweight is a poor predictor of longevity, the biology of that is more complicated but we are starting to make progress with it.

Dr. Wilson: I am going to ask a question from a point of ignorance. I wonder if you could tell me, on a population basis what is the fraction of risk attributable to these

birth and early growth differences for the diseases you have been discussing? How does the attributable risk vary from one population to another?

Dr. Barker: A recent policy document of the National Institute of Child Health states that antenatal nutrition is more important than adult behavior in determining coronary heart disease. Attempts to prevent heart disease by modifying adult lifestyle have been fantastically disappointing. I can't answer your question because the figures are not there. If you review everything that is known, as the UN committee did, you come to the conclusion that the point of action is conception to 24 months, but that doesn't mean that adult lifestyle doesn't play a part. It may be important for vulnerable people and may be unimportant for nonvulnerable people. In the Helsinki study the men with low incomes have more heart disease which is a general phenomenon across all Western societies. When you break it down that relationship is confined to one quarter of men defined by being thin at birth having a low ponderal index (birthweight/ length^2). If they have a ponderal index of <26, their income is linked to heart disease, strongly; lower income, more heart disease. For the three quarters of men who are not thin at birth it makes absolutely no difference, it's completely unrelated. We know something about the biology of this. Current ideas focus on the psychosocial stress of having a lower place in a hierarchy, however defined, and there is direct evidence for that. People who are small at birth have enhanced stress responses. Before birth cortisol plays a dominant role in the maturation of tissues and after birth the settings of the HPA axis are retained and become part of the stress responses.

Dr. Mantaring: You showed us a slide with the z scores for length, weight and BMI in the first 10 years after birth of boys who developed coronary heart disease. I am interested in the comparison group. How different are they from the z scores of those who did not develop coronary heart disease?

Dr. Barker: I am sorry, I didn't explain that slide clearly. There were 4,000 boys. The zero point was set from the whole cohort and the standard deviations were calculated from this. The body size of boys who went on to develop coronary heart disease was calculated as standard deviations in relation to the mean of the entire cohort. The boys who went on to get coronary heart disease were small up to the age of 2 in relation to all other boys, and then they had rapid weight gain after 2 in relation to all the other boys.

Dr. Björkstén: You mentioned two of the major causes of death: coronary heart disease and stroke. Is there anything known regarding very early infant nutrition in relation to either disease or particular forms of cancer?

Dr. Barker: There is a limited literature on hormonally related cancers, people who develop breast cancer tend to have slightly higher birthweight as a group. What that means, we don't know. There is a theory that exposure to maternal estrogens in some way leads to breast cancer in later life. This is a field that is just starting to wake up because the data are available. The model of cancer says that there is some stressor in adult life which only triggers it in a certain group of vulnerable people. The vulnerability may not be genetic for breast cancer; it may be an acquired vulnerability in early life. I think that over the next 3 years there will be a lot advances made.

Dr. Al Ghamdi: Those babies born with a low birthweight who remain with a low bodyweight for the rest of their lives, are they at the same risk of having diabetes and hypertension?

Dr. Barker: A component of increased risk for coronary heart disease is rapid weight gain after the age of 2. If that does not happen, as a group the children will be at lower risk. Coronary heart disease is a disease of affluence, it increases as countries become more affluent but it settles in the poorer people. We have always been looking

for two things in the causation of coronary heart disease: one is an affluence factor and one is a poverty factor. The poverty factor may be events before 2 years, and the affluence factor may be abundance and rapid weight gain after 2 years.

Dr. Al Ghamdi: What can we do for those children who had a low birthweight and increased their bodyweight after the age of 2 years? When we see them at 10 years, is there anything we can do to decrease those risk factors?

Dr. Barker: The long-term solution is to invest in better nutrition for young girls and adolescent girls. In the short-term the protection of infant growth, not necessarily the promotion of infant growth, but the protection of it from obviously adverse influences, like recurrent minor infections and poor weaning practices, is the quickest fix. At present we are bit stuck on preventing rapid weight gain in childhood: childhood obesity is rising and we do not understand why.

Dr. Malka: Low birthweight is associated with hypertension. You say that birth weight is not the major determinant for childhood blood pressure, but that the current weight is more important. Systolic blood pressure has been measured in adolescents and compared to their birthweight, and no significant relation was found.

Dr. Barker: There are studies of children which have shown that the relationship with birthweight is small and as you say the relationship with current weight is dominant. When you come to adults with actual hypertensive disease, which is the real issue, the birthweight relationships are very strong. It seems that if you acquire a lesion antenatally, say fewer nephrons, you are still able to maintain homeostasis through childhood into adult life. The differences associated with birthweight in 18-year-olds are only 3 mm Hg of systolic pressure; they are small. It is not logical, however, to say that birthweight doesn't matter, because birth weight matters a lot for the ultimate need to take drugs to lower your blood pressure when you are 50.

Dr. R. Bergmann: In your low birthweight high risk group, did you control for cigarette smoking? We know that social status and smoking prevalence are inversely related.

Dr. Barker: It's interesting, a lot of studies have addressed that question because cigarette smoking in the mother does slow fetal growth, and while smoking has a short-term effect it does not have long-term effects. The evidence is that, surprisingly perhaps, smoking which presumably acts through hypoxia, does not have the kind of long-term effects that we are seeing in association with differences in the mother's body composition and dietary intake.

Dr. R. Bergmann: I am interested in the adult smoker, the person of low social status, the poor worker, who is smoking and therefore more at risk of coronary disease and stroke.

Dr. Barker: But clearly it is possible to take these into account. Where they have been taken into account, in the American Nurses study, for example, they simply do not contribute to the relationship between low birthweight and later stroke. During the past 15 years this question must have been examined by just about anybody who had a set of data, and that is how it works out. It's convenient to blame the poor for their own ill health, it's what politicians like to do. But it doesn't work, the poor get sick because they are vulnerable not because they are mischievous.

Dr. Walker: Given the importance that you put on antenatal development, antenatal growth, should we be routinely following babies in utero from a grid looking at their growth so we know what we can anticipate? Given the fact that chronic disease takes a long time to develop, should we be looking at biomarkers of these diseases so we can get on top of it in a preventive way?

Dr. Barker: I think everyone would agree with both those thoughts. What is an optimal dynamic of postnatal growth must presumably be linked to something more subtle than the body size at birth because the same body size at birth can be acquired by growing fast early and slow later or slow early and fast later. In India the path of

growth seems to be characterized by rapid early growth which cannot be sustained in late gestation, and the results of that would be different from what is the Chinese path of growth, which is slow growth sustained from the beginning to end of gestation. The long term consequences might be different although it may lead to the same birthweight.

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The Role of Growth in Heart Development

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Abstract

While it is established that the quality of the perinatal environment is critical in sculpting the developing individual, the mechanisms by which this occurs remain poorly defined. The growing fetus is dependent on the nutrients (including oxygen) it receives from the mother via the placenta. When this supply line is compromised, heart growth patterns are altered. In addition, hormones, other circulating factors, and the hemodynamic environment in which the fetus develops are important in determining outcomes for organ structure and function. Numerous studies in sheep have demonstrated that heart development can be modified in a number of ways, and the nature of the change differs between types and gestational timings of insults. Embolization of the placenta leads to the cessation of proliferation and maturation of cardiomyocytes; this may be due in part to changes in circulating insulin-like growth factor-1 levels. Such insults may be the underlying cause of cardiovascular disease in adults. Insults that modify the maturational timeline, final myocyte number, vascularity and endothelial responsiveness in the heart can have effects that persist long after the insult has been ameliorated.

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Introduction

Epidemiological data show that slow growth during fetal life is associated with an increased risk of type 2 diabetes, hypertension, stroke and ischemic heart disease later in life [1]. Retarded growth before birth appears to be caused by depressed transport of nutrients and/or oxygen to the fetus or increased levels of maternal glucocorticoids in the fetal circulation [2], and rarely, genetic abnormalities. All of these causes can occur simultaneously.

Animal models of maternal undernutrition or low oxygenation show that fetal malnutrition leads to permanent structural and physiological changes in

the fetus that increase its susceptibility to adult-onset diseases including hypertension, type 2 diabetes and endothelial dysfunction [3]. The nutritional status of the fetus influences the production rates of circulating growth factors, hormones and cytokines, which are key regulators of organs within the embryonic and fetal body. However, the mechanisms by which these factors cause changes within specific organs are not known.

The sheep fetus is a commonly used model of cardiovascular development. It is an attractive model for studying nutritional effects on the developing heart because the fetal sheep heart grows at a rate similar to that of the human fetal heart and because its growth can be easily monitored throughout gestation. During the first two thirds of gestation, the heart grows by hyperplasia; mononucleated cardiomyocytes proliferate by normal mitosis. The heart then undergoes a maturation period during the last third of gestation when cardiomyocytes gradually undergo binucleation characterized by karyokinesis without cytokinesis. This process is known as terminal differentiation. Thus, the proportion of working myocytes that contain two nuclei can be used as a clear indicator of the progression of maturation. Binucleated cells cannot divide but they can readily enlarge. During late postnatal life most of the growth of the myocardium is driven by the enlargement of binucleated cardiomyocytes. The complexities of the growth and maturation of the myocyte population in sheep has been described [4].

The heart has critical windows of development when it is most vulnerable to specific types of stress. In the pre-implantation rat embryo, nutritional cues affect embryo growth patterns and may increase the vulnerability of the offspring to late life hypertension [5]. In early gestation, nutritional stressors may interrupt the cell proliferation process and reduce the rate at which cardiomyocytes replicate. It is not known whether the loss of heart cell numbers due to low rates of proliferation in the first half of gestation will lead to a permanent reduction in cardiomyocytes for life or whether cell numbers can somehow be replenished when conditions are better. In late gestation, when cardiomyocytes are rapidly becoming binucleated, nutritional stressors may interrupt this maturation process. If the late gestation fetal myocardium is subjected to a period of inadequate nutrition, at least two outcomes are possible. If the binucleation process is impeded the fetus may be born with a heart containing mostly immature mononucleated myocytes that must go through their maturation process at some later time. If cardiomyocyte binucleation rates become augmented, the numbers of mononucleated cardiomyocytes will be relatively fewer before the full complement of cells has been generated. This outcome would reduce the generative capacity of the myocardium as cells are exiting the cell cycle prematurely and would leave the myocardium with a less than optimal number of cardiomyocytes.

The outcome of altered cell numbers could affect the right ventricle differently from the left ventricle. As the postnatal right ventricle becomes the pump for the low pressure pulmonary circuit, right ventricular cell numbers decline.

At birth, the left ventricle must eject its stroke volume into the systemic aorta which becomes a high pressure system upon the loss of the low resistance placenta. To accomplish this task, the left ventricle must already have a sufficiently thick wall, round shape and be metabolically prepared for efficient work. The left ventricle normally has proliferative capacity for a period of time after birth as well [4]. Having too few cells could compromise the function of the ventricle over a lifetime. The ability of the adult heart to make new cardiomyocytes, especially in areas of stress has been clearly demonstrated [6]. However, it is also clear that the regenerative capability of the heart is inadequate to replace cardiomyocyte loss following infarction or failure.

The fetal heart and brain are special organs that may be partially protected under conditions of chronic oxygen shortage [7]. For example, under conditions of acute hypoxemia, blood is shunted away from abdominal organs in order to favor blood flow to the fetal heart and brain. This has been shown in a number of experimental circumstances ranging from uterine blood flow reduction to global reduction in maternal oxygen [8, 9]. Thus, babies living in suboptimal environments in the womb may grow asymmetrically with 'spared' head and heart sizes but reduced abdominal girth.

Growth Factor Support of the Heart

During fetal life, insulin-like growth factor-1 (IGF-1) is an important regulator of organ growth and is influenced by fetal nutrient supply [10, 11]. Fetal IGF-1 levels are downregulated under conditions of maternal protein/calorie deprivation or placental reduction in sheep if fetal growth is suppressed. Birthweight has a greater influence than maternal nutritional status on glucose tolerance, blood pressure, and IGF-1 levels in fetal sheep [12, 13]. Offspring born to malnourished mothers are at the low end of the growth spectrum and have decreased heart to bodyweight ratios compared to fetuses that have been exposed to elevated levels of IGF-1 before birth. Mice that are null for IGF-1 or null for the IGF-1 receptor have birthweights that are approximately 50% of normal [14]. When IGF-1 is overexpressed in transgenic mice, the heart to bodyweight ratio and the absolute heart weight are increased by increased cardiomyocyte numbers.

Much of what is known about IGF-1 effects in the fetus comes from experiments in sheep. The IGF-1 analog (IGF-1 LR3) has been used to study fetal growth in sheep [15]. When IGF-1 LR3 was infused into near-term sheep fetuses for 7 days, heart weight increased by 35% over vehicle-infused control hearts and the heart weight to bodyweight ratio was also increased. This observed growth could have been either through the stimulation of cardiomyocyte enlargement, cardiomyocyte proliferation or both. However, it appears that IGF-1 LR3 stimulates an increase in the ratio of mononucleated to binucleated cells in the myocardium as was shown in fetuses exposed for 7 days to

IGF-1 LR3; cardiomyocyte dimensions did not change in these experiments. Thus, IGF-1 is a powerful regulator of cardiac growth in the fetus and it appears that malnourished fetuses have decreased levels of IGF-1 and that cardiomyocyte proliferation rates are thus depressed.

The signaling mechanism by which IGF-1 regulates sheep cardiomyocyte growth has been studied in cardiomyocytes in vitro [15]. It appears that IGF-1 signals through the membrane-bound IGF-1 receptor and stimulates both the extracellular signal-regulated kinase (ERK) and phosphoinositol-3 kinase (PI3K) cascades. IGF-1-induced protein synthesis requires ERK activation and there is evidence that IGF-1-stimulated hypertrophy in neonatal rat cardiomyocytes signals through the ERK cascade. If data from the rat apply to the large mammal (and that has not been proven), then IGF-1 may have roles to play in hypertrophic as well as hyperplastic growth.

Placental Insufficiency, Nutrient Deprivation and Heart Growth

The placenta is the organ through which all nutrients must pass to reach the fetus. Poor placentation leads to poor nutrient transport, poor oxygenation and inadequate support for optimal fetal growth [16]. It is now known that subnormal fetal growth is associated with depressed expression of many transport proteins normally found on the microvillous membrane of the placenta, including the transport systems for several amino acids, the sodium hydrogen ion exchanger, sodium potassium ATPase and lipoprotein lipase [17].

One model of fetal malnutrition has been widely used – the placental insufficiency model in sheep [18, 19]. Inert microspheres of some 50 μm can be administered to the placenta via the umbilical arteries. Gradually the exchange area of the placenta is reduced. This model causes global placental insufficiency where the transport of nutrients, including oxygen, is reduced. Typically, fetal bodyweight is reduced compared to controls. Brain growth is mildly protected with an increased brain to liver weight ratio. This mild 'relative brain-sparing' pattern of growth has been well described following 20 days of placental embolization [20, 21].

The most surprising finding of placental embolization experiments is that heart tissue is often not spared [18, 22]. If the heart were to grow out of proportion to body size, one would expect to find vigorous growth and normal maturation (binucleation) of the cardiomyocytes. However, with embolization heart weight is normal for bodyweight. In essence, the heart stops growing in parallel with the body and the suppression of growth is profound. Both the proliferation of myocytes and the rate of binucleation of myocytes are reduced significantly while cardiomyocyte size is not. The smaller hearts contain fewer working myocytes compared to normal-sized controls and the maturation of the heart is suspended. Thus, the fetal response to placental insufficiency is one of overall fetal organ growth suppression and lack of maturation

accompanied by little or no special protection of the heart. This condition presents a physiological problem for the small heart, especially at birth when the heart needs to eject blood against a high resistance, high pressure circuit.

Only a few studies have reported the effects of intrauterine growth restriction (IUGR) on cardiomyocyte development in humans. Mayhew et al. [23] suggested that myocyte maturation is delayed in IUGR fetuses, with some 'catch-up' later in gestation. In the offspring of rats fed low protein diets during pregnancy, the heart weight/bodyweights of the offspring were not different from controls at birth. Nevertheless, there were fewer cardiomyocyte nuclei in the hearts of offspring born to mothers that were on a low maternal protein diet during pregnancy [24]. These authors conclude that the suboptimal prenatal environment leads to reduced proliferation and fewer cardiomyocytes in their model.

The fetal sheep hypothalamo-pituitary-adrenal axis may be activated when the mother is undernourished, leading to augmented fetal cortisol concentrations and early delivery [25]. In experiments where sub-pressor levels of cortisol were infused into well-nourished sheep fetuses, the cell cycle activity of cardiomyocytes was increased and cardiac mass increased [26]. Thus, one might expect that the increased fetal cortisol levels that usually accompany placental insufficiency would stimulate heart growth [19, 21]. However, this does not happen. In spite of increases in cortisol and renin in fetuses with placental insufficiency [19], both of which may stimulate cardiomyocyte proliferation in well-nourished hearts, heart growth is depressed. These data may indicate that undernourished hearts are not able to grow when nutrients, oxygen and IGF-1 levels are depressed.

Undernutrition and Fetal Blood Pressure

In the sheep, blood pressure is maintained at normal levels with placental insufficiency [19, 22]. However, under conditions of acute hypoxemia, sheep fetuses and presumably human fetuses show profound increases in blood pressure. Increases in pressure load to the heart ventricles cause accelerated maturation of cardiomyocytes [27]. This leads to a condition where the fetal myocardium has a decreased proportion of mononucleated cardiomyocytes, a larger proportion of cardiomyocytes that have two nuclei and binucleated cells that are larger than normal (fig. 1). This condition appears to lead to a myocardium with fewer cardiomyocytes and consequently fewer capillaries. This may lead to a long-term vulnerability of the heart for coronary artery disease and heart failure.

The effect of maternal and fetal undernutrition on fetal blood pressure regulation is complicated by the fact that different models lead to different outcomes. Arterial pressure may or may not be altered in fetal sheep being carried by mothers that are undernourished. In the undernourished ewe model, fetal

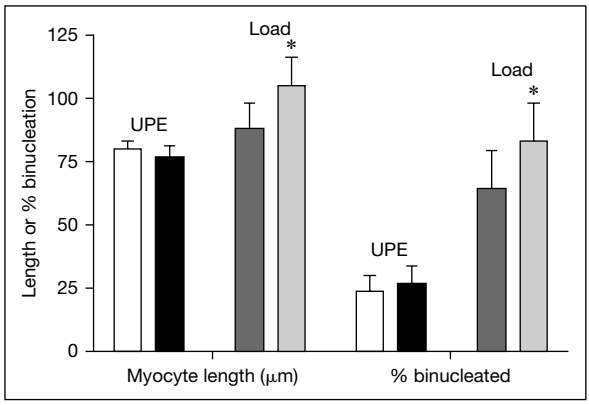

Fig. 1. Changes in cardiomyocyte length or percent binucleation under two experimental conditions in the sheep fetus, each of which were of 10 days duration. UPE = Umbilical placental embolization. Nutrition was reduced in 125-day fetal sheep via reduced placental surface area. Neither cell size nor percent binucleation changed with 10 days of UPE. However, following 10 days of pressure load to the right ventricle in 135-day fetal sheep (normally nourished), cell size was increased and percent binucleation was increased. The increase in proportion in response to fetal hypertension will alter the cardiomyocyte composition at birth.

blood pressure can be decreased [28] or increased [29]. However in the carunclectomy model of fetal undernutrition, blood pressure is maintained at a normal level through adrenergic support [30]. Changes in endothelial function in various organs may predetermine cardiovascular effects in later life [31].

Low Content of Oxygen in Fetal Blood

Davis et al. [32] have demonstrated that the coronary tree is highly plastic during the perinatal period. This finding is important because it shows that the coronary tree can respond in a dramatic fashion to changes in the fetal environment. When near-term fetal sheep are made anemic by reducing their circulating red cell mass by about 50% over several days, the cardiovascular system responds by dramatic adaptations. Heart size increases rapidly. Cardiac output goes up. But most importantly, coronary artery conductance doubles. Coronary conductance is the maximal level of blood flow, at any given pressure, that can be supplied to the myocardium when the coronary arteries are fully dilated by adenosine. Thus, at the normal fetal driving pressure across the myocardium (about 40 mm Hg), flow will rise to more than two times higher than normal after a few days of anemia. Figure 2 shows that maximal coronary blood flow increases dramatically over a few days when

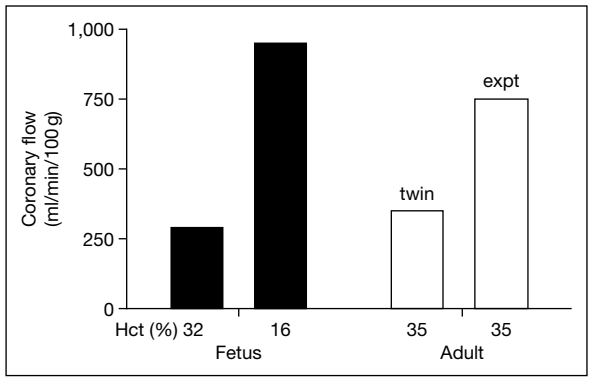

Fig. 2. Maximal coronary blood flow at normal arterial pressures for the fetus and the adult sheep. Maximal flow was obtained during continuous adenosine infusion. For the fetus, flow data are shown in the same near-term fetus at two hematocrits (Hct): normal 32%, and following 6 days of anemia with Hct at 16%. Both fetal flows were measured when arterial pressure was 40 mm Hg and the coronary vessels were maximally dilated. For the adult, flow data from a 6-month-old adult sheep after having been made anemic as a fetus for only 6 days (expt; pre-partum blood transfusion returned Hct to normal levels) compared to its normal twin that was never anemic. Arterial pressure was 100 mm Hg and the coronary vessels were maximally dilated.

hematocrit is reduced; this increase remains in the adult that was once anemic in utero. Because the coronary vessels are fully dilated by adenosine with each measurement, the only way that flow can increase under similar pressure conditions is if the resistance to flow is reduced. This can be done by making more parallel vessels in the microcirculation or by enlarging existing ones. Therefore, it is clear that the coronary tree can be remodeled over the period of just a few days and once remodeled, the increased coronary conductance remains even into adulthood. These data demonstrate that the coronary tree is very plastic during late fetal life and that the architectural changes that it makes during that plastic period are permanent.

Malnutrition versus Hypoxia

While oxygen may be considered a nutrient in one sense, it is reserved for its own special category in this discussion. Li et al. [33] showed that the adult male offspring of maternal rats that were exposed to low oxygen levels during gestation had cardiovascular abnormalities. While the hearts were of normal appearance, they showed defects when studied in an isolated chamber: (1) the hearts had fewer and larger cardiomyocytes upon histological examination, and (2) they had larger infarctions and poorer contractile func-

tion following ischemia reperfusion injury. The authors of this study surmised that the abnormal histology and function were an outcome of fetal hypoxemia. However, it has been shown that when maternal rats are placed in an environment where ambient oxygen levels are decreased, their food intake is also decreased. Thus the studies of Li et al. [33] are showing the combined effect of hypoxia and undernutrition.

Experiments from the laboratory of Xu et al. [34] have shed some light on the oxygen versus nutrition issue. Rats that developed in utero under conditions of (1) undernutrition or (2) with depressed oxygen levels, and its accompanying depressed maternal nutrition, were found to grow more slowly than normal before birth. Xu et al. [34] showed increases in the expression of several genes that are known to be associated with cardiac pathology. The hearts from the two protocols may have followed different pathways to reach a similar pathological outcome. The offspring that were once hypoxemic had cardiac hypertrophy at birth whereas the nutritionally deprived offspring did not. However, the growth and functional deficits that accompany slow intrauterine growth were apparent in both groups by 7 months of age. Thus parallel but different pathways seem to lead to the same pathophysiology.

These rodent findings may not apply perfectly to large mammals. Fetal sheep that are growth restricted due to maternal undernutrition have enlarged hearts [35]. However, as mentioned above, in sheep that are both nutritionally deprived and are hypoxemic, heart weight is normal for bodyweight at birth. Thus, it is not possible to predict the level of vulnerability for disease that is contained within the myocardium by observing the newborn heart. Complex gene expression patterns may set into motion a series of pathological processes that arise within the process of aging.

Mechanisms of Nutrient Action

To the biologist the relationships between malnutrition, growth in early life and late life cardiovascular disease beg for explanation. Animal experiments have been highly successful in demonstrating potential links between early life adversity and late life chronic disease. Yet, an integrated and thorough explanation of the mechanisms that underlie the early life origins of disease awaits further study. It is clear that fetuses whose growth is reduced because of maternal and/or fetal undernutrition face some common anatomic and physiological deficits. These include low nephron numbers, reduced numbers of β cells in the endocrine pancreas, enlarged hearts, and resetting of the hypothalamic pituitary adrenal axis. However, the effects of undernutrition may be at once subtle and powerful. The anatomic changes listed may not occur with milder forms of nutrient deprivation. Nevertheless, the vulnerability for disease may be significant. Epigenetic regulation of the genome through methylation of DNA or methylation or acetylation of histones may set

the course for vulnerability for the immediate offspring as well as for future generations. The roles of epigenetic mechanisms are only now coming into view but it is certain that fetal nutritional status affects the epigenetic regulation of genes that have life-long consequences. Because it is now clear that the health of future generations depends upon a deeper understanding of gene regulation and diet in the mother and fetus, it is unlikely that other areas of research will have a greater impact on the health of humankind.

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Discussion

Dr. Bier: It seems to me we have an ongoing nutritional experiment everyday in the US and other countries dealing with potentially altering cardiac developmental programming. After birth very low birthweight infants, who were born at 60% of normal gestation, get parenteral nutrition. If they had stayed in the womb their cardiac myocytes would have been working on a glucose fuel. They are born and right away they are presented with a fatty acid fuel, and it seems to me that this is an extraordinary change in potential programming. Do we know anything about this and what the long-term consequences are?

Dr. Thornburg: Thank you for raising that issue; I didn't have time to talk much about the metabolic effects in the heart. Some of you may not know that at the time of birth the heart switches from using carbohydrate fuel to free fatty acid fuel; mature hearts prefer free fatty acids as a primary fuel. However, when babies are born prematurely the enzymes that are present are not able to use free fatty acids properly. Nevertheless, when we treat these small babies we continue to give them high levels of

lipid. Those of us in the fetal science world are worried about this approach. In other words, if you don't have the right fuel mix for your car, it doesn't run right. We think that the baby's heart also needs the proper fuel mixture. Unfortunately, no one has yet performed the appropriate biochemical studies to observe heart function in premature babies receiving different fuel mixtures of lipid and carbohydrate to determine the degree to which we should change the lipids in commercial nutrient solutions according to the level of maturity of the heart.

Dr. Ogra: These are very elegant observations. I would like to pursue two areas of your presentation; one relates to the hematocrit-related database. How does hematocrit impact on the oxygen-caring capacity for red cells in these experiments? As a corollary, have you looked at situations of polycythemia, for example in people living in high altitudes; what happens under those circumstances in terms of tissue architecture?

Dr. Thornburg: These studies are begging to be done. No one has looked at polycythemia during this period; the data we are showing you are about the only data available in terms of modifying the architecture in the coronary tree. What we do know is that the heart is very hungry for oxygen; it is able to remodel its architecture and it can dramatically increase blood flow so that oxygen delivery can be approximately maintained. This is one way the heart protects itself. It is known that babies who are born small from hypoxemia have protected hearts, larger hearts and larger coronary trees than babies who are severely malnourished.

Dr. Ogra: Is the incidence of heart disease lower in Scottish Highlanders?

Dr. Thornburg: I don't know of any data where this has been followed. There is a cohort in New Zealand where the babies had low hematocrits and our colleagues are looking at these individuals who are now young adults to see whether their coronary trees are altered for life.

Dr. Ogra: The second question is related to endothelial dysfunction. Has anyone looked at the endothelial architecture in terms of integrin expression which is very critical for function? Such functional aspects may be related to disease expressions later on.

Dr. Thornburg: That's a good question. I didn't have the time to show you that undernourishment, for any significant window of time, will cause endothelial dysfunction in virtually every organ of the body. This appears to be related to the generation of more reactive oxygen species. Antioxidants can in large part reverse that endothelial dysfunction. We believe that's also true in humans who carry with them endothelial dysfunction based on a low birthweight. But that in itself has not been studied well in humans; we know a lot more about it in animals.

Dr. Prentice: I am still struggling to understand the likely direction of later effects that we would anticipate from very profound early effects you showed by the induction of anemia. Can you help me to resolve this?

Dr. Thornburg: Yes, we have studied this quite a bit. What happens is that a fetus makes what we call a super-coronary tree in response to anemia; it will have larger coronary vessels and early in adult life, in what we would call up through adolescence in sheep life, these hearts seem to be very capable; if you reduce oxygen levels they are able to maintain their contractile force even though their oxygen levels go down. At first we thought this was really good and all babies should be anemic just for a while so they will have a tree that is super-functional. But it is likely that these hearts will actually be more vulnerable to infarction, and when they have an infarction they will have an infarction that is actually larger than normal. We are now worried that these hearts might be more vulnerable than we thought, and that the message might be that these coronary trees, although you have more of them, may not behave normally under conditions of ultimate stress, which is when they are deprived of oxygen or are ischemic later in life.

Dr. Giovannini: Could you speculate whether the lower hematocrit levels commonly found in breastfed compared to formula-fed babies could be protective from cardiovascular disease in later life?

Dr. Thornburg: I wish that I could comment on that because your question is very important. We have also been wondering about this and we have looked for clues in the literature without success. We hope that someone will now take this up because how hematocrit affects the heart and cardiovascular function could be important.

Dr. Makrides: I want to ask a question from the opposite view: have you any information about the hemo concentration in pregnancy and what impact that may have on the fetal heart?

Dr. Thornburg: First of all we know that when vascular resistance increases in the fetus, for any reason, the heart has to generate pressure against the higher load. We know that this cardiac load stimulates heart growth, hypertrophy and premature maturation of the myocyte. Pressure can be increased by two different mechanisms, either by increasing placental vascular resistance or just by increasing the load by a mechanical method (vascular occluder). With hemoconcentration there is an increase in blood pressure partly because of viscosity and partly because of vasoconstriction, and the same kind of hemodynamic load that occurs with a very small placenta (autoregulation). Hemodynamic load then brings about changes in the growth of the coronary tree and the heart muscle.

Dr. Makrides: So with that could you speculate on what the optimal hemoglobin range in the late pregnancy might be and if a pregnant woman's hemoglobin was too high whether that would be a negative thing in terms of over-supplementation with iron?

Dr. Thornburg: That's a very good question, and what I have been referring to of course is fetal hemoglobin levels. It turns out that in spontaneously polycythemic animals when hematocrit gets to about 55% we see arterial pressure going up and cardiac load going up. This will, undoubtedly be true for pregnant women as well.

Dr. R. Bergmann: I wonder what happens if a nonanemic pregnant woman receives iron or an anemic mother receives too much iron. Would the oxidative stress of pregnancy be aggravated?

Dr. Thornburg: You raise a good point. If a woman is iron-deficient, then giving iron is important for trying to reverse the anemia to maintain hematocrit. However iron goes across the placenta via transferring and ferritin, and high iron levels in the fetus are harmful.

Dr. R. Bergmann: Perhaps the best thing would be to start iron supplements before becoming pregnant [1].

Dr. Thornburg: Absolutely, the take-home point in this is that pre-pregnancy nutrition and good nutrition over the life time will promote good health for both the mother and the fetus.

Dr. Batubara: You said that a critical period in heart development is near term. What if the insult occurs in early pregnancy; in the first 3 months of pregnancy?

Dr. Thornburg: If you remember from embryology, the heart goes through a tube stage and then it loops, and when it goes through this looping state it is very vulnerable to blood flow abnormalities determined by the establishment of the placenta. Therefore, if the placenta in the very early stages is too small and the blood flow going through the heart is too low, the embryo will have a different shaped heart. We believe that many heart defects derive from these early stage blood flow deficiencies. If venous blood flow patterns going into the embryo heart are changed, the blood flow pattern going through the heart will be changed and that blood flow pattern will then change the way the four chambers of the heart are formed; the heart may have atrial septal and ventricular septal defects.

Dr. Hanson: When the placenta is formed, its trophoblasts grow into the mucosa of the uterus. Thus the cells come from different individuals to some extent so that there is an immune reaction, and it seems as if this difference enhances the formation of the placenta and the growth. In some areas of the world where marriages between cousins are common, in some cases possibly between cousins for many generations, would the placenta form in a different way; would it be less efficient or the fetus grow less?

Dr. Thornburg: I can't give you an answer. But I can give you some reassurance that scientists are looking at the interaction between immune cells and the establishment of trophoblast invasion. It has been shown in particular populations where there tends to be more inbreeding that the genetic makeup of the partners has a great influence on how many immune cells actually migrate to the site of implantation. So your point is well taken.

Dr. Guinto: As obstetricians, we do Doppler studies in growth-restricted babies. We use the uttering arteries to predict intrauterine growth restriction and the umbilical arteries to monitor patients whose babies already have growth restriction. We use abnormalities in the fetal venous Doppler studies and the demonstration of fetal coronary arteries through color flow mapping to guide us as to when the babies should be delivered. So perhaps this is similar to what you have been telling us, that there is an increase in the coronary artery flow, and thus a demonstration of the coronary arteries on Doppler ultrasound, in growth-restricted fetuses in the end stages of compensation.

Dr. Thornburg: Thank you for that comment. One of the great things that we as a scientific community can look forward to is increasingly expert individuals who will be able to image the heart using MRI and ultrasound to obtain images of the fetal coronary vessels. We have been imaging fetal hearts using MRI and ultrasound. We believe that with time we will become much better at predicting those hearts that are likely to have a very poor outcome in terms of their vulnerability for disease. I am very pleased to hear that you are using all these tests to make clinical decisions.

Dr. Walker: Can you reverse the damage that you described by injecting insulin-like growth factor or blocking renin or corticosteroids? In other words, is it a direct effect or is it just a marker of the effect?

Dr. Thornburg: In New Zealand, Jane Harding has infused IGF-1 into fetal sheep. Her group has shown that some of the negative effects accompanying slow fetal growth can be reversed. The real question is whether or not IGF-1 treatment can cause a fetus to outgrow its nutrient supply and you may actually lose more than you gain.

Dr. Smith: Lovely work on the cardiac myocytes. I wonder whether the fetal endothelium loses some of its plasticity as well, and if you are looking at markers loaded on the cardiac endothelium, for example, does the endothelium go up and stay up? Is the dipeptopeptidase, which might be important in heading a glucose load after a feed, changed with early load?

Dr. Thornburg: We have done a little bit and we know that in anemic animals endothelin-1 is not actually increased. However, coronary arteries from fetuses that have been malnourished show endothelial defects; they don't dilate very well in the presence of normal vasodilators, they don't generate nitric oxide very well, and their function is improved a great deal by antioxidants.

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Growth and Bone Development

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Abstract

Osteoporosis is a major cause of morbidity and mortality through its association with age-related fractures. Although most effort in fracture prevention has been directed at retarding the rate of age-related bone loss, and reducing the frequency and severity of trauma among elderly people, evidence is growing that peak bone mass is an important contributor to bone strength during later life. The normal patterns of skeletal growth have been well characterized in cross-sectional and longitudinal studies. It has been confirmed that boys have higher bone mineral content, but not volumetric bone density, than girls. Furthermore, there is a dissociation between the peak velocities for height gain and bone mineral accrual, in both genders. Puberty is the period during which volumetric density appears to increase in both axial and appendicular sites. Many factors influence the accumulation of bone mineral during childhood and adolescence, including heredity, gender, diet, physical activity, endocrine status, and sporadic risk factors such as cigarette smoking. In addition to these modifiable factors during childhood, evidence has also accrued that fracture risk might be programmed during intrauterine life. Epidemiological studies have demonstrated a relationship between birthweight, weight in infancy, and adult bone mass. This appears to be mediated through modulation of the set-point for basal activity of pituitary-dependent endocrine systems such as the hypothalamic-pituitary-adrenal and growth hormone/insulin-like growth factor-1 axes. Maternal smoking, diet (particularly vitamin D deficiency), and physical activity also appear to modulate bone mineral acquisition during intrauterine life; furthermore, both low birth size and poor childhood growth are directly linked to the later risk of hip fracture. The optimization of maternal nutrition and intrauterine growth should also be included within preventive strategies against osteoporotic fracture, albeit for future generations.

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Introduction

Osteoporosis is a skeletal disorder characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture [1, 2]. The risk of osteoporotic fracture ultimately depends on two factors: the mechanical strength of bone and the forces applied to it. Bone mass (a composite measure including contributions from bone size and its volumetric mineral density) is an established determinant of bone strength, and the bone mass of an individual in later life depends upon the peak attained during skeletal growth and the subsequent rate of bone loss. Several longitudinal studies attest to the tracking of bone mass through childhood and adolescence, and mathematical models suggest that modifying peak bone mass will have biologically relevant effects on skeletal fragility in old age. 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 [3]. Environmental influences during childhood and puberty have been shown to benefit bone mineral accrual, but the relatively rapid rate of mineral gain during intrauterine and early postnatal life, coupled with the plasticity of skeletal development in utero, offer the possibility of profound interactions between the genome and early environment at this stage in the life course. There is a strong biological basis for such a model of disease pathogenesis. Experimentalists have repeatedly demonstrated that minor alterations to the diet of pregnant animals can produce lasting changes in the body build, physiology and metabolism of the offspring [4]. This is one example of a ubiquitous phenomenon (phenotypic or developmental plasticity) which enables one genotype to give rise to a range of different physiological or morphological states in response to different prevailing environmental conditions during development. Its essence lies in the critical period during which a system is plastic and sensitive to the environment, followed by a loss of that plasticity and a fixed functional capacity. The evolutionary benefit of the phenomenon is that in a changing environment, it maximizes phenotypic diversity and enables the production of phenotypes that are better matched to their environment than would be possible with the production of the same phenotype in all environments. This review will address the role played by influences during intrauterine or early postnatal life, in establishing the risk of osteoporosis in later years. It will cover the patterns of normal skeletal growth during intrauterine life, infancy and childhood; the epidemiological evidence linking the risk of low bone density and fracture to environmental influences during early development; the impact of maternal nutrition and lifestyle on intrauterine bone mineral accrual, and the mechanisms underlying the relationship between developmental plasticity and osteoporosis.

Normal Skeletal Growth

Peak Bone Mass

At any age, the amount and quality of an individual's skeleton reflect their experiences 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 bodyweight, 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, peaks earliest (prior to age 20 years) at the proximal femur, while total skeletal mass peaks 6–10 years later [5]. However, total skeletal mass does not reflect the considerable heterogeneity in mineral accrual at various skeletal sites [6, 7]. Thus, the growth velocity of total body length is high immediately after birth, but slows rapidly during infancy; it accelerates again at 12 months of age, due to more rapid longitudinal growth of the legs, but not the spine. The growth velocity of the legs continues to remain around twice that of the axial skeleton until puberty. Thus, there is a disassociation between the peak velocities for height gain and bone mineral accrual in both genders [8].

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 [9]. 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 [10].

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. The second is responsible for the formation of the bones which 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 mineralized. The development of this cartilage model can be seen by 5 weeks gestation with the migration and condensation of mesenchymal cells in areas destined to form the bone [11]. These pre-cartilaginous 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, pre-hypertrophic 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 mineralization. The major regulator of the proliferation of

chondrocytes is parathyroid hormone-related protein (PTHrP) [12], which is secreted by the perichondral cells; other proliferative stimuli include cytokines of the growth hormone (GH)/insulin-like growth factor (IGF) axis [13]. 1,25-$(OH)_2$ vitamin D_3 [14] and tri-iodothyronine [15] 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 [16], osteoblasts from the perichondrium invade and lay down matrix which is then mineralized.

During the period of a normal human pregnancy the fetus accumulates approximately 30 g of calcium; the majority of this is accrued during the third trimester [17]. 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 [18]. This materno–fetal gradient emerges as early as 20 weeks gestation [19]. It is mainly influenced by low levels of fetal parathyroid hormone activity; a lack of fetal parathyroids in mice leads to low fetal calcium levels and decreased mineralization [20]. The main effect of PTHrP seems to be on placental calcium transport [20]. Additionally there is evidence that PTH and PTHrP differentially affect the mineralization of cortical and trabecular bone [21, 22], and thus are attractive candidates for the physiological, mediation of intrauterine skeletal programming.

The rate of materno–fetal calcium transfer increases dramatically after 24 weeks, such that around two thirds of total body calcium, phosphorus 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. 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 [23]. This observation is concordant with lower cord serum 25$(OH)_2$ vitamin D concentrations observed during the 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 [24].

Developmental Origins of Osteoporosis

Epidemiological studies of coronary heart disease performed over a decade ago demonstrated strong geographic associations between the death

rate from the disorder in 1968–1978 and infant mortality in 1901–1910 [25]. 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 hypercholesterolemia [26]. These associations are explained by a phenomenon known as programming [27]; 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 [28].

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 characterizing 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.

Population Studies

The first epidemiological evidence that osteoporosis risk might be programmed came from a study of 153 women born in Bath during 1968–1969 who were traced and studied at age 21 years [29]. Data on childhood growth were obtained from linked birth and school health records. There were statistically significant ($p < 0.05$) associations between weight at 1 year and BMC, but not density, 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 mineralization. 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 subsequent cohort studies of men and women aged 60–75 years, who were born and still lived in Hertfordshire [30, 31]. These studies showed highly significant relationships between weight at 1 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 [32], and 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 polymorphism in the gene for the vitamin D receptor, birthweight, and bone mineral density (BMD) have been published from the same cohort study [33]. These suggest that genetic influences on adult bone size and mineral density may be modified by undernutrition in utero. Subsequent studies from the United States, Australia and Scandinavia have replicated these relationships between weight in infancy and adult bone mass. Finally, a recent twin study [34] evaluated the relationship between birthweight and bone mass among 4,008 white female twins aged 47.5 years. Statistically significant relationships were found between the intra-pair differences in birthweight and in BMC, after adjustment for height and weight, even among monozygous twin pairs. These data suggest that even in genetically identical subjects, a relationship can be detected between birthweight and adult bone mass.

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 [34–36]. 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 mineralization, and to predispose to an accelerated rate of bone loss during later life [35–37]. 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 [38]. 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 a significantly lower basal GH concentration, lower baseline delayed neuronal death and accelerated bone loss (fig. 1). As with polymorphism in the gene for the vitamin D receptor, a significant ($p = 0.02$) interaction was observed between weight at one year, allelic variation at this site and bone loss rate.

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

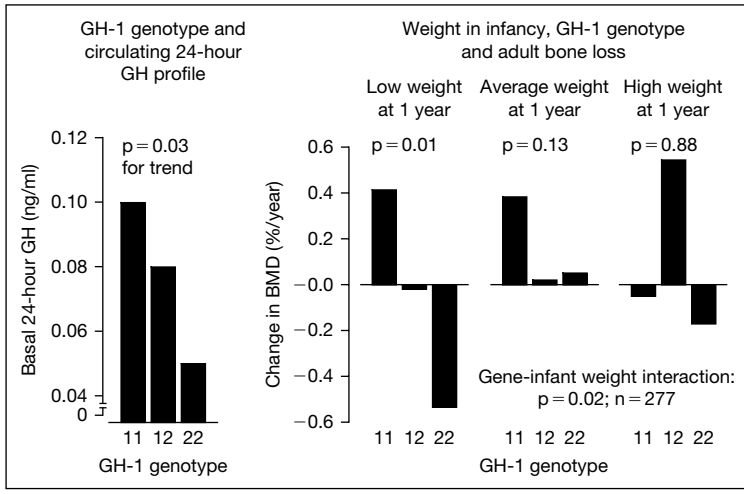

Fig. 1. GH-1 genotype, 24-hour GH concentration, weight in infancy and adult bone loss: Hertfordshire Cohort Study. BMD = Bone mineral density. Data derived from Dennison et al. [38].

series of mothers through pregnancy; anthropometric and lifestyle maternal characteristics were related to the bone mineral of their newborn offspring [39]. After adjusting for sex and gestational age, neonatal bone mass was strongly, positively associated with birthweight, birth length and placental weight. Other determinants included maternal and paternal birthweight, and maternal triceps skinfold thickness at 28 weeks (fig. 2). Maternal smoking and maternal energy intake at 18 weeks gestation were negatively associated with neonatal BMC at both the spine and whole body (fig. 3). 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.

In the most recent data from mother/offspring cohorts, body composition has been assessed by dual energy X-ray absorptiometry in 216 children at age 9 years [40]. 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 characterized 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

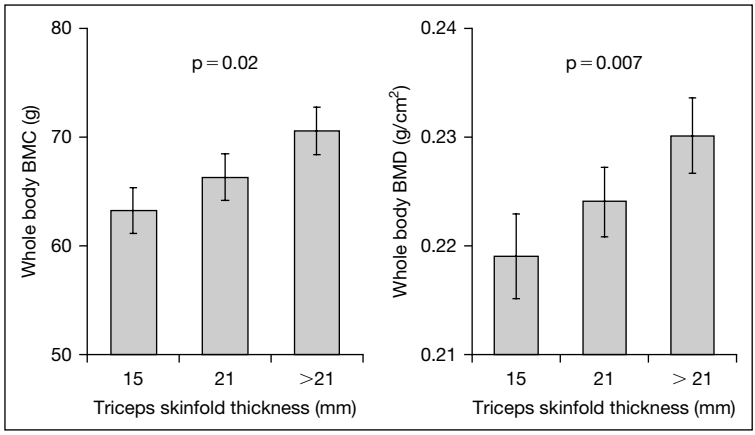

Fig. 2. Maternal triceps skinfold thickness in early pregnancy and neonatal bone mineral content (BMC) and density (BMD) among 144 term neonates. Values are mean ± SE. p values adjusted for sex and gestation. Data derived from Godfrey et al. [39].

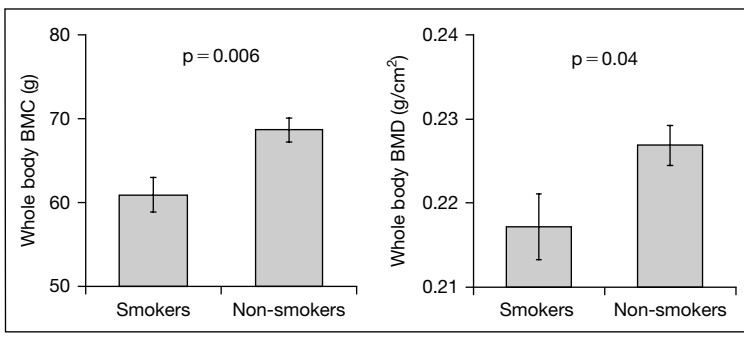

Fig. 3. Maternal smoking during pregnancy and neonatal bone mineral content (BMC) and density (BMD) among 144 term neonates. Values are mean ± SE. p values adjusted for sex and gestation. Data derived from Godfrey et al. [39].

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. A lower ionized 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 socioeconomic status on the bone mass of the children at

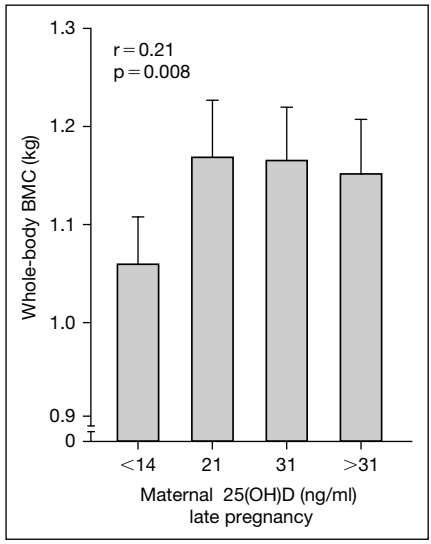

Fig. 4. Maternal 25(OH)D during later pregnancy and whole-body bone mineral content (BMC) of her offspring 9 years later. Data derived from Javaid et al. [56].

age 9 years. Around 25% of the mothers had suboptimal vitamin D status as assessed by the serum 25-hydroxyvitamin D concentration (fig. 4). 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 [40]. These data suggest that the placental capacity to maintain the materno–fetal calcium gradient is important in optimizing the trajectory of postnatal skeletal growth. They are in accord with the results of follow-up studies relating vitamin D supplementation in infancy to BMD in later childhood. In one such study, prepubertal Caucasian girls aged 7–9 years, who had received vitamin D supplementation during infancy, had greater areal BMD at the radius and proximal femur than a group of female controls of similar age [41].

Childhood Growth and Hip Fracture

Most evidence relating the intrauterine environment to later osteoporosis stems from studies utilizing noninvasive assessment 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 [42]. 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 7,000 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 a low rate of childhood growth (height $p = 0.006$; weight $p = 0.01$). The effects of maternal height and childhood growth rate were statistically independent of each other, and remained after adjusting for socioeconomic status. More important, hip fracture risk was also elevated ($p = 0.05$) among babies born short. These data suggest that hip fracture risk might be particularly elevated among children in whom growth of the skeletal envelope is forced ahead of the capacity to mineralize, a phenomenon which is accelerated during pubertal growth.

Developmental Plasticity and Osteoporosis

The observed relationship between osteoporosis risk and size at birth or during infancy does not imply a causal role of being born small, but reflects the sensitivity of fetal growth to adverse intrauterine influences. The term 'maternal constraint' encapsulates those environmental factors that influence birth size even in healthy pregnancies, for example maternal size, age, parity and multiple pregnancy. Among modifiable mechanisms limiting nutrient supply to the fetus, maternal nutrition has received the most attention, but other early environmental factors such as smoking, infectious exposure, and season of birth, may have long-term effects.

Experimental evidence that the prenatal or perinatal environment can influence adult postnatal physiology is available in several mammalian species [43, 44]. These studies demonstrate that manipulation of the periconceptual, embryonic, fetal, or neonatal environment can lead to altered postnatal cardiovascular and/or metabolic function. Although the environmental triggering cues are not yet fully understood, most manipulations have been dietary and include maternal pan-undernutrition [45, 46], low protein diet [47], or high fat diet [48, 49]. 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 [50]. Maternal protein restriction also downregulated the proliferation and differentiation of bone marrow stromal cells [51] as assessed by fibroblast colony formation at 4 and 8 weeks.

'Developmental plasticity' provides organisms with the ability to change structure and function in response to environmental cues; these responses usually operate during critical time windows and then become irreversible. Such plasticity permits a range of phenotypes to develop from a single genotype in response to environmental cues. In *Daphnia*, helmet formation (a defensive, morphological change) is dependent on the early environment and

risk of predation. In the locust, *Locusta migratoria*, the wing shape and metabolic pathways are determined in the larval stage by pheromone signals indicating population density. In the axolotl, early environmental conditions determine whether the mature form will be purely aquatic or amphibious [52]. Developmental plasticity sets the template on which continued postnatal homeostatic and homeorhetic (maintaining a time-dependent process, e.g. growth trajectory) adaptation can occur.

There are several mechanisms by which environmental cues can influence the developmental program. First, they can exert effects prior to implantation and affect gene expression, particularly by inducing epigenetic changes in the DNA. In the *agouti* mouse mutant, maternal dietary folate supplementation at conception alters the expression of the imprinted *agouti* gene by altering the capacity for methylation [53]. Non-imprinted genes can also undergo epigenetic change in response to the environment – the choice of exon usage in the glucocorticoid receptor gene is altered both by prenatal glucocorticoids and neonatal behavioral manipulation owing to changes in histone acetylation and DNA methylation in a transcriptional factor binding site [54]. These changes persist throughout life as manifested in altered hypothalamic-pituitary-adrenal axis activity. Second, tissue differentiation may be altered. Prolonged in vitro culture of the rodent or ruminant embryo affects the allocation of blastocyst stem cells to inner cell mass or trophectoderm lineages [55]. This influences the relative growth trajectories of the placenta and fetus, thus affecting fetal development in late gestation.

Developmental responses to environmental stimuli need not provide immediate advantages, but may alter the sensitivity of the organism to an anticipated future environment [44]. Such predictive adaptive responses are made during the phase of developmental plasticity to optimize the phenotype for the probable environment of the mature organism, and epigenetic change is likely to be the mechanistic basis. Where there is a match between the predicted and actual mature environment, these responses are appropriate and assist survival. In contrast, inappropriate predictions increase the risk of disease. A key issue thus becomes the relative importance of early life events in informing intervention strategies during human development, rather than during adult life. Increasing awareness of the need to promote the health and nutrition of women of reproductive age is one important element for the prevention of osteoporotic fracture in future generations across the globe.

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Discussion

Dr. Thornburg: Could you help us understand the trade off that you were discussing about increasing vitamin D sources from others?

Dr. Cooper: That's a very good question and one which taxed us enormously when we were going through the ethical approval for the vitamin D trial. If you look at the literature there is undoubtedly a high prevalence of vitamin D insufficiency in mothers in Western populations. Some of the estimates from northern Europe and the USA would reach 40%, being less than 20 ng/ml, and 25–30% being less than 10 ng/ml, so a high prevalence of low biochemical values. The data are very scanty on the relationship between vitamin D status and later cardiovascular health, cognitive function, the likelihood of allergy and a predisposition to infection. Broadly speaking, there is a possible role for vitamin D deficiency in diabetogenesis; a possible protective effect against respiratory infection, and an association with allergy and IgE-related hypersensitivity disorders. So in our cohort we studied those outcomes and basically there was no relationship between 25-OHD and adiposity or glucose tolerance; there was no relationship with cognitive function, there was a weak positive relationship with respiratory infection, and there was no relationship with pulse wave velocity.

Dr. Barclay: That was a beautiful presentation. I was very interested to see the results of this prudent diet study which are quite similar in fact to the results of the Dash study, the dietary interventions to stop hypertension in which the intakes of fruits, vegetables and dairy products was increased for adults. Not only was the blood pressure lower at the end of the study, after several months, but also the indicators of bone status had improved, which is going in the same way as your mother to infant relationship. In the Dash study one of the hypotheses was that it could be the calcium, magnesium and potassium versus sodium ratio. Do you think this is a valid hypothesis and do you think this has some value in nutrition during pregnancy and lactation?

Dr. Cooper: I am not in the least bit qualified to answer the relevance of those nutrients to blood pressure, but I think that there has been quite a lot of work on the two divalent cations and the monovalent cations and their relationships to bone health. I would have said that the best explanation of the data was actually linked to the ionized calcium itself rather than to the relative calcium/magnesium and sodium/potassium fluxes which would be involved in hypertension. There is some (circumstantial) evidence that urinary acidification modifies both calcium and magnesium balance and that this influences skeletal status through secondary hyperparathyroidism.

Dr. Walker: About mechanisms, you suggested that it is possibly an epigenetic process. Have you considered looking at polymorphisms? If you look at other micronutrients, folic acid is a classic example, there are polymorphisms in the metabolic pathways of these micronutrients, which could very well be the case in vitamin D or calcium, and could cause a much higher supplement to be given to prevent osteoporosis.

Dr. Cooper: Of course with the preface that Dr. Barker gave on the genome, the notion that fixed genetic variation would explain the observations is unlikely. However, we looked at candidate genes and we have actually published two papers: one looking at polymorphism in the vitamin D receptor [1], and the other looking at polymorphism in the promoter region of the growth hormone gene, the GH1 gene [2]. In both of those when you look at the adverse polymorphism, there is an interaction with birthweight in determining bone mass and the bone loss rate 70 years later. It is difficult to explain the rapidity of the secular trends that we are talking about in these diseases through altered frequencies of fixed genetic variation so that mathematically you would look at gene expression rather than fixed variation as the explanation.

Dr. K. Bergmann: In our childhood and adolescent health examination survey in Germany we found a very strong seasonal effect of low vitamin D, perhaps about 35% of the students have this problem between December and April and a very small percentage have the same problem during July to October. Do you think that matters and, as far as your results are concerned, are there any seasonal effects in what you observed?

Dr. Cooper: We have used broadband ultrasound attenuation (BUA) in the heel, which is a far less precise methodology than DXA, to look at what happens throughout pregnancy in women. When the 11–28 weeks of gestation occur in the summer months there is hardly any change in BUA. The pregnancy that has the 11–28 weeks of gestation in the winter months shows the steepest rates of BUA loss, with spring and autumn in between.

Dr. K. Bergmann: If we observe this in adolescents, do you think it matters? Should we do something about preventing low vitamin D values between December and April?

Dr. Cooper: My view is that the phenotypic expression of low circulating 25-OHD is not strong enough to treat, unless the calcium-phosphate product is low and the alkaline phosphatase is high.

Dr. K. Bergmann: In the United States there is no fortification. Does this change the picture?

Dr. Cooper: That is a very important question, and this needs to be evaluated in a randomized controlled trial.

Dr. Wilson: I would like to follow up on Dr. Walker's question briefly. You suggested that the secular trend is incompatible with any sort of genetic basis, which is probably true, but might the secular trend be dependent on underlying genetic differences that may contribute and give rise to a future epidemic of osteoporosis because of aging? You also talked about some methylation data. Of course methylation data have the problem of what is the chicken and what is the egg. Do you have any chicken–egg experiments to suggest that there is a causal modification?

Dr. Cooper: When I was referring to the term, secular trend, I meant after adjustment for demographic changes in the population; so after adjustment for age changes, for cardiovascular disease and for osteoporotic fracture there has been a rise. If you look at the Rochester data, fracture incidence data for the 7th to 8th decades, there is a rise from about 19.30, then a plateau for 10–12 years and then the beginning of decline. Age period cohort modeling on these shows that there is clearly an age effect, clearly a period effect but, very interestingly for this discussion, clearly a residual birth cohort effect. So there is something going on not just in the current environment and in the late stage of life, but also some experience that is differentially undergone by different birth cohorts. Data exploring methylation of the acetylcholine receptor gene and vascular reactivity suggest that alteration in folate intake might modulate gene expression.

Dr. Shaalan: During the last 3 years I have implemented a multidisciplinary project for osteoporosis in Egypt and the results will be available by the end of this year.

The preliminary results show that we have a very high incidence of osteoporosis in Egypt, although it's a sunny country. During the next 3 years we will study nutritional intervention with micronutrients and one of the main domains of this intervention will be vitamin D supplementation. You said that you are interested in new future studies on micronutrient supplementation before and after pregnancy. I am also interested in the peak bone mass attainment. Do you think it would be feasible and beneficial to unify our research to see the differences between our two countries, especially with regard to the different environmental and genetic pools?

Dr. Cooper: Yes, of course.

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The Role of Genes in Growth and Later Health

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Abstract

Genetic factors are of importance for the development of the metabolic syndrome and type 2 diabetes, but despite extensive research the identification of the underlying genes has not been fruitful. This report focuses on the interactions between intrauterine growth and genes in relation to adult health outcomes based upon findings from the Helsinki Birth Cohort Study. Candidate genes for type 2 diabetes and the metabolic syndrome have been focused upon and we report on interactions between polymorphisms of the peroxisome proliferator-activated receptor (PPAR)γ-2, plasma cell glycoprotein (PC-1) and the glucocorticoid receptor (GR) genes and - prenatal growth in relation to adult health outcomes. In elderly individuals the effects of the Pro12Pro/Pro12Ala polymorphisms of the PPARγ-2 gene depend on their body size at birth. Individuals, who had a small body size at birth and were carriers of the Ala allele, seem to be protected against insulin resistance and type 2 diabetes in later life. Similar gene environment interactions will be described in relation to the PC-1 and the GR genes. We propose that these findings reflect gene–early environment interactions and can be attributed to the phenomenon of developmental plasticity.

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Epidemiological studies from various geographical regions have clearly established that there is a strong association between size at birth and later health. It has been repeatedly shown that, for instance, an increased coronary heart disease risk associated with a small body size at birth is a consequence of growth restriction during fetal life – not prematurity. These findings support the view that maternal and fetal undernutrition are important underlying risk factors for cardiovascular disease. In fact, not only fetal growth but also growth during infancy seem to be of major importance in the programming of adult health and disease [1–11].

According to the original fetal origins hypothesis put forward by Barker [12], fetal adaptations to an adverse intrauterine environment involve programming of different pathways and functions in the body, leading to lifelong metabolic changes. These changes predispose to metabolic and cardiovascular disease in adult life. Since the fetal adaptations include reduced intrauterine growth, a small body size at birth has been used as a proxy for the early intrauterine environment [12].

An alternative explanation for the association between a small birth size and adult health was put forward in 1999 [13]. The 'fetal insulin hypothesis' proposed that one genotype could be the common denominator altering intrauterine growth as well as influencing adult health outcomes. In other words it was proposed that a small body size at birth and, for example, impaired glucose regulation in adult life could be different phenotypes of the same underlying genotype. This hypothesis was originally based upon findings in monogenic forms of diabetes, for instance in diabetes caused by glucokinase gene mutations leading to maturity onset diabetes of the young (MODY). These MODY-linked mutations were known to cause diabetes, but the carriers of the mutations also had a lower birthweight. However, MODY is a rare form of diabetes and this specific gene defect is therefore not likely to explain the association between birth size and type 2 diabetes observed in epidemiological settings. More recently a study focusing on common variants of the glucokinase gene showed that the gene is associated with fasting glucose and fetal growth – although the impact on birthweight was small [14].

Several other genes associated with growth and glucose-insulin metabolism have been focused upon. For example, the insulin-like growth factor-1 and insulin genes have been suggested to be simultaneously related to both fetal growth and adult health outcomes. The 'thrifty genotype hypothesis', on the other hand, suggests that genotypes promoting survival during nutritional adversity would increase the risk of type 2 diabetes later in life [15].

One strong 'thrifty gene' candidate is the insulin gene and variation in the insulin gene variable number of tandem repeats (VNTR) polymorphism has been suggested to modify birth size and diabetes susceptibility. Insulin is a strong candidate gene as it is a major growth factor in fetal life and infancy, and is closely linked to glucose metabolism. The insulin VNTR polymorphism has mostly been studied in relation to its effects on early growth and diabetes – with inconsistent results [16–18].

Today, there is no strong evidence to suggest that any single common gene or gene variant would explain the common association between birth size and later health outcomes. In other words support for the original fetal insulin hypothesis is small [19]. However, the genetic field is making rapid progress and it is important to keep in mind that there might be important gene–environment interactions not easily identified in genetic studies with little or no information on early growth. The intrauterine environment might well interact with genes affecting health later in life by different mechanisms. Little is

known about the possible interactions between the intrauterine environment and genes associated with growth and adult health. This field can be studied using different measures of body size at birth as markers of the early environment. This report will focus on the interactions between intrauterine growth and genes in relation to adult health outcomes. The health outcomes focused upon will primarily be type 2 diabetes and its established risk factors and comorbidities.

The Helsinki Birth Cohort Study

The Helsinki Birth Cohort Study (HBCS) comprises two study cohorts consisting of 15,846 individuals born in Helsinki, Finland. The older cohort includes 7,086 individuals born 1924–1933, with information on birth characteristics as well as growth data between 7 and 15 years of age obtained from birth records and school healthcare records. Besides information on growth, these include information on health and socioeconomic factors. A younger cohort, born 1934–1944, consists of 8,760 individuals and includes growth information from birth to 11 years of age obtained from birth records, child welfare clinic and school healthcare records. Both cohorts have been followed up from 1971 by register linkage to national Finnish registers providing epidemiological information on both morbidity and mortality.

Clinical examinations of 500 individuals from the older cohort and 2,003 individuals from the younger cohort have provided more detailed information on metabolic and genetic aspects and their associations with growth and adult health outcomes. The results presented here are based upon findings from the 500 individuals born 1924–1933 and who participated in a clinical study at the age of ~70 years.

Genetic Studies in HBCS

A number of candidate genes mostly related to insulin and glucose metabolism have been focused upon in the HBCS. This overview will focus upon the peroxisome proliferator-activated receptor-γ-2 (PPARγ-2), the plasma cell glycoprotein (PC-1) and glucocorticoid receptor (GR) genes in relation to early growth and adult health outcomes.

Peroxisome Proliferator-Activated Receptor Genes

The PPARs play an major role in the regulation of glucose, lipid and energy metabolism. A common missense mutation in the functional domain of the human PPARγ-2 gene resulting in a substitution of proline by alanine in

Table 1. Mean fasting insulin concentrations (pmol/l) according to PPARγ-2 gene polymorphism and birthweight

	Birthweight, g			
	<3,000	3,000–3,500	>3,500	p value*
Pro12Pro	84	71	65	0.003
Pro12Ala/Ala12Ala	60	60	65	0.31
p value#	0.008	0.02	0.99	

p = 0.03 for interaction between birthweight and genotype.
*p value for the difference among birthweight groups.
#p value for the difference betweeen the Pro12Pro and Pro12Ala/Ala12Ala genotypes.

codon 12 has been found to modulate the transcriptional activity of the gene [20]. In meta-analysis the Pro12Ala variant of the gene has been found to be associated with improved insulin sensitivity and a lower risk of type 2 diabetes compared with the carriers of Pro12Pro genotype [21, 22]. However, in general the published findings in relation to the PPARγ-2 genotype have been inconsistent in relation to glucose and insulin metabolism.

We have reported associations between the PPARγ-2 gene and birth size in relation to metabolic characteristics associated with the metabolic syndrome. In the HBCS we observed that elderly carriers of the Ala allele had lower fasting insulin and glucose concentrations, i.e., they were more insulin-sensitive compared to the carriers of the Pro12Pro genotype [23]. There were no differences between the groups in body size at birth or childhood body size. Interestingly the association between a small body size at birth and insulin resistance was observed only in individuals with the high risk Pro12Pro genotype (table 1). In other words the Ala allele was protective against the negative effect of a small body size at birth. There was a strong interaction between birth size and PPARγ-2 genotype (p = 0.03).

We have also examined the combined effects of the same PPARγ-2 gene polymorphisms and birth length on the occurrence of type 2 diabetes. The Pro12Pro genotype was associated with a higher cumulative incidence of type 2 diabetes (p = 0.08). This association was confined to people who were ≤49 cm in length at birth, among whom the cumulative incidence of type 2 diabetes was 24.5%, compared with those >49 cm in length at birth, in whom the cumulative incidence was 14.3% (p = 0.02) [24].

A small body size at birth is associated with insulin resistance as well as other features of the metabolic syndrome. Consequently low HDL-cholesterol concentrations have been reported in association with a small body size at birth. A protective effect among the carriers of the Ala allele of the PPARγ-2 gene – even in the presence of a low birthweight – was observed in relation to HDL-cholesterol concentrations as shown in table 2 [25].

Table 2. Mean fasting HDL-cholesterol concentrations (mmol/l) in elderly individuals from the HBCS according to birthweight groups and PPARγ-2 gene polymorphism

Birthweight, g	Pro12Pro	Pro12Ala	p value#
<3,000	1.37	1.48	0.16
3,000–3,500	1.42	1.44	0.68
>3,500	1.48	1.47	0.94
p value*	0.02	0.66	

p = 0.01 value for interaction between birthweight and genotype.
* p value for the difference among birthweight groups.
#p value for the difference betweeen the Pro12Pro and Pro12Ala/Ala12Ala genotypes.

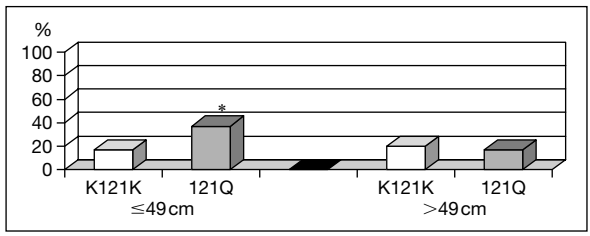

Fig. 1. Prevalence of type 2 diabetes according to length at birth and the PC-1 gene polymorphism. *p = 0.005; p < 0.05 for interaction between genotype and birth length.

Plasma Cell Glycoprotein Gene

Being an important regulator of the insulin-signaling pathway, the PC-1 gene is another candidate gene for type 2 diabetes. PC-1 inhibits autophosphorylation of the insulin receptor and impairs insulin signaling downstream of the insulin receptor. The 121Q variant of the PC-1 gene has a greater inhibitory action on the insulin receptor than the 121K variant and is consequently associated with insulin resistance [26]. We have investigated whether the K121Q polymorphism of the PC-1 gene association with insulin sensitivity, type 2 diabetes and hypertension in adult life depends on body size at birth. In the HBCS, those individuals carrying the 121Q allele had a significantly higher prevalence of type 2 diabetes and hypertension combined, but only in the presence of a small body size at birth [27]. Figure 1 shows the prevalence of type 2 diabetes in relation to birth size and the K121Q polymorphism of the PC-1 gene. Only the carriers of the high risk 121Q variant had a higher diabetes prevalence in association with a small body size at birth.

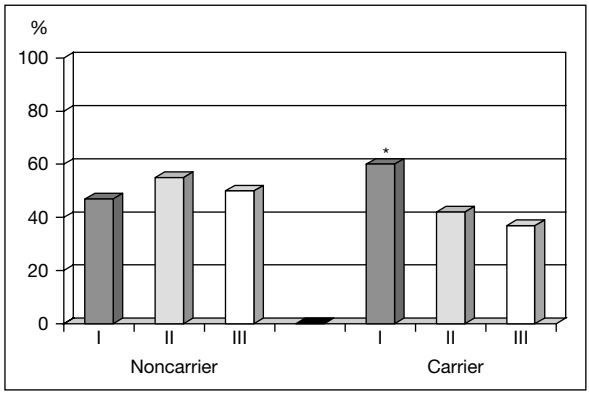

Fig. 2. Prevalence of impaired glucose tolerance and type 2 diabetes according to length at birth and glucocorticoid receptor gene haplotype. *p = 0.007; p = 0.02 for interaction between birth length and GR haplotype on the cumulative incidence of glucose intolerance.

Glucocorticoid Receptor Gene

Glucocorticoids are important regulators of fetal growth and development as well as regulators of glucose metabolism. The glucocorticoids mediate their cellular action by complexing with the cytoplasmic GR. The GR gene is another candidate gene that could partly explain the associations between early growth and later health outcomes [28]. Certain haplotypes of the GR gene modify the association between birth size and adult phenotypes in the HBCS. These findings suggest that a common GR haplotype could modify the association of short length at birth with glucose tolerance in adult life [29]. Figure 2 shows that carriers of the GR gene haplotype 3 had the highest prevalence of impaired glucose tolerance and type 2 diabetes but only in the combination with a short birth length. The interaction between the effects of length at birth and GR haplotype on glucose regulation was highly significant (p = 0.02).

Conclusion

The findings described here could be interpreted as manifestations of gene early environmental interactions and illustrate the importance of the early environment in relation to risk factors for type 2 diabetes and related disorders. But what does this mean, can we take these findings further and make some clinical implications? Acknowledging the interactions between early growth and genotypes might help us to design individual therapies as well as plan lifestyle interventions. We need to take into account individual variability

not only in the genetic setup but also in early growth phenotypes. Further studies focusing on growth during infancy and childhood – and potential interactions with high risk genotypes – are needed in this field.

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Discussion

Dr. R. Bergmann: The peroxisome proliferator-activating receptor (PPAR)γ2 gene is the master gene for the terminal differentiation of adipocytes, which is promoted by arachidonic acid [1]. Ailhaud and Guesnet [2] proposed that nutritional imbalances in n-6 and n-3 fatty acids during the last decades were determinants for the increasing prevalence of adiposity in childhood, i.e. gene–nutrition interactions.

Dr. Eriksson: I think it's quite possible but we still know very little about these and there might be gene–nutrition interactions. Another aspect that is quite important when looking at the PPARγ gene is that it has an important co-activator, PGC-1 that influences its activity, consequently there could also be important gene–gene interactions. Anyway, this field is so new and we have not had the time yet to look at any gene–nutrition interactions, but we are certainly planning to do that. We have adult food frequency data on 2,000 individuals and we are also following them up prospectively.

Dr. Batubara: Did you say that in a person with a low birthweight or who is obese but carrying this Ala allele, the gene has a protective effect on diabetes type 2?

Dr. Eriksson: Yes. In our older cohort those with low birthweight and carrying the Ala allele were protected against the negative influence of a small birth size. However, life is obviously much more complicated and if you look at all the meta-analyses in relation to the PPARγ gene, they are not really consistent. In general you can say that people who carry the Ala allele are more insulin-sensitive and they are at a lower risk of getting type 2 diabetes.

Dr. K. Bergmann: One thing that impresses me very much is that with higher birthweight there is a lower risk of later diabetes type 2. In our own study and in several other studies, we see that a high birthweight is a great risk factor for later obesity, and obesity being the basis in about 95% of cases of type 2 diabetes, you should also find effects of high birthweight on this phenomenon.

Dr. Eriksson: This is a really important. When we are looking at birth size in relation to type 2 diabetes, we can study them both using epidemiological or clinical data. If we do this mainly on epidemiological data, we usually identify only those with the worse type of type 2 diabetes and they simultaneously have coronary heart disease. So

I think that is one reason why we see an association between a low birthweight and type 2 diabetes mainly from the epidemiological data. We have been looking at those people with a higher birthweight and what you say is completely true. Both people with low and high birthweight can develop type 2 diabetes, they do it through a different early growth pathway.

Dr. Ogra: There is a certain disparity between birthweight and type 2 diabetes. Clearly obesity does contribute to the development of type 2 diabetes. Are we looking at a heterogeneous population in type 2 diabetes? Is there any relationship to early colonization with different microbial flora or diet with type 2 diabetes or those who develop non-alcoholic hepatitis associated with type 2 diabetes? Are we looking at multiple triggers? Birthweight may be only one of those factors, and gene polymorphism may also be one of the factors in this whole process.

Dr. Eriksson: As a diabetologists we know that type 2 diabetes is extremely heterogeneous in adult life. We have been studying the different subtypes of diabetes that develop in those people born with lower birthweight compared to that seen among those born with a higher birthweight. There is a study on the Pima Indians that has clearly shown that gestational diabetes is a major and important risk factor for the high birthweight route [3]. There was another study done in schoolchildren in Taiwan that also showed a strong relationship between birth size and later risk of type 2 diabetes [4], and the risk associated with type 2 diabetes in the high birthweight group in Taiwan was mostly explained by a strong family history for type 2 diabetes.

Dr. Thornburg: Has anyone looked to see whether or not the effect of polymorphisms related to birthweight are actually due to genetic modifications through epigenetic means? It seems to me that this might be a possibility that hasn't been ruled out yet.

Dr. Eriksson: It certainly hasn't been ruled out and I very much agree with you that it could be a very likely explanation for this, but as far as I know nobody has looked yet at the human population.

Dr. Ogra: The PGC-1 gene, is this a polymorphism which has been identified for a long time or is it something very recent? We are seeing a tremendous increase in type 2 diabetes in certain population groups in the USA.

Dr. Eriksson: I am sorry to say that I don't have the information on the distribution of the PGC-1 gene in various populations.

Dr. Ogra: What about the PPAR gene?

Dr. Eriksson: It is quite similarly distributed, at least in the Western population.

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Maternal Nutrition Before and During Pregnancy

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Abstract

In humans, the link between the maternal diet and the outcome of pregnancy is best illustrated by the classic study of wartime famine in Holland. During the famine it is likely that a low food intake reduced the glucose stream from the mother to fetus and gave rise to smaller size at birth. Maternal glucose production is also influenced by the type of carbohydrate in the diet. Even when famine and starvation are not issues, a low dietary glycemic index can alter maternal blood glucose production and the area under the glucose curve, and give rise to reductions in fetal growth and infant weight at birth. Reduced food intake in famine areas would also reduce the concentration of micronutrients in the maternal diet. Two micronutrients (iron and folate) have effects on pregnancy outcome that have been shown with some consistency in pregnant women. Emerging evidence now suggests that use of micronutrient-containing prenatal vitamins before and during pregnancy is associated with reductions in the risk of congenital defects, preterm delivery, low infant birthweight, and preeclampsia.

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The relation between birthweight and risk of chronic disease in later life was noted by Barker et al. [1] in their study of a cohort (1915–1938) born in Hertfordshire, England; they suggested that an increased risk of early and later adverse events was linked through poor maternal nutrition during pregnancy.

In animal models maternal starvation reduces the amount of metabolic substrate produced by the mother and supplied to the fetus and diminishes intrauterine growth [2]. Likewise, a diet low is micronutrients is associated with a pregnancy complicated by congenital defects, preterm delivery and other adverse outcomes [2]. However, apart from exceptional circumstances

such as famine, the influence of the maternal diet on fetal growth and gestation duration is controversial.

Famine and Undernutrition

Studies in developing countries and among different ethnic groups have been virtually unanimous in showing a positive relationship between pre-gravid weight or body mass index, and gestational weight gain with birthweight and gestation duration [2]. Maternal underweight and inadequate weight gain are associated with lower birthweight, decreased gestation duration and increased risks of low birthweight reflecting fetal growth restriction and/or preterm delivery [2]. Consequently, it is widely thought that many pregnant women are underweight or gain weight poorly because they are poorly nourished. Thus, one reason that maternal pre-gravid weight and weight gain correlate to adverse pregnancy outcomes may be through diet and energy balance.

In the classic study of wartime famine well-nourished Dutch women experienced serious food shortage in the course of pregnancy [3]. It was observed that first trimester exposure at the peak of the famine, in combination with infection or another unknown factor, was linked to an excess of preterm birth and to an increase in infants weighing <2,000 g. There was also a rise in the frequency of malformations of the central nervous system including spina bifida [3] which is consistent with a deficiency in folate intake.

In ewes a short interval of food deprivation around the time of conception – from 2 months before to the first month after conception – increases the risk of preterm birth. Restriction was brief, reduced maternal weight by about 15%, and was followed by ad libitum feeding for the remainder of gestation [4]. Since the nutritional demands of the fetus early in gestation are modest, it was unclear if preterm delivery was triggered by the shortage of essential nutrient(s), a lack of calories or other factors. Likewise, in Gambia there is a fluctuating food supply between the dry and rainy seasons. This is reflected by seasonal changes in maternal weight. Pregnancies conceived in the months when women are at their lowest weights are significantly shorter (38.6 versus 39.0 weeks) than pregnancies conceived when food is more abundant [5]. Apart from food shortages, periods of extended fasting (13 h or more during the second and third trimesters) are associated with increased production of corticotropin-releasing hormone, which is related to an increased risk of preterm delivery [6].

Glucose and the Glycemic Index

Glucose is the main energy substrate for intrauterine growth and is transmitted in a steady stream from mother to fetus [7]. Glucose is produced by maternal metabolism principally from carbohydrate in the diet and from the

gluconeogenic amino acids. In turn, the hormone insulin regulates glucose. Dietary restriction reduces metabolic fuels that give rise to slower fetal growth; maternal hypoglycemia is also associated with an increased risk of fetal growth restriction [7–9].

The Dutch famine is best known for its effects on fetal growth, and third trimester exposure to intense famine resulted in decreased maternal postpartum weight and reduced infant birthweight [3]. The largest deficits in birthweight, which amounted on average to 300 g, occurred with famine exposure during the last half of pregnancy. Infants returned to their usual weight after the famine ended. During the famine, reduced maternal intake following small, infrequent meals would have resulted in lower circulating levels of maternal glucose. Reductions in maternal body mass and fat stores from famine would have altered glucose disposal and left less maternal fat to oxidize as an alternative fuel. Thus, a reduced glucose stream from mother to fetus would have given rise to slower fetal growth, smaller birth size, and an increased risk of fetal growth restriction.

In addition to the amount of food (energy) that is eaten, maternal glucose is influenced by the type of carbohydrate in the mother's diet [8, 10, 11]. The glycemic index is a relative measure of the blood glucose response to a given amount of carbohydrate that represents the quality of the carbohydrate that is eaten. The glycemic index is defined as the incremental area under the glucose response curve following the intake of 50 g of carbohydrate from food compared to the glucose area generated from a similar amount of white bread or glucose [10, 11]. Some carbohydrates are absorbed more slowly than others and thus may have a weak effect on blood glucose levels. Although there is variation within and between individuals, on average foods with a lower glycemic index give rise to a smaller blood glucose response than foods with a higher glycemic index [10, 11].

During pregnancy a mother's dietary glycemic index is positively and significantly related to circulating levels of glucose; in urban, low-income women from Camden there was a 2% difference in glycosylated hemoglobin and a 4% difference in plasma glucose between gravidae at the extremes (quintiles) of the glycemic index [12]. In the same cohort, a low dietary glycemic index was associated with lower infant birthweight – a reduction of 116 g and a 2-fold increased risk of bearing a small for gestational age infant [12]. These observations were in accord with the results of a small clinical trial (n = 12) where exercising women eating a low glycemic index diet had a lower plasma glucose response to a standard meal than women on a diet with a high glycemic index diet and infants with birthweights that were substantially lower by 1,000 g [13]. A recent randomized trial in which Australian women (n ~30/group) were randomly assigned to a low or higher glycemic index diet also showed changes [14]. Women in the low glycemic index group again had infants with lower birthweights (−236 g) and lower ponderal indices, and the risk of large for gestation births was substantially reduced (3.1 vs. 33.3%), but a smaller

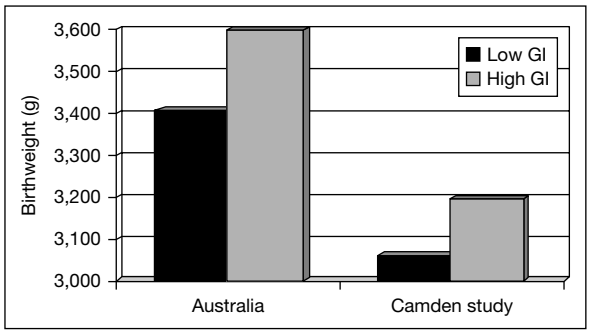

Fig. 1. Infant birthweight with high versus low maternal dietary glycemic index (GI).

increase (9.4 vs. 6.7%) in the risk of fetal growth restriction was not statistically significant. Even in situations where starvation is not an issue, the mother's glycemic index can alter blood glucose production and the area under the glucose curve, reduce fetal growth and infant weight at birth (fig. 1). It should be noted that several epidemiological studies have produced inconsistent results on the influence of the glycemic index or load on risk of type 2 diabetes. However, the evidence from pregnancy, although not yet definitive, suggests that further study may be fruitful and important.

Micronutrients

Reduced food intake in famine areas would also reduce the concentration of micronutrients in the maternal diet. A low intake of micronutrients has the greatest potential to do harm during times of rapid tissue growth such as pregnancy. But, unlike animal models that involve experimental manipulations of single nutrients, in humans inadequate intake of a single nutrient usually does not occur in isolation.

Although there are exceptions, many studies now demonstrate that the use of micronutrient containing prenatal vitamins before and during pregnancy is associated with increases in birthweight and reductions in the risk of preterm delivery, low infant birthweight, gestational hypertension and preeclampsia [15, 16]. Two micronutrients (iron and folate) have effects that have been demonstrated with some consistency, and for a third (vitamin E) data on pregnant women are beginning to emerge.

Iron is a micronutrient that is essential for the formation of hemoglobin to transport oxygen, and for the synthesis of enzymes that use oxygen to provide cellular energy. Anemia (low hemoglobin levels) and iron deficiency anemia also serve as indicators of overall maternal nutritional status during pregnancy. When overall dietary intake is inadequate, the risk of anemia is increased [2].

Folic acid functions as a coenzyme in the transfer of single-carbon atoms from donors such as serine and histidine to intermediates in the synthesis of amino acids, purines, and thymidylic acid. Inadequate intake of folate leads to impaired cell division and alterations in protein synthesis.

Vitamin E is a lipid-soluble chain-breaking antioxidant that is dietary in origin. In addition to its antioxidant actions, vitamin E enhances the release of prostacyclin, a metabolite of arachidonic acid that inhibits platelet aggregation, quiets uterine contractility and increases vasodilation thus potentially improving blood flow between the fetus and placenta.

Iron and Anemia

Maternal anemia is linked to an increased risk of adverse outcomes during pregnancy [2]. During the first and second trimesters, the hemoglobin concentration declines, reaches a low point early in the third trimester, and rises thereafter. Depending on the stage of gestation when anemia is assessed, it can be difficult to separate truly anemic women from those whose anemia results from hemodilution. The best time to detect risk associated with maternal anemia may be early in pregnancy before the plasma volume is fully expanded. This was originally examined in Camden, separating maternal anemia by time, i.e. early vs. late (week 28), and etiology (iron deficiency anemia (IDA) vs. anemia from other causes) [17]. IDA at entry was associated with greater than 2-fold increases in the risks of low birthweight and preterm delivery, while anemia from other causes was associated with a small but nonsignificant increase in risk. In the third trimester when the effects of hemodilution are profound, the risk of preterm delivery was reduced for women with IDA, and there was no increased risk for women with other anemias [17]. The association of maternal anemia, based upon early pregnancy hemoglobin with preterm delivery extends to the time before conception. Anemic Chinese workers who later experienced a pregnancy had 5- to 6-fold increases in the risk of low birthweight infants and fetal growth restriction, and gave birth to infants weighing 140–200 g less than other women. When anemia was attributable to iron deficiency, the birthweight reduction was greater still amounting to a decrement of approximately 250 g [18].

Two clinical trials conducted among low income iron-replete women from the United States suggested that iron supplementation can reduce associated risks. Gravidae were enrolled early in pregnancy (<20 weeks) and randomly assigned to supplemental iron (30 mg/day as ferrous sulfate) or to multivitamins with and without iron (ferrous sulfate 30 mg) [19, 20]. In both instances infant birthweight was increased and supplemented women had either significantly longer gestations or a lower risk of delivering preterm. Plausible biologic mechanisms underpinning the effects of iron during pregnancy include chronic hypoxia that initiates a stress response with the

release of placental corticotropin-releasing hormone and an increase in fetal production of cortisol, increased oxidative stress that damages the maternal–fetal unit and reduced immune function which increases the risk of maternal infection [21].

Folic Acid

It is recommended that pregnant women in the US consume 600 μg folic acid/day, which includes 400 μg of synthetic folic acid from supplements or fortified cereals, to reduce the risk of neural tube defects. Fortification of flour and cereal products in the US with folic acid (since 1998) has been associated with a 19% decline in the risk of live-born infants with neural tube defects, along with changes in biomarkers of folate status, including increases in serum and red cell folate and a decline in homocysteine levels [22].

An absolute deficiency of folate (from a diet inadequate to meet the needs of pregnancy) will interfere with the growth of the conceptus [2]. The influence of dietary and circulating folate on preterm delivery and low infant birthweight was studied in women from Camden, one of the poorest cities in the continental US. Low folate intake (<240 μg/day) was associated with a greater than 3-fold increase in the risk of low infant birthweight and preterm delivery. Circulating folate at week 28 was also associated with risk; the adjusted odds for low birthweight increased by 1.5%, and preterm delivery increased by 1.6% per unit decrease in concentration [23, 24]. Lower dietary folate intake (<500 μg/day) at 24–29 weeks gestation was associated with an approximately 2-fold increased risk of preterm delivery in women from North Carolina. Low levels of serum or RBC folate at the same gestation were each associated with an increased risk of preterm birth [25].

In Camden, we demonstrated an interaction between a pregnant woman's dietary folate intake and the presence or absence of a deletion allele in a folate-metabolizing gene that codes for the production of dihydrofolate reductase (DHFR) [24]. DHFR is an enzyme that converts folic acid used in supplements, and fortifies the US food supply to the reduced folate forms used by cells. The presence of the DHFR deletion allele increased the risk of preterm delivery 3-fold and, when folate intake was also low (<400 μg/day), increased the risk of low birthweight 8-fold and the risk of preterm birth 5-fold.

A metabolic effect of folate deficiency is an elevation of homocysteine [2]. Hyperhomocysteinemia can occur when dietary folate intake is low or a nutrient–gene interaction increases the metabolic requirement for folate. Homocysteine levels measured in more then 5,800 women aged 40–42 were linked to past data on pregnancy outcome contained in Norwegian birth registries [26]. Women with high homocysteine levels were more likely to have had a past reproductive history that included preeclampsia, preterm delivery, low birthweight, or fetal growth restriction.

Two recent studies supported these adverse effects with measurements of homocysteine taken before or during pregnancy. In China, pre-conceptional levels of maternal homocysteine were associated with a 4-fold increased risk of preterm delivery [27]. An evaluation of homocysteine in 93 Spanish women before and during pregnancy showed that maternal and fetal levels were positively correlated ($r = 0.5$ to $r = 0.7$); and reduced by the use of folate-containing vitamins. The birthweights of infants exposed to high levels of maternal homocysteine before conception, at 8 weeks gestation or at delivery were approximately 200 g below the birthweights of infants born to mothers with lower homocysteine levels [28].

Vitamin E

In Camden, we found that plasma concentrations of α-tocopherol, the most common isomer of vitamin E, were positively related with increased fetal growth (birthweight for gestation), a reduced risk of small for gestation births and an increased risk of large for gestation births [29]. Concentrations of α-tocopherol were positively related to the use of prenatal multivitamins before and during pregnancy and to the intake of vitamin E in the maternal diet. Although vitamin E is well studied in cardiovascular disease, there is little evidence of its effectiveness during pregnancy. However, more than a decade ago von Mandach et al. [30] studied more than 300 women and their offspring and correlated lower vitamin E at delivery with low birthweight at term ($n = 12$), a measure of fetal growth restriction. Thus, emerging evidence suggests that circulating concentrations of vitamin E may be associated with increased fetal growth possibly via increased blood flow and nutrient supply to the fetus.

In summary, maternal nutrition and nutritional status before and during pregnancy are associated with decreased birthweight and increased risk of low birthweight, measured either as preterm delivery or restricted fetal growth. This is particularly germane in the developing world where much of the low birthweight that occurs is related to the mother's past and present nutritional status. By affecting fetal growth and gestation it is plausible that maternal nutrition may have a long-term influence on the risk of chronic disease in later life [1].

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Discussion

Dr. Cameron: Given all the work you have done with teenager pregnancies, can you give us some sense of how these deficiencies interplay with growth during that time in terms of outcome, in terms of birthweight outcome?

Dr. Scholl: The deficiencies are more prevalent in teenager mothers. About 10 years ago we demonstrated that roughly half of the teenage women from Camden were still growing during pregnancy; but that maternal growth was not demonstrable from usual clinical measurements taken in the course of pregnancy [1–4]. Growing adolescents have lower levels of micronutrients (ferritin, folate), larger gestational weight gains but smaller babies. That is, they do not show the usual relationship between weight gain and birth weight, where women who have larger gestational weight gains have babies with higher weights at birth. The maternal diet buffers the effect of the mother's growth on the growth of the fetus that is not seen in mature women or teenagers who do not grow. When a still-growing mother has a lower caloric intake the birth weight deficit is quite large, 500 g or so. When caloric intake is higher (>2,400 kcal/day) the deficit is less, on the order of 200 g. The still- growing mother gains and retains more fat than the others, and has a 5–fold increased risk of new overweight/obesity in the postpartum. The competition for nutrients between a growing mother and her fetus has also been demonstrated in animal models including the rat dams and ewes [5, 6]

Dr. R. Bergmann: Could we overdo micronutrient supplementation? I am thinking about a study by Poston et al. [7] who gave very high, but still advisable doses of antioxidants, vitamin E and vitamin C, and in some risk groups there was a higher rate of preterm and in others of low birthweight newborns.

Dr. Scholl: The study you refer to supplemented gravidae at high risk of pre-eclampsia with higher levels of Vitamins C (1000 mg) and E (400 IU), and Poston et al. did show reduced birth weight at this level of micronutrient supplementation [7]. However, I was commenting on usual prenatal multivitamin mineral preparations. Poston et al. also demonstrated that babies in the placebo arm of their trial were larger when the mother used prenatal vitamins and minerals. Other studies [8, 9] have not demonstrated adverse effects on birth weight or gestation in supplemented women who later developed pre-eclampsia.

Dr. Bier: You showed us some data on the glycemic index of diets but the amount of carbohydrate in the mother and the fetus is not strictly based on the glycemic index, it is the glycemic load and this requires measuring the amount of the carbohydrate taken. What were the differences in glycemic load of these diets?

Dr. Scholl: The glycemic load had a weak effect on hemoglobin A1C. But in general it was the glycemic index that seemed to be associated with a stronger maternal blood glucose response, with hemoglobin A1C and with a response in fetal growth. The recent trial of the glycemic index among Australian gravidae [10] gave both groups the same amount of carbohydrate making the glycemic index and glycemic load equivalent.

Dr. Bier: If the glycemic load is no different, I don't understand how that has an effect on insulin secretion, glucose transport or anything else. That's what is moving, the amount of glucose.

Dr. Scholl: All of the women in the study were glucose tolerant; none of them had pre-gestational or gestational diabetes so that insulin sensitivity and beta cell function were normal; glucose homeostasis was balanced. But, I suggest that such a response might be demonstrable under experimental conditions i.e. with a hyperglycemic clamp.

Dr. Makrides: I want to ask your views on vitamin and mineral supplementation in pregnancy. The Cochrane Systematic Reviews bring together the relevant randomized

trials, whether they are to do with the antioxidants, vitamin E, vitamin C, magnesium, iron, and generally conclude that there is no clear beneficial effect [8, 9]. I wonder whether you could comment about specific subgroup analyses and whether we should be looking in more detail at population differences as you have some great data that suggest that some people may benefit more than others.

Dr. Scholl: I think there are large population differences. If you do a clinical trial in middle class women from the UK or the US, I would think that the results would be much more attenuated than the results of women who are poor, who eat diets that are high in fat, and who basically eat no fruits and vegetables. Certainly there must be huge differences between populations.

Dr. Makrides: Do you have any suggestion on how we might be able to group the populations across trials?

Dr. Scholl: You could stratify according to risk status of the underlying population. In other words does the population underlying the sample have a high risk of low birth weight, of pre-eclampsia, of preterm delivery? In effect, you could use the known prevalence of an adverse outcome as the gauge of an expected response.

Dr. Ogra: Thank you for a really very thoughtful presentation, Dr. Bier raised the issue about the glycemic index. Does it correlate well with the actual levels of insulin or C-peptide in the mothers?

Dr. Scholl: No, our unpublished data suggest a correlation with fetal levels of insulin and IGF-1. But, in his trial Clapp et al. [11] demonstrated differences in the area under the maternal insulin and maternal glucose curves.

Dr. Ogra: I was wondering if one might be able to reconcile the concerns voiced by Dr. Bier by looking at insulin levels? The second question is related to IGF-1. Do you think there is a change taking place at the transcriptional level; perhaps there are changes in other transcription factors beside IGF-1 and others, possibly NF-κB?

Dr. Scholl: I would not speculate. We measured only IGF-1 and insulin in the cord blood, but it's quite possible.

Dr. Ogra: Why do you think IGF-1 is in fact altered in this situation?

Dr. Scholl: The reason why I think the IGF-1 is altered is because the fetus is growing more. The women who ate a diet with a higher glycemic index had babies who grew larger, and insulin and IGF-1 are related to fetal size, but part of the stimulus came from the maternal diet.

Dr. Malka: Can you say anything about fish oil supplements in the third trimester that improve neonatal neurodevelopment?

Dr. Scholl: We have never measured fish oil consumption in Camden. I do know that there was a clinical trial done throughout Europe, a multicenter trial that showed reductions in the risk of preterm delivery but not in pre-eclampsia, that was another arm of the trial. So they seem to be very important but we haven't worked with fish oils.

Dr. Malka: What about women who gained more than 16 kg, were they at risk of macrosomia?

Dr. Scholl: We do have some macrosomia in Camden but prevalence is low. The women with gestational diabetes and the women who are obese have an increased risk of macrosomia, but on the whole our babies are small.

Dr. Walker: Just a point of clarification, it is my understanding that when zinc and vitamin A are given to HIV-positive mothers who are transmitting the virus to their babies who developed AIDS, they are effective only under conditions in which they were insufficient. The mothers were being given quantities of these micronutrients to make them sufficient, rather than excessive amounts having an anti-HIV effect, is that right?

Dr. Scholl: That's right, thank you for that.

Dr. Makrides: May I contribute to the discussion about glycemic index and the question that Dr. Bier raised. I understand that there was an issue with the Australian

study in that the women allocated to the high glycemic index group also happened to have a higher BMI at study entry, and that may have contributed to the results because BMI at study entry was not adjusted for in the statistical analysis [10]. I understand that this group is now planning to do a much larger study that will address this particular issue. With regard to fish oil supplementation in pregnancy, we have recently finished the Cochrane Systematic Review including all relevant trials with over 6,000 women, and the effect of fish oil supplementation in pregnancy was to increase the length of gestation by an average of 2.5 days, and have no effect on low birthweight or preeclampsia [12]. The effect on gestation length is of questionable clinical significance. There are a number of ongoing studies in Mexico, Australia and the USA trying to address the issue of neurodevelopment, but in terms of major pregnancy outcomes, such as preterm birth and preeclampsia, fish oil does not seem to play a large role.

Dr. Guinto: From the studies you cited, were the micronutrients given for the entire pregnancy or are there critical periods during pregnancy during which these micronutrients must be given?

Dr. Scholl: Women were provided with micronutrient supplements, it was an observational study. Some women used them, some did not. Some of the women used them during the first, second and third trimester, others used them erratically, and all of this was taken into account. I didn't show the data on the supplements which did have an effect on preterm delivery. They reduced the risk of preterm delivery depending upon when they were started, how often they were taken, and how often they were used.

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The Diabetic Pregnancy, Macrosomia, and Perinatal Nutritional Programming

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Abstract

Health and diseases are generally perceived to be caused genetically. It is meanwhile accepted, however, that alterations in the intrauterine and early postnatal nutritional, metabolic, and hormonal environment may also predispose to disorders and diseases throughout later life. Studies in the offspring of diabetic mothers (ODM) have decisively contributed to this perception and our understanding of causal mechanisms. It has long been known that hormones are environment-dependent organizers of the developing neuroendocrine-immune network, which regulates all fundamental processes of life. When present in non-physiological concentrations during critical periods of development, induced by altered intrauterine and/or neonatal environment, hormones can therefore also act as endogenous functional teratogens. Fetal and neonatal hyperinsulinism is the pathognomic feature in ODM. Epidemiological, clinical, as well as experimental data obtained by our group indicate that insulin itself, when occurring in elevated concentrations during perinatal life, may program the development of obesity and diabetes. Similar situations may occur due to maternal overweight accompanied by increased fetal food supply, and neonatal overfeeding. From a clinical point of view, general screening and therapy of all types of diabetes during pregnancy as well as avoidance of early postnatal overfeeding are therefore recommended. These measures might serve as causal approaches to a genuine primary prevention.

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Introduction

The impact of the intrauterine and early postnatal environment on lasting determination of fundamental processes of life is becoming more and more accepted. Especially investigations and hypotheses by the groups of Hales and Barker [1] have led to the postulation of a so-called small baby syndrome

which is explained by a thrifty phenotype acquired by poor fetal nutrition. This concept has contributed mainly to the worldwide attention to the phenomenon of early epigenetic conditioning, and terms such as nutritional programming or imprinting have been proposed to describe it. It was Günter Dörner, however, who in 1974 was the first to postulate a general etiological concept of epigenetic, perinatal programming of the lifetime function of fundamental regulatory systems and, thereby, the possibility of perinatal prophylaxis [2, 3].

Hormone-Dependent Perinatal Programming

Already in the early 1970s in a series of clinical as well as experimental studies Dörner [2, 3] demonstrated that especially hormones are environment-dependent organizers of the neuroendocrine system, which finally regulates all fundamental processes of life. When present in non-physiological concentrations, induced by alterations in the intrauterine and/or early postnatal environment, hormones can therefore also act as endogenous functional teratogens by malprogramming the neuro-endocrine-immune system (NEIS), leading to developmental disorders and diseases throughout life. This means that the classical science of teratology, as the discipline which addresses macroscopic malformations, should be supplemented by the science of functional teratology as the discipline of perinatally acquired malfunctions [2, 3].

Diabetes in Pregnancy, Perinatal Hyperinsulinism, and Perinatal Programming

Evidence for the existence of the biomedical phenomenon of fetal programming originates mainly from the fields of reproductive behavior and stress [3–5], with research addressing the significance of altered concentrations of the respective steroid hormones (sexual steroids, or gluco- and mineralocorticoids) during critical periods of perinatal development for a permanent malprogramming of the affected subsystems of the NEIS. However, the results of clinical investigations and animal experiments on the long-term effects of maternal diabetes during pregnancy in the development of the offspring have for a long time provided key support for the concept of fetal programming of disposition to diseases.

Clinical Observations

Pregnancy is a diabetogenic situation per se. Women with gestational diabetes, just as pre-gravid diabetic women, are classed as risk pregnancies, and

their offspring show increased perinatal morbidity and mortality. The disturbances manifested during the neonatal period, apart from a tendency to hypoglycemia, hyperbilirubinemia, and neonatal respiratory distress syndrome, are characterized above all by an increased prevalence of macrosomia. This is caused by the virtually pathognomic fetal and perinatal hyperinsulinism, which arises because of the materno-fetal hyperglycemia and consequent overstimulation of the fetal pancreatic B cells.

Already in the 1970s it was shown in a cohort of 4,000 diabetic patients that type 2 diabetes was inherited more frequently through the mother than the father, and the difference was highly statistically significant [6]. The offspring of a mother with gestational diabetes show an increased tendency to be overweight or obese already in childhood [7–9], accompanied by disturbances in glucose tolerance, insulin secretion, and insulin sensitivity [10–12].

It is particularly noticeable that these alterations may even occur independent of genetic influences and the type of maternal gestational hyperglycemia [9, 11, 13, 14]. However, they do show marked correlations with fetal metabolic alterations of the affected children, namely the degree of fetal and perinatal hyperinsulinism [10–12]. In the Pima Indian study it was shown that the prevalence of juvenile diabetes and obesity among the offspring of mothers who had diabetes already during pregnancy was several times higher than the prevalence among the offspring of normoglycemic or also pre-diabetic mothers (the latter being those who showed a genetic predisposition to diabetes but only became diabetic after delivery) [13]. This observation was later confirmed impressively by Dabelea et al. [14].

In particular, a positive correlation was found between the level of amniotic insulin or perinatal hyperinsulinemia and the increase in relative bodyweight and risk of impaired glucose tolerance (IGT) in later life for children of diabetic mothers [10–12]. The latter has to be interpreted as a decisive indication of a persistent influence of the diabetic intrauterine milieu and consequent hyperinsulinism in the sense of a hormonally initiated malprogramming.

Experimental Observations

Animal experiments have confirmed that maternal gestational hyperglycemia leads to overweight, IGT, hyperinsulinemia and insulin resistance in the juvenile and adult offspring, regardless of any genetic disposition [15–17]. Remarkably, the female F1 offspring of gestationally diabetic dams spontaneously develop gestational hyperglycemia. In the F2 offspring exposed in utero this can then in turn lead to diabetogenic disturbances in later life, and therefore an epigenetic, materno–fetal transmission of increased disposition to diabetes is possible through a number of generations in sequence even without any genetic predisposition [15–18].

A permanent influence on the function of pancreatic B cells has been proposed, on the one hand, as an etiopathogenetic mechanism of this prenatally acquired malprogramming, in particular a persistent B cell hyperplasia and hyperactivity leading to permanent impairment of insulin secretion in the offspring [15]. On the other hand, studies have shown that permanent alterations of the programming of neuroendocrine and vegetative functional systems play a key etiopathogenetic role [18–20]. Thus the experimental induction of gestational hyperglycemia not only leads to perinatal hyperinsulinemia in the offspring but also to increased insulin concentrations within the immature hypothalamus, followed by the morphological characteristics of permanent, i.e. lifelong, dysplasia of central nervous control centers for metabolism and bodyweight. In particular this affects the ventromedial hypothalamic nucleus (VMN), which develops a permanent dysplasia and neuronal hypotrophy as a result of the exposure to increased insulin concentrations during critical periods of development [16, 18, 20]. Furthermore, as an expression of perinatally acquired hypothalamic resistance to the peripheral satiety signals, insulin and leptin, there is a permanent dysorganization and malfunction of specific neuropeptidergic neurones in the arcuate nucleus. Particularly important seems to be a lifelong increased activity and number of neurones which express the orexigenic peptides, galanin and neuropeptide Y [19, 21]. The extent to which these neuroendocrine functional impairments are attributable to perinatally acquired permanent alterations in gene expression remains an open question. Interestingly, however, the promoter regions of the neuropeptides and receptors involved show an increased CpG content [22] and are thus potential candidates for methylation-dependent alterations of the expressivity.

All this is accompanied by a permanently increased disposition for diabetes and obesity, characterized by hyperphagia, overweight, basal hyperinsulinemia, insulin resistance, and IGT. It should be emphasized that, both clinically and experimentally, these permanent disturbances occur independent of the birth weight and can also be observed in animals treated neonatally with insulin, experimentally applied either peripherally or only intrahypothalamically [16, 18, 23, 24].

Finally, even type 1 diabetes susceptibility is increased in the offspring of diabetic mothers. Multiple low dose streptozotocin (STZ) treatment is a well-known model for type 1-like diabetes in rats accompanied by cell-mediated immune responses which closely resemble the autoimmune processes associated with infantile type 1 diabetes in the human. In maternal-side F1 and even F2 offspring of STZ-treated gestational diabetic mother rats (F0) spontaneous gestational diabetes, basal hyperinsulinemia from birth into adulthood, indicating persisting basal overstimulation of the pancreatic B cells, and, most important, a severe insulin-deficient type 1-like diabetes after a single low dose STZ treatment were observed in contrast to the offspring of control mothers [16]. The offspring of mother rats with gestational diabetes responded to multiple low dose STZ treatment with increased spleen cell cytotoxicity to syngeneic

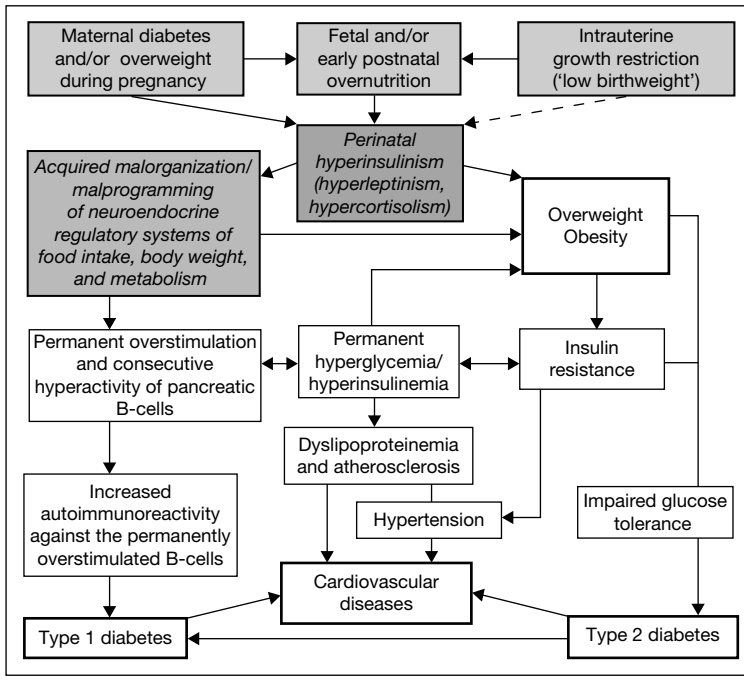

Fig. 1. Proposal of pathogenetic mechanisms and consequences of perinatal malprogramming, showing the etiological significance of perinatal hyperinsulinism for excess weight gain, obesity, diabetes mellitus (type 2 as well as type 1) and associated cardiovascular diseases (CVD) in later life. Adapted from and modified to Dörner and Plagemann [18].

B cells. Exogenous insulin treatment of newborn rats, even when only intrahypothalamically performed, was also followed by increased susceptibility to low dose STZ type 1-like diabetes in further life [23, 24]. Moreover, these experimental data were accompanied by some clinical and epidemiological observations indicating that prevention of gestational diabetes in the mother may prevent increased type 1 diabetes susceptibility in the offspring [18].

Conclusions

Taking together the epidemiological, clinical, and experimental observations, it seems obvious that fetal hyperinsulinism induced by maternal hyperglycemia has functionally teratogenic significance for a permanently increased disposition to obesity, diabetes, the metabolic syndrome, and subsequent cardiovascular diseases in the children affected (fig. 1). Given that gestational diabetes has in the mean time probably reached a prevalence in excess of 10% in developed industrialized countries, it is urgent that all pregnant women are screened for glucose intolerance and adequately treated as a primary prevention measure.

Birthweight, Neonatal Nutrition, and Lasting Programming

The widely discussed data and hypotheses of the groups working around Barker and Hales have led to the postulation of a small baby syndrome, according to which fetal undernutrition, growth restriction, and low birthweight predispose to the later development of alterations in metabolism, bodyweight, and the cardiovascular system in terms of type 2 diabetes, metabolic syndrome, and cardiovascular diseases [1, 25].

Clinical Observations

Since the early 1990s, an impressive number of studies in various populations have been published which clearly show a persuasive link between a low birthweight and a subsequently increased risk for aspects of the metabolic syndrome. Even in the Pima Indian study, a long-term investigation in a North American population with a particularly high disposition to diabetes and obesity, it was shown that type 2 diabetes associated with overweight in adulthood was more prevalent in patients who had been overweight at birth, but also in patients who were neonatally underweight [26]. By means of meta-analysis of a variety of studies beyond that in the Pima Indians, we were recently able to confirm these observations [27]. This leads to the postulation that, in fact, no linear-inverse but a U-shaped relationship exists between birthweight and subsequent diabetes, obesity, and the metabolic syndrome.

Whereas the pathogenetic context seems more or less obvious for neonatal overweight, no clear etiopathogenetic link has been established for reduced perinatal weight [18, 28]. In particular it should be emphasized that no causal link has been established between intrauterine growth restriction and a subsequently increased disposition to obesity, i.e., the pathophysiological key for subsequent diabetes and cardiovascular diseases. It remains to be clarified whether fetal growth restriction and neonatal underweight, per se or rather the quality and quantity of early postnatal nutrition and weight gain in early infancy have pathophysiological significance for the prospective risk.

The central pathogenetic importance of later overweight is clear, for example, within the context of the metabolic syndrome, but although a positive correlation has frequently been demonstrated between weight at birth and weight or overweight in later life, an independent inverse relationship has never been shown [29]. Increased weight gain in early infancy, on the other hand, leads to increased disposition for obesity in later life [30]. It also seems remarkable that increased weight gain in early childhood, in particular in underweight newborns, leads to early manifestation of insulin resistance [31, 32]. Finally, it has been variously shown that increased weight gain in early childhood is a predictive factor for a disposition to the metabolic syndrome

and cardiovascular risk in adulthood, in particular in the case of low birthweight [33–35].

Experimental Observations

Against the background of the thrifty phenotype hypothesis, investigations on the small baby syndrome frequently use animal models of maternal underfeeding during gestation and lactation, which leads to a pronounced intrauterine and neonatal growth restriction in the offspring [36]. However, examination of the results on the long-term effects obtained with these and similar models showed no congruence with the observations after intrauterine growth restriction and low birthweight in humans.

Thus, for example, the animal experiments of Hales' group [36] have shown that offspring born to rat dams that were malnourished during gestation and lactation do not become overweight, but rather show a life-long persistence of low weight. This is associated with a permanently reduced food intake [36]. The animals predominantly show increased instead of decreased glucose tolerance. In contrast to the metabolic syndrome in humans, hyperinsulinemia and insulin resistance do not occur, but rather lower insulin secretion. All these findings persist even after dietary provocation [36, 37].

In contrast, it has early been postulated that transition from fetal malnutrition to early postnatal overfeeding could play a key role in the etiopathogenesis of the small baby syndrome [18], especially since it seemed quite possible that growth-restricted neonates, also in the epidemiological studies, had been overfed and possibly fattened. Similar hypotheses on the possible significance of early postnatal nutrition for the long-term outcome of underweight neonates have since been formulated by other authors, around Barker and Hales [32, 34].

The influence of this early postnatal nutritive situation on the later outcome of metabolism and bodyweight has often been investigated using the small litter model. Rats which were overfed in the early postnatal period show phenotypic alterations through juvenile age into adulthood, such as overweight, hyperphagia, glucose intolerance, hyperinsulinemia, dyslipidemia and increased blood pressure, which correspond in important aspects to those of the metabolic syndrome in humans [38]. This is all the more remarkable because clinical findings suggest that early postnatal overfeeding in humans also predisposes for an increased risk of metabolic syndrome in later life (see above). But here too the causes are not clear.

As already mentioned, neuropeptidergic hypothalamic centers play a key role in the regulation of food intake, bodyweight, and metabolism. It is of note that, very similar to the offspring of diabetic dams, neonatally overfed small litter rats show persisting disorganization and malprogramming of these regulatory systems, including malfunction of the VMN and especially resistance of the arcuate nucleus to the satiety signals, insulin and leptin, which may explain their neonatally acquired long-term risk [16, 18, 38, 39].

Conclusions

From an epidemiological point of view there is a clear phenomenological link between reduced birthweight and subsequently elevated risks. The critical integration of epidemiological, clinical, and experimental observations, however, cast doubt on a causal relationship. However, neonatal overfeeding and rapid early weight gain with increased fat deposition could be of lifelong pathophysiological importance especially for underweight newborns (fig. 1). Therefore, prophylactic recommendations should focus on the recognition, avoidance, and optimal treatment of the causes of intrauterine growth restriction (nicotine, alcohol, stress, gestosis, etc.), and also on the avoidance of neonatal overfeeding.

Synopsis

Globally, diabetes mellitus, obesity, and the metabolic syndrome are life-shortening diseases, and the continual dramatic increase in their prevalence represents a health problem of the greatest relevance, so that there is an urgent need for prevention strategies.

Generally, complex pathogenetic processes, in particular those relating to the so-called diseases of civilization, originate from an impaired interaction or an imbalance between environmental factors and the genetic matrix. From a practical clinical viewpoint, therefore, it is extremely important to characterize epigenetic risk factors with long-term malprogramming effects which can be influenced by preventive measures in critical periods of early development.

At least every tenth pregnancy in developed industrialized countries is probably affected by a disturbed glucose tolerance. The great majority of cases go unrecognized and thus untreated, because there is no universal screening for glucose intolerance of all pregnant women.

Fetal or early postnatal hyperinsulinism (and also hyperleptinism), induced as a result of maternal gestational hyperglycemia and/or early postnatal overfeeding, acts as a functional teratogen during critical periods of differentiation and maturation, especially of the NEIS. This can lead to irreversible, lifelong malprogramming of fundamental control systems and hypothalamic regulation centers for metabolism, food intake, and bodyweight. The result is a disposition to become overweight, and the development of obesity and associated metabolic disturbances such as hyperinsulinemia, insulin resistance, IGT, type 2 diabetes and metabolic syndrome, including undesirable clinical outcomes such as cardiovascular disease (fig. 1). Even an increased disposition to the manifestation of insulin-dependent type 1 diabetes may be pre-programmed in this way [18, 40, 41]. Here once again, the complex malprogramming of the NEIS is probably of causal significance, so that, for example, an under-functioning of the VMN as well as basal hyperglycemia lead to a permanent basal

overstimulation of the pancreatic B cells. But a permanent basal B cell overstimulation not only contributes to hyperinsulinemia, but also to increased autoimmune reactivity to the constantly hyperactive B cells [42, 43], particularly in otherwise predisposed individuals and/or together with exposure to noxae (e.g. viruses), with the consequence of increased susceptibility to type 1 diabetes [18]. Remarkably, these aspects have also been substantially supported in recent years by observations and interpretations according to which accelerated growth and overweight in childhood can have pathogenetic significance for the manifestation of type 1 diabetes [44].

Finally, regardless of the quality and quantity of the neonatal nutrition of underweight newborns, it is probable that they are exposed to a further form of hormone-dependent malprogramming. They probably show considerable pre- and perinatal alterations of the glucocorticoid levels, at least in the form of temporary hypercortisolism, with the potential consequence of glucocorticoid-induced malprogramming of the hypothalamic-pituitary-adrenal axis. This, in turn, may substantially contribute to long-term risk of central obesity and accompanying metabolic and cardiovascular disorders.

In all, from a clinical point of view this suggests the possibility of primary prevention of increased disposition for overweight, diabetes mellitus, and cardiovascular diseases by preventing fetal and/or early postnatal hyperinsulinism, and also hyperleptinism and hypercortisolism, during critical periods of development. A key to the approach is the prevention of any glucose intolerance during pregnancy, and probably also the avoidance of early postnatal overfeeding and thus consecutive hyperinsulinism during critical periods of early development. In particular, universal screening of all pregnant women for glucose intolerance seems to be urgent.

Prospects

The aspects presented here have a model character and, by way of example, show the long-term pathophysiological significance of abnormal nutritive, metabolic, and first of all hormonal conditions during critical fetal and perinatal periods of development, implying at the same time that primary prophylactic management is possible by optimizing the fetal and early postnatal environmental conditions. In this context, the general etiopathology should be extended to include epigenetic dispositions, as exemplarily illustrated in figure 2. For example, the molecular causes could lie in perinatally acquired alterations in the DNA methylation pattern of receptors and/or neurohormones which are involved at a cybernetically key position in the regulation of the NEIS. All this may be of critical importance for the development and lifelong functioning, or permanent malfunctioning, of fundamental regulatory systems and life processes, and in the future should therefore be taken into account in research into etiopathogenesis and preventive medicine.

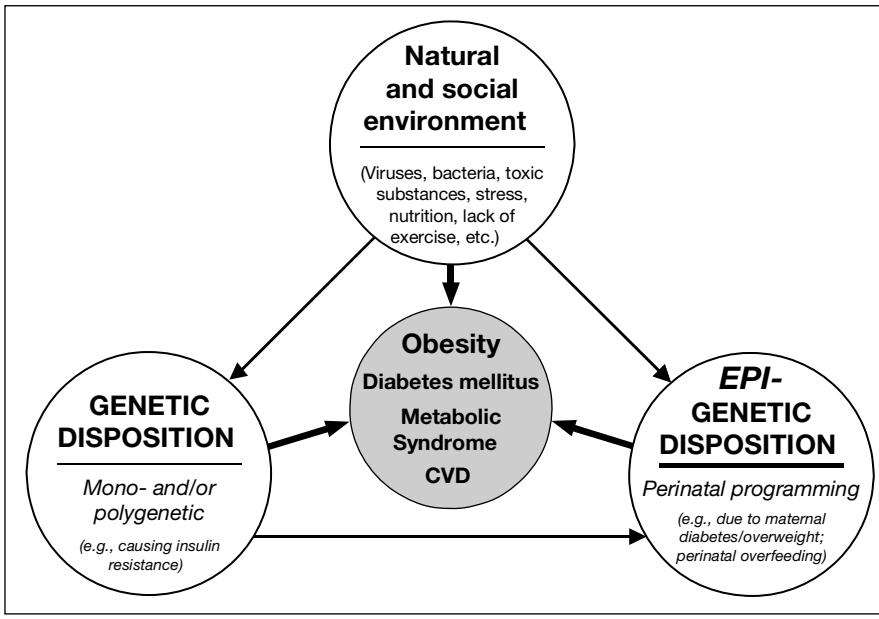

Fig. 2. Fundamental concept on the multi-etiological origin of obesity, diabetes mellitus, the metabolic syndrome, and subsequent cardiovascular diseases (CVD), pre-programmed critically by the pre- and perinatal nutritional conditions.

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Discussion

Dr. Spivey-Krobath: Which substrates play a role in early postnatal overfeeding and subsequent obesity and diabetes: protein, energy or both?

Dr. Plagemann: I would like to suppose that both increased energy intake as well as increased protein supply might play a crucial role. Clinical and experimental data clearly suggest that increased energy intake itself, when leading to rapid neonatal growth and, particularly, increased neonatal fat deposition accompanied by respective hormonal alterations, like hyperleptinism and hyperinsulinism, is critically involved in processes of perinatal programming of obesity and diabetes. That means, nutritional malprogramming may occur regardless of the composition of the diet but due to general overfeeding. However, increased protein content might indeed be particularly problematic since increased levels of amino acids are capable of inducing overstimulation of developing pancreatic β cells, as it occurs in utero as a result of increased glucose levels in the case of maternal diabetes. This may cause perinatal hyperinsulinism, which appears to be a pathophysiological key factor in perinatal programming of obesity and diabetes, as I illustrated in my talk. In this sense, a potential role of the protein content of the neonatal diet for the long-term risk of obesity is also supported by data from animal models. For instance, in rats a maternal low protein diet during gestation and lactation leads to a decreased susceptibility in the offspring to develop obesity during later life, while the offspring of rat dams fed a high protein diet have an increased risk of developing overweight. In recent years a maternal high fat diet has also been intensively discussed as playing an important role in programming the offspring for later overweight. Altogether, increased glucose, protein, as well as fat may be unfavorable during critical time windows of early development, and it remains to be determined by future research at which time which components of the maternal and neonatal diet have the most important long-term influence, and especially which of them are most easily and effectively modifiable in terms of perinatal nutritional prevention of later health risks. In my opinion, this could become one of the most important aspects and challenges in the field of infant nutrition and, thereby, primary preventive medicine in the future.

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Undernutrition and Growth Restriction in Pregnancy

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Abstract

Newborn size is the result of intrauterine growth. Premature, low birthweight of <2,500 g, small for gestational age (SGA, <10th percentile), or intrauterine growth-restricted (IUGR) newborns may have similar weights. Serial fetal biometry (ultrasound), required for the diagnosis, timing and severity of intrauterine growth restriction in the individual infant, is still not common in epidemiological studies. SGA newborns have less lean body mass, but they particularly lack fat mass. The most important etiological determinants of intrauterine growth restriction in developed countries is cigarette smoking, while in developing countries it is usually longstanding food deprivation. Follow-up studies of SGA newborns consistently showed a positive association between birthweight and later lean body mass, whereas associations with adiposity were more variable. Most SGA infants had catch-up in length/height. Signs of the metabolic syndrome accompanied the catch-up in bodyweight and central adiposity. So far, no overarching model is available to explain how the epigenetic and hormonal tunings, which accompany intrauterine malnutrition from preconception through pregnancy, can program the regulatory systems of fundamental life processes. The theoretical concepts of a thrifty phenotype (Hales and Barker) and of a predictive adaptive response (Gluckman and Hanson) offer a comprehensive approach to understanding the empirical and experimental findings.

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We owe it to Professor David Barker and his colleagues in Southampton for having called attention to the association between low birthweight and coronary death [1]. His pioneering ecological study in England und Wales showed a geographical relation for ischemic heart disease in 1968–1978 and infant mortality rates in 1921–1925. A follow-up study of men and women in Hertfordshire showed that those who had low birthweights had relatively high death rates from coronary heart disease in adult life [2, 3]. Another of

their studies pointed out that persons who were small at birth as a result of growth retardation rather than those born prematurely were at increased risk of coronary disease [4]. Similar trends were observed for cardiovascular disease risk factors [5]. Associations between birthweight and adult blood pressure were found in each social group and were independent of smoking, alcohol intake and obesity in adult life [3, 5]. To complete the signs of the metabolic syndrome, the prevalence of an impaired glucose tolerance and type 2 diabetes were inversely related to birthweight in a dose-response manner [6, 7]. These studies triggered an avalanche of publications worldwide, challenging and proving the Barker hypothesis of the 'fetal origins of adult disease'.

Malnutrition in Utero – Intrauterine Growth Restriction, Definition and Diagnosis

A high proportion of premature infants do not grow properly in utero, particularly those delivered for medically indicated reasons [8]. Where gestational age is not known, as is common in developing countries, a low birthweight of <2,500 g has been used as an indicator for impaired fetal growth [9]. But even considering gestational age, newborns below the 10th percentile value of the corresponding population are defined as small for gestational age (SGA). Newborn size is only the result of intrauterine growth. Unless this is known, SGA according to a fixed (or ideal) growth standard is used as a proxy for intrauterine growth restriction (IUGR). But even these SGA newborns may be normally grown, and not pathologically small, and vice versa, a birthweight above this cutoff value may still be the result of IUGR. Customized antenatal growth charts take 'physiological' variables into account, such as fetal sex, height, parity, ethnic group, and pre-pregnancy weight of the mother [10]. Although we can argue over the sense of 'normalizing' the smallness of a newborn of an undernourished or smoking mother, customized, population-based birthweight standards improved the prediction of adverse perinatal outcomes better than non-adjusted standards [11–14]. But until now, customized birthweight standards have not been used to predict the long-term outcome of growth-retarded infants.

For over 20 years in utero analysis of fetal growth patterns has been possible by sonographic weight standards [15]. Even customized fetal weight percentiles are available, although they are less accurate than calculated intrauterine growth velocities [16]. A combination of serial fetal biometry and other biophysical measurements is used to determine the optimal time point for premature delivery of a growth-restricted fetus. Quantitative estimates of fetal body composition can currently be achieved using ultrasound tools [17]. Future studies will show if these methods are useful in predicting the long-term outcome.

Table 1. Low birthweight (LBW) and intrauterine growth restriction (IUGR): rates in developing countries

Country	LBW (<2,500 g), %	IUGR (<10th percentile), %
Argentina	6.3	9.7
China	4.2	9.4
Colombia	16.1	17.8
Cuba	8.1	14.7
Gambia	12.1	13.5
Guatemala	12.5	25.3
India (Pune)	28.2	54.2
Indonesia	10.5	19.8
Lesotho	10.3	13.0
Malati	11.6	26.1
Myanmar	17.8	30.4
Nigeria	12.4	22.2
Nepal, rural	14.3	36.3
Sri Lanka	18.4	34.0
Thailand	9.6	17.0
Vietnam	5.2	18.2

SGA and IUGR, Prevalence Rates and Determinants

In the last 20–30 years an increase in the prevalence of premature births has been observed in wealthy countries [18–20]. A major part of this increase is associated with the birth of multiplets after the use of assisted reproduction [18, 19, 21]. But around 25% of all preterm births in the USA were delivered for medical reasons, e.g. severe fetal growth restriction and fetal distress, the rest is spontaneous [20].

Low birthweight and IUGR are significant problems in developing countries. It was estimated that at least 13.7 million infants are born every year at term with a birthweight below 2,500 g, representing 11% of all newborns in developing countries, a rate that is 6 times higher than in developed countries [21, 22]. Of all newborns, not only of those born at term, 20.5 million were low birthweight. According to the 10th percentile of a sex-specific single-twin growth chart for Californian newborns, about 24% were IUGR, or approximately 30 million newborns per year. Nearly 75% were born in Asia, a smaller part in Africa and Latin America (table 1) [21]. The most important etiological determinants for IUGR in developing countries and the population-attributable risks in developed countries were calculated by Kramer et al. [23–25], and are listed in table 2. The major determinant in developed countries is cigarette smoking during pregnancy, but the main determinant in developing countries is a low energy intake (low pregnancy weight gain as a proxy) and

Table 2. Determinants of intrauterine growth-restricted newborns (IUGR) in developing and developed countries, listed in decreasing order of importance

Developed country	Developing country
Cigarette smoking	Low energy intake, low gestational weight gain
Low energy intake, low gestational weight gain	Low pre-pregnancy BMI
Low pre-pregnancy BMI	Short stature
Primiparity	Malaria
Low stature	Cigarette smoking
Pregnancy induced hypertension	Primiparity
Nonwhite	Pregnancy-induced hypertension
Congenital anomalies	Congenital anomalies
Other genetic factors	Other genetic factors
Alcohol, drugs	

Adapted from Kramer et al. [22, 23].

the poor nutritional status of the mother (low pre-pregnancy body mass index, BMI, and short stature as proxies). Usually, weight changes during the second and third trimester of pregnancy were considered the most important determinants of the birthweight of the newborn. But by using accurate measurements of pre-pregnancy weights rather than relying on reported weights, it could be shown that the weight change in first trimester of pregnancy more strongly influenced newborn size than the changes in the second and third trimester [26].

In order to identify the determinants of a birthweight below the 10th percentile (according to our own fixed growth standard), we analyzed the Berlin perinatal data of the years 1993–1999 (n = 169,000), independent of whether they occurred often or not (table 3). The most prominent determinant is a multiplet pregnancy, followed by hypertension. Although we have significant underreporting, cigarette smoking during pregnancy is an important determinant of IUGR and is closely related to a low social status. Other proxies for a low social status may be low gestational weight gain, short stature, binge drinking, and strenuous physical work, but also high weight gain and high pre-pregnancy BMI [22–24]. Being unmarried and having a short maternal educational period somewhat increased the risk for mild IUGR in Canadian newborns [25]. SGA clusters in families; the association is stronger for female than for male relatives, which can be attributed to environmental conditions, e.g. intrauterine ‘maternal constraint’ [27, 28]. But paternal as well as maternal height have been correlated with birth length, and to a lesser degree with birthweight, e.g. in Indian infants, which points to an involvement of fetal genes in the regulation of fetal growth, partly explaining ethnic differences [29, 30].

Table 3. Risk factors for a birthweight <10th percentile (according to a local growth for gestational age fixed standard) in Berlin newborns, utilizing perinatal data of 1993–1999 (n = 169,000)

Risk factor	OR	95% CI
Multiplet delivery	6.67	6.27–7.11
Hypertension in pregnancy (chronic or acute)	2.90	2.64–3.18
Smoking in pregnancy	2.31	2.22–2.39
Mother's height ≤ 160 cm	1.86	1.79–1.93
Firstborn infant	1.83	1.76–1.89
Pregnancy weight gain		
<250 g/week	1.71	1.64–1.77
≥400 g/week	0.61	0.59–0.64
Female infant	1.70	1.65–1.76
Pre-pregnancy BMI		
<20	1.67	1.65–1.78
>30	0.51	0.47–0.55
Malformation	1.60	1.44–1.78
Mother's age		
<20 years	0.95	0.89–1.03
>30 years	1.10	1.07–1.14
German	1.04	1.00–1.08
Diabetes in pregnancy	0.64	0.56–0.73
Constant	0.03	

Logistic regression, adjusting for all other variables.

Prevention of IUGR by Reducing the Risk

Although fetal nutrition is not directly dependent on maternal nutrition – the maternal metabolic and endocrine status, uterine blood flow, placental transport and metabolism, and the metabolic and endocrine status of the fetus are translating and modulating the dietary effect – mean birth weights were higher by about 20–30 g, if mothers received calorie or balanced protein/calorie supplements [31, 32]. There is a tradeoff in nutrient partitioning between the mother and the fetus, evidently with the goal of sustaining maternal fertility: Malnourished women in Guatemala taking a nutritional supplement gained weight from one delivery to the next consecutive delivery (reproductive cycle), but their second (study) infant tended to weigh less than their previously born infant [33]. In contrast, just marginally nourished women lost weight during their reproductive cycle, and their second infant tended to weigh more at birth than the first born. In rural Gambia, a high energy supplement, consumed by chronically undernourished women from mid-pregnancy (in the presence of birth attendants), reduced the risk for low birthweight babies, and increased the mean birthweight significantly by an average of 201 g

in the hungry season, and by 136 g in the harvest season [34]. But high protein supplements may increase the risk of IUGR [32]. In stunted populations with a long history of scarce food resources nutrient supplementation of the pregnant mother has to be carefully balanced. A more prudent approach is to improve the growth and nutrition of females as early as possible.

In most studies micronutrient supplements to combat deficiencies ('hidden hunger') in pregnant women have not shown striking effects on the prevalence of IUGR. But maternal iodine supplements in deficient areas significantly increased the mean birthweight by 157 g [35]. Multiple micronutrient supplementation beginning in mid-pregnancy in Nepal, compared to routine iron and folic acid supplements, resulted in a significant increase in birthweights by 77 g, and a fall in the proportion of low birthweights by 25% [36]. Multi-nutrient supplementation of anemic women in Guinea-Bissau increased birthweights by 218 g and decreased the risk of low birthweight [37]. Birthweights in women who gave up smoking in pregnancy were significantly higher by 292 g compared to continuing smokers [38].

Body Composition of Small and Growth-Restricted Infants, Tracking

In the human fetus lipid is deposited quite rapidly during the last trimester of pregnancy [39]. Premature infants therefore especially lack adipose tissue. The body composition of SGA newborns is different from appropriately grown (AGA) and from large for gestational age (LGA) newborns of similar gestational age, as could be shown recently by dual-energy X-ray absorptiometry (DXA) measurements (table 4) [40]. While lean body mass (LBM) is lower by 22% in SGA, and 20% higher in LGA compared to AGA newborns, there is 51% less fat mass in SGA, and 128% more fat mass in LGA newborns. In the malnourished fetus preferential blood flow to the brain and heart may deprive other organs from oxygen and nutrients. In growth restricted newborns the volumes of liver and kidney are more decreased than the body as a whole [41]. Maternal smoking in pregnancy selectively reduces LBM but not fat mass [42]. Although being born 800 g lighter at birth, Indian newborns have similar subscapular skinfold thicknesses as British newborns, but for each ponderal index unit the skinfold value is higher, a phenomenon described as the thin-fat Indian baby [43].

European children born short (rather than light) for gestational age had a 6- to 7-fold increased risk of adult short stature; about 10–14% remained short [44, 45]. When adjusted to mid-parental height (target height) small for gestational length children remained 4–5 cm shorter [45]. But former premature AGA children in the Netherlands attained normal height at 10 years of age [46]. The strongest predictor of stunting in malnourished African infants was small birth size [47].

Table 4. Body composition of small for gestational age (SGA), appropriate for gestational gage (AGA), and large for gestational age (LGA) German and Swiss newborns, measured by dual-energy X-ray absorptiometry [40]

	SGA (n = 26)	AGA (n = 118)	LGA (n = 15)
Gestational age, weeks	38.2 ± 2.7	38.3 ± 3.0	39.1 ± 1.8
Age at study, days	5.0 ± 2.4	4 ± 2.2	4.7 ± 2.1
Birthweight (BW), g	2,320 ± 660	3,150 ± 680	4,430 ± 630
Birth length, cm	46.1 ± 4.8	49.7 ± 3.5	52.3 ± 2.5
BMI	10.4 ± 1.4	12.2 ± 1.5	15.0 ± 1.7
Fat mass, g	210 ± 100	430 ± 190	980 ± 510
(% of BW)	(8.6 ± 3.1)	(13.1 ± 4.3)	(22.2 ± 8.2)
Lean body mass, g	2,080 ± 520	2,650 ± 520	3,170 ± 360
Bone mineral content, g	39.2 ± 16.0	54.5 ± 15.8	81.1 ± 16.7

Those of the European SGA children who as adults remained short had a slightly increased risk of being overweight [48, 49]. Former SGA infants of the cross-sectional survey NHANES III in the USA continued to remain lighter and LGA infants heavier through early childhood, but the discrepancies in weight up to 4 years were primarily attributable to differences in muscularity (LBM) and only to a limited extend to fatness [50]. In a sample of British 7-year-old children and adolescents LBM but not fat mass was associated with the birthweight z score [51]. Older Englishmen (in a small study) who had a birthweight below the 25 percentile compared to those above the 75 percentile had significantly less LBM [52]. They were not different in fat mass (assessed by DXA) but differed slightly in favor of a more central fat distribution. The birthweights of 9- to 10-year-old children of the large ALSPAC study in Bristol were positively associated with both LBM, but also with fat mass (DXA determination) [53]. In these children, weight and length at birth did not predict central adiposity. A similar, but smaller study in Spanish adolescents found an association between birthweight and LBM (as well as bone mass), but not with fat mass, and only a marginal (not significant) association with truncal adiposity [54]. These follow-up studies have consistently shown a positive association between birthweight and LBM, whereas associations with adiposity were more variable.

Catch-Up Growth in Length/Height and Weight

Catch-up growth in length/height was observed in European SGA infants, most of it occurring in the first 12 months of life, birth length and mid-parental height being significantly related to its magnitude [55–57]. Consequent to

catch-up weight gain in the first two years, SGA infants in a recent study in Barcelona showed a dramatic transition towards central adiposity [58]. Infants of maternal smokers, who were small at birth, showed complete catch-up growth during the first 12 months; likewise the infants of primiparous women, who overshoot other infants and were significantly heavier and longer at one year [59]. Maternal smoking was a significant risk factor for overweight and obesity in 3- to 7-year-old children (BMI and skinfold thickness), especially when mothers smoked in early pregnancy, when they had a higher catch-up gain in the first year, and when being bottle-fed [60–63]. Interestingly, in the Dutch famine study, obesity in women was more prevalent if their mothers had suffered from famine in early gestation [64]. Weight in former extremely low birthweight infants (25% SGA) declined up to three years, only thereafter they started to catch-up [65]. In the small Indian children, greater weight gains in late infancy and adolescence were associated with increased adult, especially central adiposity [66].

Catch-Up Growth and the Metabolic Syndrome

A follow-up study of Chilean SGA and AGA infants from 48 h of life to 3 years showed that, according to the catch-up in weight and BMI, a marked transition occurred from lower pre-feed insulin and increased insulin sensitivity at birth to insulin resistance at 3 years [67]. In the thin-fat Indian newborns already at birth glucose and insulin concentrations were higher [68].

Concomitant with their catch-up growth and the transition towards central adiposity, the SGA infants of a study in Barcelona developed increasing insulin resistance between 2 and 4 years of age [58]. A large follow-up study of young adults in France who had been SGA and AGA newborns of 32–42 gestational weeks showed that the mean waist to hip ratio was significantly higher in the SGA group [69]. The SGA group had significantly increased triglyceride, lower HDL-cholesterol, increased plasma fasting and 120 min after-load glucose concentrations, and an increased insulin resistance index. While catch-up in height was not significantly related to any of the parameters of the metabolic syndrome, catch-up in BMI was inversely related to BMI at birth, i.e. thinness at birth.

In a systematic review of 80 studies, blood pressure was shown to increase with decreasing size and head circumference at birth, while accelerated postnatal catch-up growth increased blood pressure [70]. An early alteration in the hypothalamic-pituitary-adrenal axis as a mediator of elevated blood pressure has repeatedly been studied in many settings. In children urinary glucocorticoid metabolite excretion was higher in those who had been light or heavy at birth [71]. This U-shaped relation persisted after adjustment for sex and current weight. In 20-year-old South Africans plasma cortisol levels in the morning and after low dose ACTH stimulation were higher in former SGA

newborns [72]. In adults from three populations the cortisol concentration fell with increasing birthweight [73].

A meta-analysis of 14 studies involving 132,180 persons found a U-shaped relation between birthweight and later risk of type 2 diabetes [74]. The risk of small newborns (<2,500 g) compared to newborns of normal weight (2,500–4,000 g) was increased by OR = 1.47 (95% CI 1.26–1.72), a value slightly higher than that for a birthweight over 4,000 g.

The Thrifty Phenotype

In 1962 the geneticist Neel [75] invented the concept of the thrifty genotype to characterize the inherited ability of subjects to store energy in times of food surplus in order to survive in times of shortage. The term thrifty phenotype was proposed by Hales and Barker [76] to describe the metabolic adaptation of a malnourished fetus that allows him to survive in a deprived environment. Gluckman and Hanson [77] elaborated the concept of the predictive adaptive response, a metabolic tradeoff to permit immediate survival even facing long-term costs. They reflected 'that the risk of disease is increased when the actual postnatal environment does not match that predicted prenatally' [78]. Prentice [79] regrets that we still do not have a definite plausible overarching biological mechanism through which empirical findings and these hypothetical models can be explained. But a wealth of empirical data, and an exponentially growing number of experimental studies, are filling the hypothetical framework.

Potential Mechanisms

The factors governing placental development have a major impact on the etiology of IUGR and later outcome [80, 81]. An epigenetic modification of the allele of a gene, i.e. 'genomic imprinting', plays a central role in the control of the fetal demand and the placental supply of nutrients to the fetus from the peri-conceptional period and the earliest stage of the feto-placental development [82, 83]. Generally, paternally derived imprinted genes enhance placental and fetal growth, while maternal imprinted genes suppress growth [83]. For example, glucose transporters can be upregulated (programmed) by hyperglycemia in the first trimester leading to accelerated fetal growth in late gestation [84]. Hypoxia can decrease expression of system A in the trophoblast, a transporter of neutral amino acids [85].

Hormones, such as insulin, insulin-like growth factors (IGFs), thyroxine and the glucocorticoids, play a central role in regulating fetal growth and development [86]. The glucocorticoids are key regulators of organ development and maturation [81]. They act directly on genes and indirectly through changes in the bioavailability of hormones [85]. Some of the endocrine

changes induced by glucocorticoids in utero are transient while others persist after glucocorticoid levels have returned to normal values [85]. In the long-term they can permanently reset endocrine systems, such as the somatotrophic and hypothalamic-pituitary-adrenal axis.

The IGFs-1 and 2 are expressed in fetal tissues from the earliest stage of pre-implantation to the phase before birth [86]. IGF-2 supports embryonic growth and IGF-1 is important in later gestation. Peri-conceptional nutrient restriction decreases IGF levels in mid-gestation, probably by an epigenetic mechanism [87]. Glucocorticoids affect the expression of both IGF genes [85]. Growth hormone has relatively little effect on the fetal IGF axis [86]. At birth a shift from IGF-2 to IGF-1 predominance occurs, and IGF-1 production becomes growth hormone-dependent to induce postnatal growth.

The hypothalamic neuropeptides that regulate energy intake and expenditure in adult life are already present in the fetal brain, e.g. neuropeptide Y has been found to be present from 21 weeks of gestation in the human hypothalamus, and at this stage there are already projections between the nucleus arcuatus and the paraventricular nuclei [88]. Nutritional or hormonal exposures during fetal or early neonatal life may be important in the subsequent development of the appetite-regulatory system, e.g. by prenatal nutritional deficiency and postnatal abundance [89]. The development of appetite control in a growth-retarded infant is supported by breastfeeding, which helps to establish the regulation of food intake in a critical period. Feeding regimens in premature infants have to balance the requirements for catch-up growth and normal development, while avoiding the hazards of overfeeding [90, 91]. The most promising approach in the prevention of adult diseases induced by disadvantageous environments in fetal and early life is to increase the personal and social resources, especially of females, including optimal nutrition, physical activity, no tobacco, no alcohol, no drugs as the basis for a bright future.

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Discussion

Dr. Ogra: Thank you Dr. Bergmann for a very elegant presentation. What is the definition of a small for gestational age or low birthweight infant in the developing and the developed world? Is it based on the total cellular mass, the DNA content in the first trimester, or does it relate to total bodyweight as a function of body fat?

Dr. R. Bergmann: What I understood from the data of Yajnik et al. [1] is that the body composition of Indian newborns was different from that of European newborns. Although they had a lower birthweight, the percentage of fat mass was higher and muscle mass lower. Even at birth they already had insulin resistance while, according to the European data, this seems to develop over the first year [2]. In the Dutch famine study, the offspring of mothers who experienced starvation during the first part of pregnancy had a higher prevalence of obesity [3]. My hypothesis is that they were programmed for small size in the first part, and had catch-up growth during the second part of pregnancy. Dr. Barker may have another explanation.

Dr. Ogra: How relevant is it to the eventual normal functioning of the human host? If the infants start with very low birthweight and continue to have a lower birthweight in Brunei as compared to English children, for example, they will remain smaller than the British children. Yet these are probably normal children. Are there any data to suggest otherwise?

Dr. R. Bergmann: You might be right. This opinion is supported by the observation that long resident high altitude populations exhibit less altitude-associated IUGR and pre- and postnatal mortality than people who have lived there for shorter periods, e.g. as in a study in Tibet [4]. When people are turning into a modern society, challenges occur that they are not designed for. So they must be careful while establishing this new way of life.

Dr. Ogra: Because malaria is very ubiquitous in many of these countries, how extensively have the low birthweight babies been screened in the first trimester for congenital infections other than malaria? Congenital infections such as cytomegalovirus or herpes simplex or some perinatal bacterial infections may also affect growth.

Dr. R. Bergmann: The main risk factors for permanent stunting are malnutrition and infections, which Dr. Hanson will deal with in his presentation.

Dr. Walker: Much of the work that has been done and a lot of the work that you reviewed suggest that it is a vicious cycle. Once you start down the road of IUGR or small for gestational age, it is propagated from generation to generation. Has anyone looked at this in reverse? As nutrition starts to improve, do the changes that occur during pregnancy as an adaptation to intrauterine starvation modify, and the infants become larger, and again later their offspring become larger? Has that been looked at?

Dr. R. Bergmann: I presented the results of the analysis by Kramer and Kakuma [5]. Balanced protein-energy supplements in pregnancy increased birthweights significantly, but the increase was rather low. We are not sure how much is due to a low compliance of pregnant women. In the studies in the Gambia this was taken care of: birthweight increased by 201 g during the hungry season [6]. I don't think it is good to force supplements on pregnant women who are not adapted to a high intake.

Dr. Prentice: It is very complex as you stated. Just as a matter of information, we are following up the offspring from those trials now. Unfortunately they are not really old enough; they are still in their adolescence at the moment so it will take time. Also the Guatemalan supplementation studies are being followed up by Dr. Martorell and others. This kind of data will come out in due course.

Dr. R. Bergmann: Analysis of the data from the INCAP study in Guatemala showed that malnourished women gained weight if they received supplements over a reproductive cycle, but their second-born infant weighed less [7]. Marginally nourished women lost weight during the reproductive cycle, but their second-born infant weighed more than their first-born, and supplements reduced their own weight loss. There seems to be a delicate partitioning of the share in energy between mother and fetus in order to maintain reproductive success.

Dr. Prentice: This field has started to use a phrase, perhaps it originated from Dr. Barker, about 'harmonious growth', a concept that I believe is very important. What we were trying to do with our supplementation program in the Gambia was to prevent fetal growth retardation. Some of these studies are done in periods when there is actually no really good evidence for fetal growth retardation, and what they are trying to do there is to promote fetal overgrowth. So it is very important indeed for us to be looking at the size of the baby in relation to the size of the mother. Just a few moments ago you mentioned the customized growth charts by Gardosi et al. [8] and the babies who have very low perinatal mortality in spite of being born very small: they are appropriate, they harmoniously grown. So it comes back to what Dr. Barker was saying yesterday, how do we get out of this cycle? We have small mothers, and in fact if they have small babies it is actually probably totally appropriate. But we want to get them out of that cycle somehow, and can they be gotten out in one generation by prenatal supplementation? We don't know. First of all it is difficult to do. You very generously cited our studies in which we had the biggest effect, but most of the studies have had a much smaller effect. So it is hard to do and then we question whether it is the right thing to do anyway.

Dr. Barker: One point about harmonious growth might be that a small mother has a small baby; now that might be harmonious or it might not be, it depends how the baby grew. If the baby started on a slow growth trajectory and held to it, which is broadly what happens in China, then what is delivered at birth is a miniature human being. That is very different from the trajectory of growth in Asia, which is broadly a more rapid initial trajectory of growth which is not sustainable in late gestation. The end product is a baby of the same size as the Chinese baby, but the body composition and the long-term consequences are profoundly different. So small mothers may have small babies because the baby senses very early on that he is just going to grow slowly or they may have small babies because they can't sustain a more rapid growth trajectory. That is one of the great mysteries out there. What is it in very early gestation that sets the fetal growth trajectory because it is an extremely important trajectory whatever it is. If it is not sustained then there are adverse consequences, and at birth the babies are disproportionate rather than the Chinese baby who is simply a proportionate miniature human being. My second comment, and I don't know how secure the factual basis for this is, but I was told recently that Pygmies are starting to get taller now. Geneticists have long held that Pygmies have a genetic basis. In the Cameroun the Pygmies are now getting taller and it may be that for Pygmies it will take many generations to escape from what was once an adaptation to profound undernutrition. We just don't know, but the literature certainly talks of many generations before you can put this right.

Dr. R. Bergmann: I have another comment regarding Dr. Prentice's proposal: customized growth charts also adapt for factors that are either not important or should

not be cleared away, e.g. the father's weight or the mother's BMI or even smoking during pregnancy. The father's weight does not matter much regarding fetal growth, but the mother's BMI and smoking are risk factors and not neutral determinants of newborn weight.

Dr. Haschke: My question is related to DHA and its influence on BMI at 21 months of age. At the Nestlé Research Center data have recently shown in a mouse model that just supplementing the animal during pregnancy with DHA results in a lower, different body composition of the offspring. This means that the offspring of the mice who received DHA had a lower body fat content and higher muscle mass, which could correspond with your findings. Were those infants whose mothers were supplemented with DHA during late pregnancy exclusively breastfed, and was there a difference in breastfeeding between the two groups? If yes, this could be attributed to the duration of breastfeeding. You showed that the body composition of breastfed infants differs at 21 months of age; what could be the cause of this?

Dr. R. Bergmann: The children we observed were all breastfed; 80% of them exclusively for 3 months. We used mixed models to adjust for influential variables [9]. We did not expect these results; they were a surprise. The findings are supported by animal experiments, e.g. bodyweight and adipocyte size of the rat pups whose mothers received an n-3 diet were higher than with a n-6/n-3 diet [10]. This could be a biological explanation for the change observed. Dr. Makrides might know more about this. We did not measure the body composition on these children, we just relied on anthropometric data as a proxy.

Dr. Malka: Smoking combined with caffeine is negatively associated with birthweight, and pregnant smokers require more micronutrients. I also want to mention environment which can have an adverse influence in later life. A low socioeconomic status and poverty and the psychosocial consequences associated with low social class are very important.

Dr. R. Bergmann: Yes, in our analysis cigarette smoking explained more of this reduction in birthweight than social class [11]. Smoking mothers may also have a poor diet which we could not control for.

Dr. K. Bergmann: There is some discussion about why IUGR and, on the contrary, macrosomia might similarly lead to increasing the incidence of type 2 diabetes and all its consequences. We speculate that IUGR produces small muscle mass which is tracked over a long time. This muscle mass is not only small, but at the same time it is programmed to somehow have impaired glucose uptake. If under affluent conditions body mass is then added, this preferably would be fat. The relation between fat mass and muscle mass is exceeded more easily if the muscle mass is low, and this produces metabolic syndrome even with lower total BMI status. The macrosomic infant has a high muscle mass plus a high fat mass and this is what is called the adiposo-gigantic type of an obese person who, because both muscle mass and fat mass are very high, produces the same phenotype of metabolic syndrome.

Dr. Hanson: With regard to IUGR, it certainly is a complex condition. One thing we found is that there is a significant reduction in the placenta of the nRNA for IL-10, an immunosuppressive and anti-inflammatory cytokine. We found this in Swedish pregnancies, but especially in Pakistan where IUGR is, as in many other similar countries, a very common condition [12]. This may well indicate that somewhere along the line inflammation is a mechanism involved.

Dr. Walker: Coming back to DHA, there are some mixed views about DHA supplementation during the pregnancy. There are people who feel that DHA is very helpful in preventing prematurity and other things. As I understood, DHA supplementation caused an initial increase in body size and then it fell off with ongoing supplementation. Is that right?

Dr. R. Bergmann: We stopped the supplementation of lactating mothers at 3 months. The BMI increased in these breastfed infants (which is typical for breastfeeding) of both DHA-supplemented and not supplemented mothers, but a little bit more (though not significantly) in the DHA group.

Dr. Walker: To me that implies that over the latter part of gestation BMI was falling off with DHA supplementation, or did I misinterpret that? What is the mechanism; what is the evidence to support that mechanism?

Dr. R. Bergmann: The mechanism, as I understood it, is that n-3 PUFAs have an antiadipogenic effect [13].

Dr. Walker: How does that occur? What is the mechanism by which those fats alter the maturation of adipocytes?

Dr. Makrides: I am not sure that the mechanisms are well sorted out; there is a lot of work going on in the area. Some other work that might be relevant was done quite some time ago [14, 15]. It was shown that muscle tissue that is higher in the long chain n-3 fatty acids has a better glucose response and is less insulin-resistant. Lean muscle tissue with a higher composition of the long chain fatty acids, specifically of the n-6 variety, also react quite differently to muscle tissue with membranes that are more saturated. We are undertaking a large scale randomized trial involving 2,500 women to test n-3 fatty acid supplementation in pregnancy; this is being funded by the National Health and Medical Research Council in Australia., Although the study is focused on neurodevelopmental outcomes, from what I have heard this meeting it signifies that we should also look at the metabolic side. We are in the process of putting together a rationale for doing this and the new data that seem to be coming out signify that we should really be planning that into the long-term.

Dr. Walker: Has anybody actually taken pre-adipocytes? Has anybody incubated pre-adipocytes with different types of fat and looked at its conversion? My understanding is that fatty acids change membrane composition, they incorporate themselves, and long chain fatty acids have a different effect and saturate in other forms. I had no idea that they actually had an effect on maturation itself.

Dr. R. Bergmann: Yes, and the hypothesis is that the increase in adiposity, this epidemic in the United States, is caused by the consumption of too much peanut butter and vegetable oils in the USA, while the consumption of n-3 PUFAs has not increased over the last decades. This has changed the fatty acid composition of breast milk as well [13].

Dr. Prentice: My understanding of this is that Dr. Ailhaud and his team have shown that some of the long chain PUFAs, in particular their derivatives PGE2, are the ancestral ligands for PPARγ, and so that is one proposed mechanism. The question I want to ask is a bit of a challenge. I am concerned that we keep propagating what I think is a myth; namely that IUGR babies end up being obese. Really the only evidence that is persuasive is the data of Ravelli et al. [3] on 19-year-olds; their data [16] on 50-year-olds is really not persuasive. There is one small subgroup which shows a tiny significant 7% increase in BMI which, if you adjust for multiple testing, disappears completely. I am persuaded much more by what Dr. K. Bergmann described, that they are likely to have a low muscle mass. But I really think we have got to stop propagating this myth that low birthweight babies become obese. The meta-analysis by Oken and Gillman [17] shows quite the reverse: it is the big babies that become obese, not the small ones.

Dr. R. Bergmann: I agree. We should be cautious and warn our colleagues here that they should not introduce our way of life into their countries too fast, because it may not be appropriate for their populations.

Dr. Maldonado: My question relates to what Dr. Ogra was talking about regarding inflammation. A few years ago, there was a large study of about 2,000 HIV-infected

pregnant women in Malawi, Tanzania and Zambia looking at the effect of subclinical chorioamnionitis on HIV transmission. In that study the women were randomized to normal prenatal care versus antibiotic treatment for presumed chorioamnionitis in 28 and 32 weeks of gestation. There was no effect on HIV transmission but there was an effect on decreasing IUGR, small for gestational age babies as well as decreasing sepsis in newborns which would be expected. It is very unclear to me what the definition of subclinical chorioamnionitis was. Are you aware studies of this nature where antibiotic treatment of potential not clinically recognized inflammatory states can influence the outcome of pregnancy?

Dr. R. Bergmann: In developed countries, besides unknown causes, the main etiological determinant for premature birth is infection of the genitourinary tract, often chronic such as bacterial vaginosis [18]. Twenty percent of premature babies in the USA are delivered prematurely due to maternal or fetal indications, e.g. severe growth restriction, and 30% due to premature rupture of the membranes, often as a consequence of chorioamnionitis [19]. Part of this growth restriction could be avoided by antibiotic treatment.

Dr. Maldonado: Do you believe that might have an effect on longer term outcomes beside just reversal of IUGR? I imagine that hasn't been looked in a longitudinal fashion.

Dr. R. Bergmann: I don't know of any study.

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Growth and Nutrition: The First Six Months

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Abstract

Today the WHO Growth Chart Standards, based on the growth of breastfed infants, are used. These growth curves solve the problem of the deviating observations for breastfed compared to non-breastfed infants using previous growth charts. Presently it is not clear how the mother's diet, especially the fat intake, influences the growth of the offspring. Animal experiments indicate that a low intake of n-3 polyunsaturated fatty acids via the milk may have short- and long-term negative consequences. There is limited information in man. It has been suggested that the mammary glands may have phylogenetically originated from glands providing innate immunity, later developing capacities for providing nutrition. This would agree with the fact that human milk contains so many major components which do not primarily function as nutrients, but seem to protect nutrition and growth. Lactoferrin, oligosaccharides, glycoproteins, secretory IgA antibodies, α-lactalbumin and the antisecretory factor have such functions.

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Growth in Breastfed and Non-Breastfed Infants

The usefulness of growth curves in the evaluation of the health status of infants and children has made them a basic instrument in clinical pediatrics and research. Growth curves are, for instance, most useful to show improvements

in measures introduced to prevent impaired health and growth in underprivileged populations. This was illustrated in our long-term follow-up of children in different social groups in and around Lahore, Pakistan. Diarrheal illness was significantly reduced by measures such as starting to breastfeed within 3 instead of 47 h after birth, an increase in exclusive breastfeeding from 5 to 80% at 1 month of age, and a reduction in the use of pre-lacteal feeds from 100 to 34%. Within 10 years postnatal linear growth increased by about 3 cm at 24 months of age [1].

Today we use the WHO Child Growth Standards [2], which were set up to demonstrate optimal growth based on infants exclusively breastfed for the first 6 months, with complementary food added thereafter. Among those previously used, the Center for Disease Control reference tables showed the average growth of all infants, including those overfed, underfed and uncared for, as well as those well-fed. Using this standard, breastfed infants showed enhanced growth during the first 2 months and reduced growth during months 3–12 compared to formula-fed infants [3]. A very large observational cohort study analyzed the effects of different modes of feeding on growth, analyzing z scores of weight for age, length for age and weight for length [4]. The investigation showed a growth-accelerating effect of formula compared with human milk and other milks throughout infancy, but especially at the age of 3–6 months. This analysis confirmed several previous investigations. The study also brought out a strong negative association between cereal intake and length, weight and head circumference gains in the 3- to 6-month interval. This may be similar to the previously demonstrated effect of offering cereals and other solid foods during that critical time period [5]. The effect on final weight and length is not known from these studies. The normative growth in the WHO chart of weight as well as height is regarded as representative of infant growth and includes the additional growth previously seen in breastfed compared to non-breastfed infants during the first few months.

We studied the association of breastfeeding to linear growth using a nonlinear model and followed the six phases, neonatal, infantile, early childhood, mid childhood, late childhood, and pubertal, introduced by Walker and Walker [6] in their model. Breastfeeding data and measurements of height were collected longitudinally for every Swedish infant born on the 15th day of any month in 1981. Data included measurements of height for 3,107 children. Information about breastfeeding was available for 2,773 of these (12.2% missing). There was a significant association between any breastfeeding and the growth rate of boys in the neonatal phase, i.e. up to 2–3 months of age [Silfverdal et al., unpublished]. The lowest growth rate was seen in those breastfed for less than 30 days. Next came the group with any breastfeeding for 30–150 days, illustrating a dose-response relationship (table 1). The same pattern could be seen for girls with a significant effect of the age at peak height velocity in the neonatal and infantile phases, which were delayed compared to those most breastfed (table 1). Further, among the girls, there was a

Table 1. Parameter estimates and 95% confidence intervals for the Walker and Walker model with the first of the five phases of growth in girls

	Parameter estimate (95% CI) for reference group (breastfed >150 days)	Estimated difference (95% CI) compared to reference group for children breastfed	
		30–150 days	<30 days
Adult height	167.6 (167.1; 168.2)	−0.35 (−1.15; 0.43)	**−1.40 (−2.41; −0.40)**
Growth phase I (neonatal)			
Age at peak height velocity	−0.12 (−0.14; −0.11)	0.01 (−0.02; 0.03)	**0.06 (0.03; 0.10)**
Growth rate coefficient	5.97 (5.76; 6.20)	**−0.66 (−0.93; −0.40)**	**−0.86 (−1.15; −0.60)**

Significant group differences are printed in bold type.

negative association with adult height for the group that was breastfed for less than 30 days compared to the group most breastfed. The reason for this is unknown, but we have previously noted that the body mass index was lower in breastfed girls [Silfverdal et al., unpublished]. Excluding infants with a low birthweight (<2,500 g), chronic disease, or of immigrant parents did not lead to any significant changes in the results. Moreover, separate analyses of groups based on birthweight categories (<2,500, 2,500–4,000, >4,000 g) did not change the conclusions as to the association with breastfeeding. A weakness is that no controls for confounding factors like smoking and socioeconomic status were included.

The WHO growth curve does not take the diet of the breastfeeding mother into consideration. Based on studies in experimental animals, as well as in man, there is evidence that the quality of the mother's fat intake plays a role in the fat content of the milk with possible consequences for the offspring. This was obvious in our investigations in rats during pregnancy, lactation and adulthood. Thus a deficiency in essential fatty acids in the maternal diet during late pregnancy and lactation caused significantly low serum leptin levels in the offspring [7]. The effect was due both to the regulation of the amount of adipose tissue and leptin mRNA expression [8]. A perinatal deficiency in essential fatty acids was also associated with increased body weight and significant changes in the trabecular and cortical bones of adult male offspring [9]. Dams eating a diet with a low ratio of 0.4 of n-6/n-3 polyunsaturated fatty acids (PUFAs), compared to those given a high ratio of 9, had offspring with lower leptin levels and body weight, length, inguinal fat pads and adipocyte size [10]. In adult age the male offspring of the mothers given the high ratio n-6/n-3 diet compared to those given the low ratio showed increased systolic blood pressure and serum triacylglycerol levels [11]. The females in that diet

group had increased cortical bone thickness and significantly longer femurs with larger cortical cross-sectional bone area, as well as bone mineral content [12].

In humans the fat content in milk varies much and can provide up to 50% of the energy intake of the infant [13]. Colostrum contains a higher percentage of long-chain fatty acids, whereas the n-6 and n-3 series PUFAs decrease during the first month. The milk fat content varies between breasts and women. In a small group of infants such variations could not be shown to affect growth [14]. Chinese mothers with a high carbohydrate but low fat, protein and energy intake had high concentrations of PUFAs in milk with a n-6/n-3 ratio of 21.6. The ratio between arachidonic and docosahexaenoic acid was as high as in vegans. The concentration of these two fatty acids correlated positively with the breastfed infants' weight gain at month 1 and length gain at 1 and 3 months [15]. Supplementing the diet of lactating women with fish oil during 0–4 months of lactation did not change the growth of their infants with regard to weight or length during the first 9 months, but had effects on body composition at 2.5 years of age. A preferential transfer of n-3 PUFA via breast milk to the infant's erythrocytes was suggested [16]. Supplementation with fish or corn oil for 3 weeks during pregnancy did not have any effect on infantile growth during the first year [17].

Breastfeeding compared to formula-feeding was found to give higher leptin but lower grehlin and insulin-like growth factor-1 serum levels in the infant [18]. Decreased milk leptin levels were related to rapid growth in small for gestational age infants; large infants instead received milk with increased leptin levels seemingly adjusting the appetite of the infants [19]. Milk leptin levels at 1 month of lactation were related to maternal plasma leptin and inversely to the maternal body mass index [20].

Phylogenetically, Milk Glands May Initially Have Provided Innate Defense, Later Adding Nutrients

According to a recent review [21], mammary glands providing nutrition and host defense may have originated from skin glands initially involved in innate immunity. Such host defense, providing broad nonspecific protection, may also have developed the capacity to provide energy for the growing offspring in incremental steps, in man primarily as lactose and fat. This seems obvious in man where the protein content is low compared to many other species and where most of the major milk proteins do not seem to be available for nutrition, but rather function in defense. The evolution of two enzymes, xanthine oxidoreductase and lysozyme, which are both expressed in and secreted from the lactating mammary epithelium, was used as examples of this development. These enzymes are thought to be involved in the evolution of the nutritional capacity of human milk and are antimicrobial [22].

Factors Affecting Growth in the Neonate and Young Infant

Early Microbial Colonization and Host Factors that May Protect/Enhance Growth

The newborn meets a very complex microflora from delivery on. Already from birth the infant is equipped with receptors on mucosal membranes and leukocytes belonging to the toll-like receptor (TLR) family. These receptors activate an inflammatory response based on the production of nuclear factor(NF)κB. This is the transcription factor which initiates the production of proinflammatory cytokines such as interleukin-1β, tumor necrosis factor-α and interleukin-6, which among many other effects induce increased levels of leptin, decreasing appetite.

In a recent study of mice, it was found that already in the birth canal neonates meet with microbes which seem to tolerize their innate immune system so that TLR4 on the intestinal epithelium was inactivated. Those born by cesarean section were not similarly tolerized [23]. In contrast the TLR4 on submucosal leukocytes remained capable of activation to defend against any Gram-negative microbes entering tissues. But there was a 1- to 3-log lesser response of monocytes from cord blood with regard to the production of tumor necrosis factor-α compared to adult blood monocytes to a ligand activating many TLRs, including TLR4, suggesting a less strong proinflammatory response in the neonate [24].

Human milk, but not formula, also contains a proteinaceous component, which can modulate TLR-mediated responses specifically and differentially. This may be a milk-mediated mechanism helping the neonate to finely tune its response to microbial exposure from delivery on, especially in the extensive gut mucosa [25]. By activation of TLRs, inflammation with subsequent symptoms like anorexia might otherwise be induced via the proinflammatory cytokines produced. These authors also showed that human milk contains soluble TLR2 which may downregulate the responsiveness via cellular TLR, which are activated by Gram-positive bacteria [26].

The microflora of the gut is extremely complex and only part of it has been defined and described. Obviously the neonate starts to become colonized by microbes from the mother already in the birth canal, and especially by being exposed to maternal stool flora by being born next to the mother's anus. In early life the aerobic microflora is more prevalent than after the anaerobic flora has expanded and, by competition, reduced the number of aerobes which contain many potential pathogens. Breastfeeding supports this form of protection by presumably promoting the strict anaerobes. A recent study demonstrated that infants delivered vaginally at home and exclusively breastfed mostly had a 'beneficial' microflora with the highest number of bifidobacteria and lowest of *Clostridium difficile* and *Escherichia coli* [27]. A study from The Gambia showed that colonization with *Helicobacter pylori* in early infancy predisposed to malnutrition and growth faltering [28]. Actually, the

weight loss caused by colonization with *H. pylori* showed a significant relation to the absence of secretory IgA antibodies in their mothers' milk against the *H. pylori*-vacuolating cytotoxin A of these bacteria [29].

Factors which May Promote or Protect Growth

Human Lactoferrin

Lactoferrin is a major milk protein present in concentrations of 5–7 g/l in colostrum and 1–2 g/l in mature milk. It is not only an antimicrobial protecting against experimental urinary tract infection in mice given lactoferrin perorally [30], it is also an anti-inflammatory. It downregulated inflammation in a model of dextran-sulfate-induced colitis in mice [31]. This effect could be further defined, showing that lactoferrin downregulated the production of the proinflammatory cytokines by reducing the activation of NFκB [32].

Further studies demonstrated the additional effects of human lactoferrin which is potentially favorable to the growth of the breastfed infant. Thus we find that it seems to function like a heat shock protein [33], suggesting that it may protect the integrity of proteins during synthesis and afterwards; it may have carrier functions and aid in the uptake of proteins in the gut. We also noted that, similar to heat shock proteins, lactoferrin unsaturated with iron binds ATP and has ATPase activity. ATP may become available when milk lactoferrin and the peptides thereof, like lactoferricin, exert their antibacterial activities on the bacteria in the gut. Furthermore, a complex is formed by lactoferrin and casein kinase protecting CK-2 [Moisei et al., unpublished], which results in phosphorylation of lactoferrin [34]. Since CK-2 phosphorylates a number of cellular enzymes and functional proteins such as transcriptional and translational factors, it can be suggested that lactoferrin may stimulate protein synthesis at the cellular level. All this indicates that human milk lactoferrin may have additional effects in the breastfed infant by the lactoferrin-CK2 complex influencing the intracellular phosphorylation process and signal transduction. Actually the phosphorylation sites for CK2 on human lactoferrin are about twice those of bovine lactoferrin [34]. The availability of some phosphorylated components or nucleotides for the infant may be enhanced by human milk lactoferrin.

All mammals can to some extent synthesize nucleotides, i.e., the essential building stones in DNA and RNA. The mere fact that all milks, including human milk, contain substantial amounts of nucleotides strengthens the assumption that nucleotides are semi-essential for newborns. As exogenous nucleotides may derive either from the diet or from the gastrointestinal microflora we performed the following experiment. At weaning, young conventional and germfree rats were put on either a nucleotide-free diet or the same diet enriched with nucleotides. After 2 weeks on these diets the rate of enterocyte mitosis was investigated [35]. No effect was found in conventional

rats, whereas an increase in the rate of mitosis was found in germfree rats (27/11, 19/18, 23/12 and 12/15% increase in the duodenum, jejunum, ileum, colon of male/female rats, respectively). In short, the results demonstrate that young animals can utilize microflora-related as well as dietary-related supplementation of nucleotide.

Milk Oligosaccharides and Glycoconjugates, Secretory IgA and α-Lactalbumin

These major milk components are, like lactoferrin, better known for their roles in host defense and protection of the host's integrity than for nutrition, again stressing the complex role of human milk in supporting the infant. For secretory IgA an additional function has just been described: after binding to the antigen in the gut it attaches to the M cells over Peyer's patches and is taken up together with the antigen potentiating the breastfed infant's defense by an immune response to an additional microbe or toxin [36]. Milk antibodies have also been found to be able to hydrolyze nucleotides [37]. α-Lactalbumin has the capacity to transform into a complex with oleic acid that makes it lethal to tumor cells (HAMLET = α-lactalbumin made lethal to human tumor cells) [38].

Anti-Secretory Factor Protects against Mastitis in the Mother and Diarrhea in the Infant

The peptide anti-secretory factor (AF) appears in body fluids including human milk, presumably in response to exposure to bacterial enterotoxins. It can also be induced by eating a specially treated cereal [39]. We were able to show that clinical mastitis was prevented by inducing this peptide in milk after intake of the special cereal by lactating mothers [40]. Presumably the effect is due to decreased secretion in the inflamed area, making the intense pain subside.

In a double-blind randomized study of diarrhea in 240 children, 6–24 months of age, in Lahore, Pakistan, in addition to oral rehydration we gave either egg yolk from hens fed the AF-inducing cereal, thus containing preformed AF in high concentrations, or ordinary egg yolk without AF [41]. Of those 120 who had been sick for <7 days 60 received the AF and 60 did not. Within 3 days we noted a striking reduction in the number of stools among the treated compared to the controls ($p = 0.0054$). The consistency of stools also normalized significantly faster ($p < 0.05$). Those 120 who had been sick for >7 days were similarly studied. The number of stools did not differ from the controls, but the stool consistency normalized faster in the active treatment group ($p < 0.008$). Since AF can easily be induced in human milk by intake of a special cereal diet and also may result from oral exposure to enterotoxin-producing *V. cholera* and *E. coli* [42], it is likely that AF provides a further explanation as to how breastfeeding may protect against diarrhea.

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Discussion

Dr. Bier: The last slide you showed us about infections and birthweight, was that corrected for the small infants, the number of days they might have spent in a neonatal ICU or a neonatal facility in the hospital?

Dr. Hanson: No, such details were not available. 1.7 million children is quite a large number, such details were not there. On the other hand, and what I am stressing, is that it was an increased problem also above 1,500 g.

Dr. Björkstén: Thank you for a very interesting and nice presentation. I have a question regarding exclusive breastfeeding starting very early. To my knowledge there

is no traditional society in which some sort of Beikost is not given in addition to breastfeeding. We usually discuss exclusive breastfeeding meaning no formulas, but mothers have always been giving something in addition to breastfeeding. As you know there are numerous bacteria present in breast milk, including almost any pathogens, but they do not cause any harm, as long as the breast milk is fresh. However if you pasteurize it, then the microbes can cause disease as when present in any food. So my question is whether addition of food proteins and microbes under the umbrella of breastfeeding is actually important for tolerance induction. Going through the literature, we did not find a single example of an infection epidemic caused by banked breast milk, provided the milk was fresh and not pasteurized. All examples of breast milk spreading disease have been related to pasteurized milk.

Dr. Hanson: This is quite an important question. What is given to the neonate is often given without realizing how small the neonate's stomach is. It is tiny and it expands only a little in the next 2 days. As far as possible the newborn should only receive the early milk, the colostrum, which has very high levels of secretory IgA antibodies, between 7 and 15 g/l (mature milk contains 0.5–1.0 g/l). These antibodies are mainly directed against the mother's intestinal microflora, which normally is the main colonizer of the newborn. When the bacteria expand in numbers they provide 'colonization resistance' against other microbes, limiting their numbers. I would assume that tolerance induction in early life is a less relevant mechanism than build up of defense.

Dr. Björkstén: My question is related to host defense mechanisms, and the idea is that you would not only induce tolerance but a regulated immune response also to pathogens. Thus it may actually be an advantage to be exposed to the antigens together with breast milk. We have an instructive example from Sweden when we changed feeding practice and introduced high doses of wheat flour rather suddenly at 6 months. The incidence of celiac disease increased rapidly to about 1 in 200 until we realized that gluten should be introduced gradually and preferably under the umbrella of breastfeeding. So my question relates not only to tolerance, but also to the actual development of a good immune response to the bacteria you are being exposed to under the umbrella of breast milk.

Dr. Hanson: It's an interesting point but I would also add that the immune system is tiny in a newborn. What makes it grow is primarily the exposure to microbes. Therefore I would regard microbial colonization, as this happens normally by being delivered next to the mother's anus, to be the most efficient way to expand the immune system and possibly also the capacity to develop tolerance. In many settings the material given instead of mother's milk is more or less contaminated with microbes other than from the mother and thus potentially risky. The role of such microbes for the development of tolerance is, as far as I know, not defined.

Dr. Walker: Celiac disease is an autoimmune disease, and unless you are talking about a very rare form of celiac disease, which is allergy to wheat, it doesn't matter. The reason that wheat is not introduced early is that it creates growth failure in infants. It is a genetic disease; I don't think it has anything to do with breast milk. There is a condition called 'breast milk colitis'. The mothers drink cow's milk and other allergens that go into the breast milk, and an infant has only small quantities that cause inflammation. So it is very controversial whether providing foreign antigens with breast milk is a good thing early on in life. The other point I wanted to make is first of all you continue to underscore the quality of breast milk, but there is something that perhaps should be pointed out: the protective factors are highest in concentration early in nursing, when the baby needs it, and then they fall off. This is a very important area.

Dr. Ogra: There are several new pieces of information which you alluded to in your elegant talk. I would like to pursue a couple of those issues with you. One relates

to the phylogenetic concept that the breast started as a skin tissue. If that is the case, are there any data to suggest that the macrophages in the milk are homologous to the Langerhans cells in the skin? Second, is there a defined process of antigen presentation or processing by these cells, similar to the skin?

Dr. Hanson: I do not know of any data that would help me answer your question. Rather little has been done on milk cells recently. There was quite an interest many years ago, but it has not been followed up.

Dr. Ogra: The second question relates to the issue of transmission of infections via breastfeeding. What you outlined in your presentation is absolutely correct. However, there are data to suggest that milk does transmit infections with the development of disease. One of them would be the HTLV and there are some data on HIV, although soft, but it is still possible to transmit infections via breastfeeding. Milk does carry a lot of infectious agents and antigens. Is there any evidence to suggest that such feeding-associated transmission of infectious agents and their antigens is protective in the long run against disease or contributes to the induction of tolerance against allergy and hypersensitivity?

Dr. Hanson: It is certainly true that normally there is a very wide variety of microbes in milk and at times pathogens pass through; HIV being a very sad example. Since HIV transfer via the milk is linked to the presence of subclinical mastitis, we thought we might possibly prevent this by using the peptide antisecretory factor which reduces the secretion of fluids over membranes, for instance preventing clinical mastitis in a controlled study [1]. However, the peptide had no effect on subclinical mastitis according to the preliminary outcome of our controlled study in Pakistan.

Dr. Ogra: The final question relates to the fascinating data on TLRs in milk. It would be interesting to see whether soluble TLRs in milk adhere to the mucosa of the breastfeeding infant. Is it possible that such TLRs in milk function as a bypassing mechanism by directly binding to the pathogen-associated molecular patterns of infectious agents and thus affect their elimination from the neonate?

Dr. Hanson: The answer is not known but it is a really interesting point you made, because it turns out that the tolerance induced in a mouse model included only the TLR on the mucosal epithelium, the submucosal sets kept the TLR. This seems to be a very interesting, well-adapted defense system. These are very new data and I am sure we will hear more about it.

Dr. Giovannini: Could exclusively breastfed infants need more nutrients in the 4- to 5-month period, and does the z score on the new WHO charts progressively decrease in exclusively breastfed infants after 4 months? In your opinion should complementary food be started before 6 months? DHA is not used for growth, but perhaps for neurobehavioral development. The kind of fish the mother eats is also very important because for instance sole has no n-3 but salmon and cod do. When speaking of allergy, it is very important that weaning be complementary, not supplementary. In southeast of Asia 78% of the mothers throw away the colostrum because the color is different, and it is very important to educate these women against throwing away the colostrum because it is the first protection against infection and disease.

Dr. Hanson: As to the colostrum, I consider that nature has after long development come up with a very specialized milk for the newborn with its very tiny stomach and a rather empty gut, which is very quickly colonized with bacteria that reach high numbers quite fast. It is an urgent matter for the baby to get as much protection as fast as possible over its large mucosal membrane surfaces, especially in the gut. I mentioned previously the very high content of the secretory antibodies in the colostrum which will likely cover the mucosa. Thus they prevent the microbes that are invading the baby's gut from reaching the mucosa, where they could potentially cause inflammation and infection. Thus I believe that colostrum is very important. One might add

its very high content of lactoferrin with its many positive functions. It seems to have been developed to try to support the baby in a critical period when aerobic bacteria, including potentially dangerous ones, reach high numbers in the gut during the first week of life. Therefore I would avoid anything other than breast milk that might bring unknown microbes: from hospitals, their staff or contaminated foods. In many traditional societies various symbolic, often heavily contaminated foods and fluids are given to the newborn early after delivery, before anything else is given.

As to breastfeeding, as you all know 6 months is indicated as the optimal period of exclusive breastfeeding. There are good reasons for that goal, and I am aware that there are many factors that can make this difficult, for instance some mothers have to go back to work much too early. Sweden has a system which permits the mother to stay home for an extended period of time so that does not become a problem. We have to put our hope into the next generation, with better support for the mother and baby during this very important period of life. If it is possible to continue to breastfeed exclusively for 6 months, then this is favorable.

Of course I am a bit sided in this because I spent the last 45 years working on components in human milk and we just keep finding new, remarkable ones, presumably energy-expensive ones for the mother. So why are they there; for nothing? I think that human milk and especially colostrum is a very elaborate and elegant system for protection of growth and development of the human offspring. Presumably we still only understand part of it. Yet I think we can conclude that human milk really gives the baby the best chance to develop and grow optimally. So in my mind the answer to your question is easy, but I am sided although based on facts.

Dr. K. Bergmann: You mentioned something very interesting, namely that there is some protection from infections up to the age of 10, but you said most of this occurs during the first 6 months. Do you also have data on the incidence of infection beyond 1 year?

Dr. Hanson: Firstly, for certain vaccines the immune responses are enhanced by breastfeeding, for other vaccines this is not seen. Secondly, there seems to be long-term protection against certain tumors in breastfed children, as against breast cancer in mothers who have breastfed. There is also evidence to suggest protection against certain immunological diseases, like celiac disease. In addition there are reports on enhanced protection against certain infections. It should be added that such long-term studies are difficult to do and control perfectly. Still there are data to suggest that there are in some instances long-term protective effects. One could consider that many of the growth factors and multiple other signals present in human milk may be involved in such long-term effects. Clearly more research is needed.

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Growth in the First Two Years of Life

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Abstract

Compared to other periods of life, infancy is a period of rapid growth, but the relative relationships among rates of linear growth, weight accretion and brain growth vary greatly during the first years of life. Additionally, while the energy requirements for body tissue deposition as a fraction of daily energy needs decrease dramatically during infancy, brain energy demands, measured as the cerebral rate of glucose utilization, increase markedly during the same period. There is now substantial evidence that postnatal growth in infancy is associated with various consequences detrimental to health in adult life, particularly hypertension, cardiovascular disease, obesity and type 2 diabetes, but the relationships vary depending on whether one takes growth to mean statural growth or ponderal growth, as well as on the specific period of infant growth. Recently, several mechanisms have surfaced that might account for the relationships observed. These include epigenetic effects on gene expression, alterations in neuronal signaling because of inappropriate dendritic pruning, and gut microbiota effects on fat storage.

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Introduction

The Oxford English Dictionary defines growth as 'the action, process or manner of growing; both in material and immaterial senses' including 'size or stature attained by growing'. The Cambridge Advanced Learner's Dictionary defines growth as an 'increase in size or amount'. The MSN Encarta Dictionary calls the growth process 'the process of becoming larger and more mature through natural development', while a textbook of pediatric endocrinology says that 'growth can be defined as an increase in size by accretion of tissue' [1]. While generally describing the same processes, differences in the precise meaning of growth to individual scientists become important for interpreting

the reported information on pathophysiology, control, and nutritional influences on normal and abnormal growth. Thus, to a pediatric endocrinologist, growth almost invariably means statural growth, an increase in length or height. To a nutritionist, on the other hand, growth often means ponderal growth, an increase in body mass. These distinctions are important because, even though length and mass increase concomitantly, some regulatory elements are common to both and others are not. Likewise, it is not unexpected, then, that some environmental influences might alter both, while others might influence weight gain, but not stature, or vice versa.

Growth during Fetal Life and Infancy

Fetal growth is under different controls than postnatal growth. In utero, fetal growth is influenced by factors extrinsic to the fetus, including placental function and maternal anatomical, blood flow and nutritional considerations. Intrinsically, fetal growth is orchestrated by various major gene families, like the homeobox and growth factor classes, and limited by nutrition supplied via the placenta [1]. Although the prenatal regulation of insulin-like growth factor-1 (IGF-1) is not entirely understood, inadequate postnatal nutrition impairs IGF-1 gene expression. Thus, the effects of fetal malnutrition on growth may be mediated, at least in part, by inadequate IGF-1 generation. Infants malnourished in the womb and infants who completely lack IGF-1 activity, either as a result of a defective growth hormone receptor or a mutation in the IGF-1 gene [2, 3], are both born small, as are the corresponding gene knockout animal models [1]. Fetal nutritional insufficiency during the first two trimesters tends to affect birthweight and length proportionately, but inadequate fetal nutrition during the last trimester impairs weight gain more than a decrement in length. There is little doubt that fetal growth is highly dependent on the environment in utero, including the effects of placental blood flow [4], and prevailing data suggest that intrauterine environment and fetal nutrition are the principal determinants of birth size. Nevertheless, despite dramatic improvements in maternal health and nutrition over the last century, there has been little increase in birthweight in societies keeping reliable records [5] and balanced maternal protein-energy supplementation during pregnancy results in only a very modest increase of 21–75 g in infant birthweight [6]. Although the fact that heavy mothers produce larger babies is well appreciated [4], the determinants of neonatal body composition are poorly understood. Recently, however, Harvey et al. [7] showed that maternal size, parity, and fat stores contributed independently to neonatal body composition, and Andreasyan et al. [8] demonstrated that a higher maternal protein intake during late pregnancy led to a lower infant ponderal index at birth.

There is only a weak correlation ($r = 0.3$) between birth size and adult size. However, by 3 years of age, the correlation between and length and adult

height reaches 0.8 [1]. Thus, genetic factors are the predominant influence on linear growth during the first 3 years of life. However, even though length velocity during the first 3 years of postnatal life is faster than that of any period other than fetal life, infants with 'tall genes' who are born short grow more rapidly during the first 3 years of life, and infants with 'short genes' who are born long grow more slowing during the first 3 years. Historically, the former circumstance is often interpreted to be the result of optimal nutrition and/or parental care while the latter circumstance is felt to reflect nutritional inadequacy and/or the consequence of disease.

Although a small minority of single gene defects affect growth or bodyweight [9, 10], both weight and linear growth are primarily polygenic traits [10–13], and genetics are the major determinants of both adult weight and height. Silventoinen [11] has estimated that only about 20% of the variation in height is due to environmental factors, predominantly nutrition and disease. Similarly, genes account for the major fraction of variance in bodyweight [14]. Only about 2% of monozygotic twins are discordant for weight and, although the heavier adult twin weighed slightly more at birth, bodyweight discordance was only seen after puberty [15].

It is equally important to appreciate that the relative increments in length and weight during the first 2 years of life are dramatically different. Thus, from birth to 24 months of age, body length increases about 75% to a value approximately 50% of final mature height while weight increases by more than 2.5-fold, but to a mass only about 20% that of adult bodyweight [1, 16, 17] (fig. 1). Remarkably, during the same interval, brain size increases to more than 80% of adult brain size and, by the age of six, its size is 95% that of the adult [1, 18] (fig. 1). The endocrine control of body compositional changes during infancy and childhood has been extensively studied [19] and energy requirements for growth [20, 21] and cerebral metabolism [22] have now been measured. Surprisingly, despite the fact that growth is especially rapid through the first 3 years of life, the energy cost of growth as tissue deposition amounts to 40% of energy expenditure at 1 month of age, but declines to about 3% at 1 year of age [20] and remains at this low level until the onset of pubertal growth [21]. On the other hand, brain glucose consumption increases rapidly during the first few months of life, continuing to rise to about 4 years of age, when cerebral glucose consumption rates are more than twice those of the adult [22]. Further, the cerebral metabolic rate of glucose consumption remains well above the adult rate until late adolescence [22].

Relevance

Until recently, despite an established interest in the effects of prenatal growth on adult disease, interest in postnatal linear growth and weight gain was primarily related to diagnosing and rectifying the causes of failure to

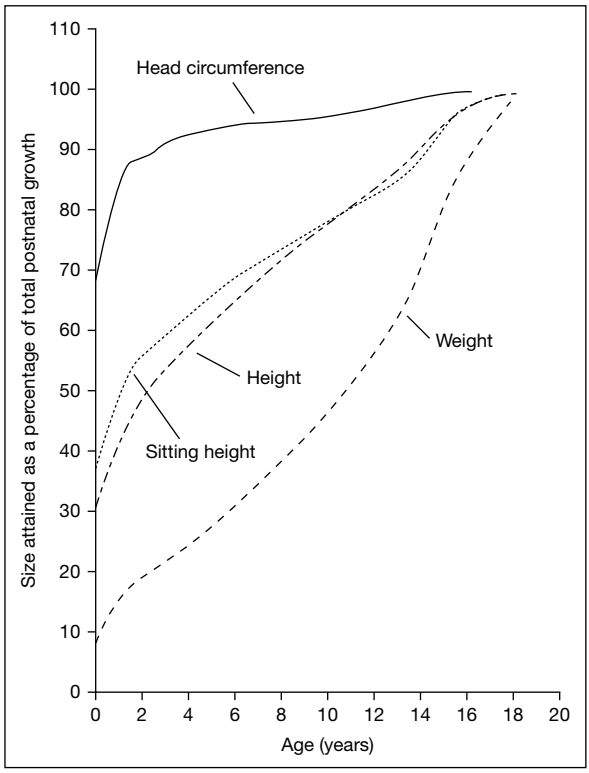

Fig. 1. Relative relationships among linear growth, growth in bodyweight and brain growth during infancy, childhood and adolescence. From Clayton and Gill [1], with permission.

thrive, including malnutrition, infection, and assorted other pathological conditions or genetic disorders. Concern about rapid growth was virtually nonexistent. This situation changed with published data that raised the possibility that rapid postnatal growth came with a 'cost', namely increased risk of chronic diseases in adult life [23–29]. In this context, rapid growth refers to increased weight gain, since there is an inverse association between height and the risk of developing coronary heart disease [30]. More specifically, in reference to coronary heart disease and diabetes, the postnatal trajectory of increased weight gain does not take place until a period of slow weight gain for the first year or two of life [31] or is the consequence of an earlier than expected second rise in the childhood body mass index curve (adiposity rebound), before the ages of 5–7 years [26]. While this model has extensive support from observational studies, it is not entirely clear whether the detrimental adult outcomes (namely obesity and its comorbidities) are related to a

Table 1. Some mechanisms for maintaining organismal memory throughout life

Permanent anatomic change in organ structure
Epigenetic DNA imprinting
Clonal selection
Neuronal pruning
Gut microbiome stability

simple mass accretion times time function. In other words, since we have had very little success in achieving and/or maintaining long-term weight loss, do the associations merely reflect the fact that individuals who start to gain excess weight at an earlier age are fatter at any given later age and more likely to suffer the consequences? An alternative model, where the critical period of rapid weight gain is the neonatal period [23, 24, 32] also has experimental support although a recent study with a contemporary cohort of infants found no relationship between weight gain in the first 6 weeks of life and height, weight, body fat or insulin resistance at 5–8 years of age [32].

Research for Resolution

Compelling questions arise from the observations reported above. First, how, precisely, do cells or organs 'remember' their environment during infancy, what they ate and how fast they grew? Secondly, what cells or organs are responsible for the integration of the information stored? Third, what does this have to do with nutrition during the first few years of life? Some plausible answers to the mechanism of memory are shown in table 1. Expected mechanisms for which there is direct evidence include genomic imprinting and CNS synaptic pruning, while a fully unexpected explanation involves the consequence of the stability of the gut microbiome.

What is now very clear is the fact that cellular environmental 'memory' can be held in the form of permanent marking of DNA via methylation (genomic imprinting), resulting in gene silencing. This process, in which gene expression is affected without a change in DNA structure is called epigenetics. Further, since the dietary nutrients methionine, folate, vitamin B_{12}, choline and betaine are responsible for the cellular 'methylation milleu', recent evidence has shown that postnatal 'methylation' diets fed during the post-weaning period can permanently alter the expression of the IGF-2 gene in adult animals [33]. The full extent of this phenomenon, the specific nutritional boundaries, and the proof-of-principle experiments in human infant early nutrition have yet to be described.

Through hypothalamic regulation, the brain is the regulatory center of both linear growth and bodyweight regulation. MRI and PET studies demonstrate the very high degree of metabolic activity in the human infant brain during

early infancy [18, 22] and the proof-of-principle experiments of nature that link gene methylation to altered CNS regulatory experiments have been described. Thus, Rett syndrome (and possibly other autism spectrum disorders), a severe developmental regression syndrome associated with impaired growth in girls, is the consequence of abnormal gene methylation in the CNS that affects neuronal transcription and synaptic activity by interfering with dendritic expansion and pruning [34–36]. Similarly, in addition to well-described fetal animal programming effects on appetite and obesity [37–40], there is now clear demonstration that hypothalamic feeding circuits are programmed during critical early developmental periods in rodents by leptin effects on the development of neuronal connections from the arcuate nucleus [40, 41], and that leptin treatment during the appropriate critical period can reverse developmental programming effects [41, 42]. Further, others have shown that variations in maternal care during infant rodent suckling can result in epigenetic alterations in hippocampal gene expression in adult animals and, perhaps more importantly, that these epigenetic markings are reversible later in life by provision of the methyl donor, methionine [43, 44].

Finally, rather surprising new developments raise the possibility that bacterial colonization of the neonatal gut may play a important role in infant weight gain. Recently it has been demonstrated that (a) microbial colonization of the gut increases calorie extraction from plant polysaccharides ingested in the diet, (b) the specific population of gut flora is unique to an individual and is remarkably stable within an individual over time, (c) the relative abundance of the two principal bacterial divisions, the Bacteroidetes and Firmicutes, regulates fat storage in the host animal by two independent mechanisms, alteration of fasting-induced adipose factor and phosphorylation of AMP-activated protein kinase, (d) that the relative population of gut Bacteroidetes in obese adults is reduced compared to lean individuals, and (e) that obese individuals subjected to dietary weight loss regimens increase the gut Bacteroidetes to Fimicutes ratio toward the value found in lean subjects [45–48]. Clearly, these observations should be pursued during the initial period of neonatal gut colonization and as a function of the types of milk fed to infants, the time of weaning and the nature of weaning foods in order determine if gut bacteria play any role in the differences in weight gain found in breastfed and formula-fed infants or in defining optimal weaning foods and regimens to help prevent childhood obesity.

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Discussion

Dr. Barker: Can I respond to your point about the adiposity rebound. In the Finnish data your point is correct in that the rates of increase in BMI after the adiposity rebound are the same whatever the age at the adiposity rebound, so it is true that you are fatter at 10 if you have an early adiposity rebound because you simply started earlier. It is like elephants being bigger because they grow for longer not because they grow particularly fast. The magic is why do you start early? Why does a thin 1-year-old get triggered into an early adiposity rebound? That is the magic and we know nothing about it.

Dr. Bier: I agree with that completely. I think it relates to more fuel, and the genetic background. We absolutely don't know why that happens, and I think that is a very important thing that we just don't understand.

Dr. Walker: We have all seen twins who were born strikingly different in size and weight and which seems to persist through life. You said that birthweight and twin genetic effects seem to be dominantly controlled. Is there something going on in utero that has affected the long-term difference in the size and height of these twins?

Dr. Bier: First a lot of twins that are born discordant are dizygotic, not monozygotic twins, so it is a different category. There are clear examples in which a twin has been affected in some way, presumably by something in the intrauterine environment, that is different than the other twin, and they maintain some of those differences. I am not sure that this is different from when, for example, twins are reared within supposedly the same extrauterine environment; we think it is the same environment but it is different. We talk a lot about the fact that the family and the environment need to be the same, but there is a significant body of data showing that the unshared environment of that individual child has a direct effect, and I think it may be the same in utero, I just don't know.

Dr. Walker: Gordon et al. did studies looking at the nature of the bacterial flora in individuals who are overweight and obese and those who are slim, which show that they might represent different microflora. There may not be just an adverse effect, an increasing weight, but there may be microflora that control a decrease or maintenance of normal weight.

Dr. Bier: The flora distribution is related not only to the weight of Ob/Ob mice compared to lean mice but obese adults compared to lean adults. The gut flora change with restricted carbohydrate or fat diets. What has also been demonstrated using molecular methods, which have not really been applied much to newborn colonization to any great degree, is that the colonization is actually remarkably stable within an individual until the diet changes or their weight is reduced, and then it changes. This has clear implications for types of formula feeding, the introduction of solid foods, and all those things that we are talking about in early infancy.

Dr. Ogra: Do you have any opinion as to what comes first, the altered microflora or the metabolic changes? Is it possible that acquisition of the altered flora is the result of the metabolic changes that have taken place in the host?

Dr. Bier: Actually Gordon et al. have now published a series of studies in *PNAS* and *Science*, and I think these are things that people who are interested should look at because they have done all the controls. They have introduced the flora to germfree animals and shown the changes as a result of that; they have taken animals which were genetic knockouts of the fat-induced adipocyte factor and showed that they don't respond to the change in bacteria. They are really elegant experiments.

Dr. Ogra: The second question relates to the memory these cells maintain for genetic information. Are there any data on programmed cell death being altered or mediated through changes in nutrition or by triglycerides, for example?

Dr. Bier: There are certainly some data on change in programmed cell death and its consequences; whether they are nutritional or not, I am not entirely sure about that.

Dr. K. Bergmann: You said that you don't know anything about what could influence the adiposity rebound, that it just occurs. In our study on the effect of breastfeeding on the emergence of overweight and obesity, the main finding was that breastfeeding postponed the adiposity rebound. Perhaps something could be done about it.

Dr. Bier: My simplistic view of the world is that you eat or you don't eat, and this could be entirely due to a difference in energy intake. We have virtually no way of telling the energy intake of free-living human beings. We can tell energy expenditure but all the data on intake are highly variable and flawed. Again I would say this could merely be the result of a small difference in energy intake in infants who are being formula-fed and getting the extra ounce in the bottle versus the breast-fed infant who stops feeding. No one has talked about this but I think one of the major benefits of breastfeeding is that it allows the infant to turn off the feeding.

Dr. K. Bergmann: In our observation there weren't any differences until the start of the rebound and that was much later than the weaning period. So it should have programmed something in the infants to perhaps not take as much milk.

Dr. Giovannini: What is your opinion on how long the programming effect of nutrition persists in later infancy? When would you suggest starting a diet controlled for lipid intake?

Dr. Bier: As far as the length of programming is concerned, I think that there are mouse data but there are no corresponding human critical periods, and we need to find a way to get those. I think one of the lessons of history in pediatrics and developmental biology has been that your are developing throughout your life. We have now very clearly seen neurologic changes in development in teenagers and things that we never thought would exist by the science we knew before. My guess is there are developmental windows all along. The ones that deal with feeding I am concerned about how these are changing permanent appetite/satiety mechanisms and whether they are related to special things in breast milk, whether they are related to the amount of food you are getting, whether there are feedback signals from the changes in adipose amount and distribution that you get with different kinds of feeding. We just need to understand that. Fat is 50% of the energy expenditure from the start, in the breast milk or formula feeding. So the type of fat may become very important. There are a significant number of studies showing that after the infant is weaned the actual fat content of the diet, as a fraction of calories, drops for a period of between 6 months and maybe 1.5 or 2 years, and that is the introduction of the low energy dense non-fat foods. We don't know whether this is good or bad.

Dr. K. Bergmann: You said there was no correlation between birth length and adult height. But the interesting thing is that there is channeling of birth height to adult size like McCance and Widdowson showed in their very early studies on channeling in the Bundy population in Great Britain. Those who were shorter at birth remained shorter until adult life.

Dr. Bier: The channeling is stronger and stronger the older you get, and at the beginning there are certainly changes in centiles in some fractions of the population.

Dr. Cameron: The change in correlation is around at 0.2 at birth to about 0.7 or so by 4–5 years of age. It was always said years ago that that was because the infant was recovering from the constraints upon growth because of the size of the mother. But over the first 2 years it is maintained that there is a process of growth assortment occurring which we see in the growth charts of the child changing in terms of their growth canal. I am not sure what you mean by channeling from birth to adulthood. Certainly channeling or canalization from about 3 years of age upwards but certainly not between birth and upwards.

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Effects of Early Environment on Mucosal Immunologic Homeostasis, Subsequent Immune Responses and Disease Outcome

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Abstract

During the neonatal period, the mammalian host is exposed through mucosal surfaces for the first time to a plethora of environmental macromolecules and microbial agents. The neonatal mucosa is endowed with all major elements of innate and adaptive immunologic repertoire. Rudimentary Peyer's patches and mucosal lymphoid follicles expressing HLA-DR+ and CD4+ cells can be observed as early as 10–11 weeks of gestation. CD5+ and IgA+ B cells can be detected in Peyer's patches by 16–18 weeks. CD7+ CD3+ T lymphocytes have been observed in fetal Peyer's patches, epithelial surfaces as well as in the lamina propria. Interestingly, however, the early neonatal period is also characterized by a relative deficiency in antigen-presenting cell functions, altered cell-mediated immune responses, and a relative increase in apoptosis and eosinophilic responses. After birth, each human being may be colonized by over 100 trillion bacteria, representing over 500 bacterial species. The ratio of bacterial to human cells in a normal adult may exceed 10:1. The nature and the species of microflora acquired in the first few months of life is determined by many factors including, external environmental microflora, introduction of cow's milk, use of antibiotics and immunomodulatory agents, and use of breastfeeding. Recent Investigations have shown that the nature of mucosal microflora acquired in early infancy determines the outcome of mucosal inflammation and the subsequent development of mucosal disease, autoimmunity and allergic disorders later in life. It appears that altered mucosal microflora in early childhood alters signaling reactions which determine

This review is dedicated to Mrs. Judith Maurino, a remarkable person, our friend and administrative assistant for many years. She has been instrumental in putting together almost every published contribution to date from this division. She has typed over 800 manuscripts for us for the past four decades.

T cell differentiation and/or the induction of tolerance. Reduced Th1 and increased Th2 cytokine expression in the respiratory tract associated with increased allergic disease has been correlated with reduced exposure to microbial agents associated with Th1 responses. In contrast, reduced exposure to helminthes in the gut associated with reduced Th2 expression appears to correlate well with dominant Th1 cytokine expression and inflammatory bowel disease. These observations suggest that the nature of interaction between the external environment and the mucosal tissues in the early neonatal period and infancy may be critical in directing and controlling the expression of disease-specific responses in later life.

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Introduction

In his fascinating science fiction novel 'Prey', Michael Crichton [1] proposes a very provocative view of distributed systems, past learning and external environment, and their impact on the course of biologic evolution. He states,

'The first life shows up four billion years ago as single-cell creatures. Nothing changes for the next two billion years. Then nuclei appear in the cells. Things start to pick up. Only a few hundred million years later, multicellular organisms. A few hundred million years after that, explosive diversity of life. And more diversity. By a couple of hundred million years ago there are large plants and animals, complex creatures, dinosaurs. In all this, man's a latecomer: four million years ago, upright apes. Two million years ago, early human ancestors. Thirty-five thousand years ago, cave paintings.

The acceleration was dramatic. If you compressed the history of life on earth into twenty-four hours, then multicellular organisms appeared in the last twelve hours, dinosaurs in the last hour, the earliest men in the last forty seconds, and modern men less than one second ago.

It had taken two billion years for primitive cells to incorporate a nucleus, the first step toward complexity. But it had taken on 200 million years – one-tenth of the time – to evolve multicellular animals. And it took only four million years to go from small-brained apes with crude bone tools to modern man and genetic engineering. That was how fast the pace had increased'.

Although the scientific accuracy of the temporal events proposed in this science fiction setting remains to be determined, it is important to recognize that evolution of mammalian immunologic functions has also been repeatedly shaped by past learning and external environmental conditions in a manner similar to evolution of life itself. The greatest impact has been on the evolution of innate and adaptive immunologic defenses, acquisition of a symbiotic relationship with highly selected microbial flora in mucosal surfaces, and on a balanced interaction between the host and the external microbial environment. The outcome of such interactions has ultimately determined the health and the survival of the species. During the past few centuries, these biologic interactive

processes have been drastically influenced by the evolution of human societal culture associated with continuing introduction of numerous man-made modalities designed to improve human life and living conditions on earth.

This review will attempt to relate microbiologic, environmental and host mucosal immunologic factors to the mechanisms of prevention and control of human diseases, as well as to the possible evolution of several newly acquired diseases in man, with well-defined or possible immunologic basis.

Host Development: Mucosal Defenses

Over the past several million years of mammalian evolution, complex mechanisms of systemic and mucosal defenses have evolved. These include mucosal surfaces and cutaneous tissues and their barrier elements, innate immune functions, and adaptive immunity.

The nonspecific mucosal barriers consist of intact mucosal surfaces, gastrointestinal digestive enzymes, mucin, glycoproteins and several other mucosal repair and protective peptides such as, the trefoil factors, paneth cells, and defensins [2–5].

Innate Immunity

The major effector mechanisms of innate immunity consist of pathogen recognition receptors (PRRs), designed specifically to recognize unique pathogen-associated molecular patterns (PAMPs). Other effector mechanisms include, several antimicrobial peptides, phyocytes, dendritic cells (DCs), and alternate pathway complement products. It is believed that innate immunologic mechanisms have appeared long before the development of specific adaptive immunity and some form of innate immunity may exist in all multicellular organisms [6, 7].

Innate immune recognition appears to be mediated by germ-line-encoded receptors, in which the specificity of each receptor is genetically predetermined. The recognition receptors appear to have evolved by natural selection with defined, albeit limited specificity for infectious microorganisms. It has been proposed that unlike adaptive immunity, the PRR in innate immunity recognize limited, but highly conserved antigens or antigenic determinants (PAMPs) present in many organisms [8]. These include bacterial lipopolysaccharide (LPS), peptidoglycan, mannans, microbial DNA, δsRNA, and other microbial determinants. It is estimated that the total number of PRRs in the innate immune system is very small and limited at the most to several hundred ($10^{2–3}$). On the other hand, the number of different somatically generated immunoglobulin and T and B cell receptors in fully developed adaptive immune responses is estimated to be in excess of 10^{14} and 10^{18}, respectively [9].

Table 1. Pathogen recognition receptors (PRRs) and innate immunity

Receptor	Ligand	Function
Secreted		
Mannan-binding lectin	Terminal mannose residues	Lectin pathway activation of complement
Lipopolysaccharide (LPS)-binding protein	LPS	LPS recognition[1]
C-reactive protein	Microbial phosphocholine	Opsonization, complement activation
Serum amyloid protein		(classical pathway)
Endocytic		
Macrophage-mannose receptor	Terminal mannose residues	Phagocytosis
Macrophage scavenger receptor	LPS, dsRNA, low density lipoprotein	Lipid homeostasis
Macrophage receptor with collagenous structures (MARCO)	Bacterial cell wall	Phagocytosis
Signaling		
Toll-like receptors (TLR)	Many microbial determinants	Many
dsRNA-activated protein kinase	dsRNA	Activation of NF-κB
CD14	LPS, peptidoglycan, monocyte, macrophage, PMN	Co-receptor for TLR[1]
Nucleotide-binding oligomerization domains (NOD) receptor	Most NOD proteins, LPS	NF-κB-activated apoptosis
RP 105 (CD180, LY78, MD1)	Many microbial molecules	B cell recognition and signaling + LPS[1] responses
MD2 (LY-96)	(mature B cells, dendritic cells)	LPS[1] signaling response

[1]Required for LPS recognition by TLR-4.

Pathogen Recognition Receptors

Important pathogen recognition-bearing structures identified to date are described in table 1. Such receptors are expressed on a variety of mucosal and other body tissues, including macrophages, DCs, and B cells. These receptors belong to several distinct protein families and function quite independently.

Secreted receptors include mannan-binding lectins, LPS-bearing proteins, C-reactive protein and serum amyloid protein. These receptors possess strong opsonic activity, and function by attaching to the microbial cell wall

and priming the cells for subsequent interaction with phagocytes, complement components, and eventual cytolysis of the target cell.

Endocytic receptors are detected on the phagocyte surface, and mediate the uptake and delivery of microbial agents to the lysosomes for eventual cell lysis. The microbial proteins or peptides can also be presented by a major histocompatibility complex (MHC) molecule present on the macrophage surface. This class of PRR includes the macrophage-mannan receptor, macrophage receptor with collagenous structures, and macrophage scavenger receptors (table 1).

Signaling receptors are able to recognize a variety of PAMPs and other antigens, and activate the signal transduction pathways necessary for expression of immune response genes and a variety of cytokines [9]. The signaling PRRs include double-stranded ribonucleic acid-activated protein kinase, nucleotide-binding oligomerization domain receptors (NODs), CD14 and other receptors expressed on several host cells which bind to microbial antigens. These include RP105 and MD2 (table 1). One group of signaling PRRs which has recently attracted considerable attention is the family of toll-like receptors (TLRs).

TLRs were first isolated as a component of signaling pathways responsible for dorsoventral polarity in fly embryos [10]. The toll gene encodes a transmembrane protein with a large extracellular domain of leucine-rich repeats. The cytoplasmic domain of TLR protein is similar to the cytoplasmic domain of the mammalian interleukin-1 (IL-1) receptor. Several toll receptors have been shown to activate transcription genes of the NF-κB, the immune response genes, and other immune and inflammatory cytokine activation genes [11]. The regulation of gene expression especially for the cytokines has been shown to occur through several adapter molecules, including myeloid differentiation priming response protein 88 (MyD88), Toll/IL-1 receptor domain-containing adapter protein, mal and TLR domain containing the adapter-inducing IFN-β [12].

TLRs which are homologous to drosophila toll receptors have also been demonstrated in mammals, including man. To date, over 10 (TLR-1–10) TLRs have been identified in humans, and about 13 in murine cells. Most TLRs initiate signaling by homodimerization, although TLR-2 also forms a heterodimer with TLR-1 or TLR-6 to initiate signaling [13, 14].

Activation of the NF-κB pathway by TLR has been associated with production of such inflammatory cytokines as IL-1, IL-6, IL-8, TNF-α, IL-12, and induction of important co-stimulatory molecules such as CD80, CD86, and CD40. Furthermore adapter molecules, including MyD88, have been shown to trigger apoptosis through caspase cascade.

The specific microbial ligands associated with and the functions observed for different members of the TLR family are summarized in table 2. The expression of TLR on human cells is widespread. All TLRs, except possibly TLR-3, are uniformly expressed on monocytes and macrophages. Myeloid DCs have been shown to express TLR-1, 2, 4, 5, 7 and 8, and plasmacytoid

Table 2. Functional characteristics of toll-like receptors (TLRs) identified to date

TLR	Ligands	Possible functions and interaction
TLR-1/LR-2 (heterodimer)	Triacyl lipopeptide, gram positive bacteria mycobacteria, Neisseria porins, *B. burgdorferi*	Regulates TLR-2 response
TLR-2/TLR-6 (Heterodimer)	Diacyl lipopeptide	Microbial lipoprotein, peptidoglycan, CD14-dependent or independent responses, NF-κB activation
TLR-3	dsRNA	NF-κB activation, induction of IFN-α
TLR-4	Bacterial LPS, RSV (F protein), Chlamydia, HSP60, extracellular matrix	Microbial LPS, CD14-dependent LPS responses, NF-κB activation
TLR-5	Flagellin	Bacterial flagellin, NF-κB, response to salmonella
TLR-6	Same as TLR-2	Microbial lipoprotein similar to TLR-1
TLR-7	dsRNA, imidazoquinolines	Interacts with small antiviral compounds (imiquimod/resiquimod)
TLR-8	Same as TLR-7	Interacts with ssRNA
TLR-9	CpG DNA	Receptor for CpG bacterial DNA, weakly similar to TLR-3
TLR-10	Not known	Homodimer or heterodimer with TLR-1 and 2
TLR-11	Not known	Recognition of protozoan pathogens
TLR-12	Not known	Not known
TLR-13	Not known	Not known

CpG = Cytosine guanine dinucleotide; ds = double-stranded ribonucleic acid; RSV = respiratory syncytial virus; HSP = heat-shock protein.

DCs and B cells selectively express TLR-3, 7, 9 and possibly 10. TLR-2, 4, 6 and 8 are frequently associated with mast cells. Recent studies have also suggested the expression of TLRs in epithelial cells on different mucosal surfaces [15, 16].

Although the precise role of TLRs in innate and adaptive immune functions is still evolving, it is clear that TLR activation is associated with increased antimicrobial activity, apoptosis of phagocytic cells, and increased expression of co-stimulatory molecules necessary for the induction of proinflammatory or immunoregulatory cellular functions.

The possible role of TLRs in the mechanisms of protection against or the pathogenesis and outcome of infectious diseases is currently under careful investigation. The precise contribution of different TLRs in the development of immunologically mediated disorders remains to be determined. However,

many TLRs have been linked to the development of such disease states as arteriosclerosis (TLR-1/2, TLR-4), allergy (TLR-4), HIV infection (TLR-2), IL-1 receptor kinase 4, deficiency involving signaling for several TLRs (TLR-2/1, 2/6, 5, 7, 8, 9), defects in NF-κB essential modulation associated with incontinentia pigmenti in females and 1-κB defects associated with partial blockage of NF-κB-signaling processes. Furthermore, many single nucleotide polymorphisms have been identified in some TLR genes. Polymorphism in CD14 and TLR-2 has been associated with the severity of atopic disease. However, other studies on the association of TLRs with various disease states have provided conflicting results [12, 17, 18].

Cellular Components of Innate Immunity: Dendritic Cells

In addition to the interaction with PRRs, a major signal expressed after exposure to microbial pathogens by the innate immune system includes the activation of the adaptive immune system, mostly via the regulation of the function of antigen-presenting cells. It has been shown that CD80 and CD86 molecules on the surface of antigen-presenting cells represent vital co-stimulatory molecules. These molecules in association with the MHC-peptide complex are essential for T cell activation, and T cell interaction with an antigen in the absence of CD80 or CD86 leads to apoptosis or inactivation of the cells. The induction of CD80 and CD86 molecules on antigen-presenting cells is controlled by the TLRs when exposed to PAMPs on specific pathogens [19]. However, it is important to note that TLR induces expression of CD80 and CD86 only after natural or acquired infections.

The DCs represent the key antigen-presenting cell and serve as a major link between innate and adaptive immune responses. As pointed out earlier, these cells recognize antigens by specific expression of PRRs that bind to different PAMPs. After antigen uptake, the DCs mature into different subsets with distinct biologic functions. Lymphoid DCs (CD8+) are often tolerogenic and myeloid DCs (CD38+) are by and large immunogenic. Mucosal DCs have now emerged as critical cells which are very important in regulating immunity to pathogens, development of mucosal inflammation and disease, and induction of mucosal (oral) tolerance. Aggregates of DCs are widely distributed in different mucosal surfaces, including lymphoid follicles, Peyer's patches, regional lymph nodes, cryptopatches, and the entire lamina propria of the small and large intestine. The lamina propria DCs are characterized by the expression of CX3 CR1 (receptor for fractalkine), CD83, CD11, and possibly other cell differentiation markers, depending on the level of functional maturity. The DC subsets can be defined by the expression of different TLRs. Isolated human plasmacytoid DCs express TLR-7 and TLR-9, whereas myeloid DCs express TLR-1, 2, 3, 4, 6, 8 [20, 21].

Mucosal DCs continually sample the environmental macromolecules, microbial antigens and other dietary antigens. Lamina propria DCs prevent dissemination of antigens to deeper tissues, and instead transport them to the

regional (mesenteric) lymph nodes to induce specific mucosal IgA response by the B cells. In addition to their activation by PAMP–PRR interactions, maturation of DCs may also be driven by stimulation via proinflammatory mediators such as TNF-α, IL-1, interferons, which are frequently released after bacterial or viral infections.

Mucosal DCs have also been shown to express c-type lectins and mannose receptors as PRRs, in addition to the TLRs. It has been shown that DCs express nucleotide-binding oligomerization domains (NOD-1, NOD-2) during antigen processing. These peptides recognize muramyl tripeptide (NOD-1), from gram-negative organisms and muramyl dipeptides (NOD-2) common to all peptidoglycans of bacterial species [22].

Based on the information obtained to date, DCs in mucosal surfaces may have several functions. The major defenses appear to be related to the production of IL-12, and type I interferons which influence the polarization of T helper cells (Th1 CD4+), the development of cytotoxic T cells, induction of antibody responses and memory cells, or the development of tolerance. The DCs possess remarkable ability for dendrite formation and antigen sampling, and migration to defined sites, in particular the T cell sites in the lymphoid organs, and sites such as skin, lung, solid organs and different areas of mucosal epithelium and lamina propria. The migration process is significantly influenced by bacterial LPS and other PAMPs, TNF-α, IL-1, and by specific microbial parasitic, or viral, agents [23].

It appears that antigen recognition in the mucosal immune system is obligatory for induction of tolerance and, depends on CCR7-mediated cell migration. More specifically, antigen presentation by lamina propria DCs appears to be critical for induction of oral tolerance. On the other hand, it has also been demonstrated that dysregulated recognition of intestinal microflora by DCs may be a major factor for the induction of mucosal disease, such as inflammatory bowel disease in genetically susceptible individuals. Mutations of NOD-2 have been associated with Crohn's disease. More recently, animal experiments have suggested that intestinal DC function can be significantly altered by certain enteric pathogens. DC activation by *Heligmosomoides polygyrus* and expression of IL-10 was found to impair host protection against *Citrobacter rodentium* infection and the development of severe mucosal injury [24]. Thus, mucosal DCs may be the sentinel cellular element responsible for the outcome of antigenic exposure in innate as well as adaptive immune responses, on the mucosal surfaces.

Adaptive Immunity

In contrast to the innate immune system, the receptor repertoire of T and B lymphocytes is generated somatically during their development. Significantly, however, since they are not encoded in the germ line, these receptors are not

predetermined for recognition of any specific pathogens, PAMPs, or antigens. A diverse receptor repertoire is generated randomly during development. However, certain lymphoid cell populations bearing receptors for selected pathogens or other antigens are selected for clonal expansion after antigenic exposure. Such receptors are not transmitted to the next generation and as a result have to be regenerated or re-invented for every newborn infant of the species.

Because of the random nature of T and B cell repertoire development, the immune response can, under certain circumstances, be directed against otherwise self (autoantigens, neoantigens), or other benign environmental agents, resulting in the development of autoimmune or other immunologically mediated disease processes.

The ability of the host to discriminate and selectively modulate the immune response (immune response vs. tolerance) to self or environmental antigens appears to be largely a function of the mechanisms of innate and adaptive immunity operating on external mucosal surfaces, the primary port of entry for most microbial pathogens and dietary antigens in the mammalian host.

Components of Mucosal Immunity

In addition to the defined elements of innate immunity described above, mammalian mucosal surfaces possess several nonspecific, but highly effective mechanisms of defense and local repair. These include trefoil peptides produced by goblet cells. The trefoil factors also play an important role in protection against bacterial toxins and effect intestinal epithelial repair following injury. Another important product is defensin generated by the Paneth cells, specialized epithelial cells derived from the intestinal stem cells. Paneth cells secrete antimicrobial lysozymes and phospholipase A2. These cells preferentially disrupt microbial cell membranes and effect cell death [2].

The organized lymphoid follicles in the intestine (gut-associated lymphoid tissue) and bronchial subepithelial regions, and nasopharyngeal tonsils are considered to be the principal inductive sites of mucosal immune responses. Under certain circumstances, the appendix, peritoneal precursor lymphoid cells and rectal lymphoepithelial tissue (rectal tonsils) may also serve as inductive sites of local immune responses [2].

Recently, the crypt lamina propria of the mouse small intestine has been shown to harbor tiny lymphoid clusters endowed with cells positive for IL-7 receptor. These clusters have been referred to as cryptopatches. The lymphoid structures may represent yet another important inductive element of gut-associated lymphoid tissue [24].

The development of Peyer's patches and other follicle-associated lymphoepithelium is first observed around 10–11 weeks of gestation (table 3). It has been demonstrated that during fetal growth, progenitor lymphoid tissue-inducer cells populate the developing lymph nodes and Peyer's patches. In

Table 3. Development of Peyer's patches in human neonate

Period of life	Age	Features of Peyer's patch lymphoid tissue
Prenatal	10–11 weeks	Rudimentary patches HLA-DR+CD4+ cells
	11–16 weeks	CD8+ cells Surface IgM, IgD+B cells
	16–18 weeks	CD5+ B cell IgA+ B cells
	18–20 weeks	Appearance of B and T cell zones
	24 weeks	Visible Peyer's patches
Postnatal	24 h to 6 weeks	Formation of germinal centers after mucosal antigen exposure
Average number of patches	24 weeks gestation Birth and perinatal period	60
	12–14 years	305
	20 years	200
	90 years	100

the adult, similar lymphoid tissue inducer-like cells support the formation of gut-associated lymphoid tissue, cryptopatches and other isolated lymphoid follicles. Interestingly, these cells are also located in close proximity to mucosal DCs [25–29].

The common features of all inductive mucosal sites include an epithelial surface containing M cells overlying organized lymphoid follicles. Their ultrastructural and functional characteristics were extensively defined in the early 1970s. The mucosal epithelium is a unique structure and in addition to M cells, it contains mucin-producing glandular cells, lymphocytes and plasma cells, DCs and macrophages. The mucosal epithelial cells express polymeric immunoglobulin receptor (PigR) and secretory component (SC), MHC class I and II molecules, other adhesion molecules, and a variety of cytokines and chemokines.

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 the transport, 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 (S-IgA) and other mucosal specific responses [2].

The luminal appearance of S-IgA in mucosal secretions results from transcytosis of polymeric IgA (pIgA) across the mucosal epithelium via binding to PigR. The receptor is eventually cleaved resulting in the association of pIgA with a substantial part of PigR. 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, DCs, and B cells especially of the IgA isotype. The interaction of lymphocytes with mucosal epithelium is important in the differentiation of some segments of the mucosal epithelium into M cells. The activation of T cells results in the release of a number of cytokines or chemokines from different T cell subsets, and recognition of plasma cells. Such differentiation involves interaction with a variety of cytokines and T cell subsets. The switch of IgM B cells to the production of IgA can also occur without T cell help.

Locally produced IgA consists mainly of J chain-containing dimers and the larger pIgA that is selectively transported through epithelial cells by the PigR. The resulting S-IgA molecules are designed to participate in immune exclusion and other immunologic functions at the mucosal surface. IgG also contributes to such surface defense. It often reaches the secretions by passive diffusion from the blood stream and, less frequently, by local synthesis. However, its proinflammatory properties render IgG antibodies of potential immunopathologic 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 defenses. 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 upregulate adhesion molecules on endothelial and epithelial cells, thereby enhancing accumulation of eosinophils and, in addition, resulting in aberrant immune regulation within the epithelium. 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 hyporesponsiveness to dietary antigens in the gastrointestinal tract. Numerous cytokines and chemokines have been shown to be intimately involved in the induction and maintenance of antigenic epitopes involving MHC class I or II molecules. 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. S-IgA antibody response is also enhanced by immunologic adjuvants such as cholera toxin which results in polarized Th2 cell response. S-IgA antibody response may also be induced through Th1 cytokines (IL-2, IFN-γ) as shown with studies on intracellular pathogens such as salmonella.

It appears that the process of isotype switching of B cells to pIgA-producing plasma cells begins in mucosal inductive sites. Such switching requires specific signals by co-stimulatory molecules including cytokines

and T helper cells. However, Th1 and Th2 type cytokines appear to contribute only minimally to the switching of B cells to surface IgA-positive B cells. Such switching is greatly enhanced by transforming growth factor (TGF)-α. 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-sensitized 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 activated lymphoid cells. Oral (intestinal) mucosal exposure to antigen seems to favor expression of α4β7 integrins, and intranasal immunization has been shown to induce expression of L-selectin as well as α4β7 integrins. However, systemic immunization is generally restricted to the expression of L-selectin. This information has been reviewed in more detail elsewhere [2].

Recent studies have provided extensive characterization of the T cell in the human neonatal mucosal tissues especially in the intestine. Peyer's patch T cells are largely CD4+, while most intraepithelial lymphocytes are CD8+, derived from CD7, CD3+ cells. The lamina propria T lymphocytes are also largely derived from CD7CD2+ cells, and over 50% of these cells are CD4+, although up to 20 and 30% of cells are CD8+ and CD4− CD8− cells, respectively [30–32] as shown in table 4 [32].

S-IgA can be detected as early as 1 week after birth and significant salivary IgA levels are detected by 4–6 weeks. However, the levels continue to rise up to 18 months of age. During the first year of life, there is also a switch from monomeric to polymeric S-IgA. At birth S-IgA subclass 1 predominates but the levels of S-IgA subclass 2 predominate by 6 months of age [33–35].

The diversity of interactions of the Fc region by the IgA molecule with specific receptors provide S-IgA with many unique functional attributes. Available evidence for the role of different IgA receptors in immunologic homeostasis is summarized in table 5. The epithelial PigR on mucosal epithelial cells transports pIgA to the mucosal surface, where in complex with the SC, immunoglobulin A (S-IgA) contributes to the exclusion of the multitude of dietary, environmental, and microbial antigens. IgA-mediated exclusion forms a part of the initial defense against infection. It also spares the systemic immune system from potentially deleterious responses to innocuous antigens which can otherwise culminate in disease. Other IgA receptors may contribute to protective immunity and prevention of disease. FcaRI is the principal myeloid IgA receptor and is responsible for effector responses such as respiratory burst, degranulation, and phagocytosis by granulocytes, monocytes, and macrophages. Furthermore, an IgA receptor specific for the SC elicits powerful effector responses from eosinophils. On DCs, FcaRI participates

Table 4. Distribution of T cells in human fetal intestine

Tissue site	Cell type		Relative proportion	
Peyer's patch lymphocytes	CD7+CD3+ αβ TCR+	>90%	CD4+ 90%; CD8+	10%
Intraepithelial lymphocytes	CD7+CD3+	99%	CD7+CD3+ CD8+	50%
			CD7+CD3+ CD4−CD8−	50%
			αβ TCR	70%
			γσ TCR	70%
Lamina propria lymphocyte	CD7+CD3+	80%	CD7+CD3+ CD4+	50%
			CD7+CD3+ CD8+	20%
			CD7+CD3+ CD4−CD8−	30%
			αβ TCR	95%
			γσ TCR	5%
	CD7+CD3−		CD7+CD3− CD4−CD8−	50%
			CD7+CD3− CD8+	50%

Table 5. Distribution of IgA associated receptors in mammalian cells

Receptor	Location	Function
Polymeric IgR	Mucosal epithelial cells	Complex with SC formation of SIgA: immune exclusion
Fc-aRI	Myeloid cells	Oxidative burst degranulation, phagocytosis
	Dendritic cells	Antigen presentation
IgA receptor	M cells	Transmucosal antigen transport
IgA/IgD receptor	T cells	Autoimmune regulation
Multiple receptors	Not known	IgA nephropathy

in antigen presentation, while on M cells another IgA receptor may function in the transport of antigens across the mucosal epithelial barrier. The expression of a still to be characterized IgA1/IgD receptor on T cells may affect the development of autoimmune disorders. The interplay of several different IgA receptors has been shown to affect immune complex deposition in IgA nephropathy [36].

Regulation of Mucosal Immune Responses

The outcome of host–pathogen–other environmental macromolecular interactions are determined by the elaboration of many potent cellular and soluble products in the host. These include proinflammatory or immunoregulatory cytokines or chemokines, specific antibodies, the development of various T cell activation processes, and natural killer (NK) cell expression. Such responses, either alone or in well-orchestrated mechanisms, cause destruction of specific microorganisms, or infected cells, tumors or autoreactive cells. An important byproduct of such events may be the expression of severe injury or death of host cells as well. As a result, a complex process of regulation of the immune response has evolved. This includes, NK cells (NKT), naturally occurring CD25+CD4+, inducible (adaptive) CD5+CD4+ T helper cells, CD8+ T suppressor cells, T cell receptor (TCR)-specific anti-idiotypic cells, and anti-ergotypic TCR-nonspecific T cell populations. The CD25+ CD4+ T cells are apparent by 13–14 weeks of gestation in the human fetus and represent about 5–10% of CD4+ T cells at birth, a proportion similar and slightly more frequent than in the adults [37–39]. Considerable attention has focused on four subpopulations of regulatory T cells, CD4+ helper T cells (Th1, Th2, Th3), and Th17/ThIL-17 cell population [40, 41].

Although the immunologic repertoire for regulatory functions in the human neonate is competent and responds effectively to microbial pathogens and other environmental antigens, certain differences are characteristic of neonatal T cell function. These include, a higher overall T cell number including CD4 and CD8+ cells than observed in the adult. Most neonatal T cells are naive in phenotype and function, over 90% of the cells are CDRA45+. Most neonatal T cells also possess high activation threshold and co-stimulation dependence for IL-2 production. Neonates exhibit lower production of IL-4 and IFN-γ, and impaired initial expression of CD40. However, these parameters return to normal adult values after activation-induced proliferation [42].

There are also subtle but significant differences in antigen-specific T cell responses in the neonatal period [42]. These include delayed skin reactivity to *Mycobacterium tuberculosis*, delayed appearance of CD4+ response after perinatal infections with herpes simplex virus or cytomegalovirus. The T cell-independent B cell antibody responses are generally absent at birth and fully mature T cell-independent B cell responses occur by 3–5 years of age. On the other hand, a T cell-dependent B cell response can be detected shortly after birth to most protein antigens (table 6). Detailed information about neonatal immune responses is provided in other presentations given here. It is, however, important to note that the delayed maturation of T cell function in the neonatal period preferentially appears to affect Th1 responses. The precise mechanisms underlying this physiologic delay remains to be determined. It has been proposed that deficient induction of co-stimulatory molecules and Th1/inducer cytokines, alone or in concert with intrinsic T cell dysfunction may be largely responsible for these observations [42].

Table 6. Functional characteristics of B lymphocyte response in the newborn period and early childhood

Age	T cell-dependent B cell responses	T cell-independent B cell responses
Birth	B cell receptor diversity Priming for B cell memory to proteins	Absent
2 months	Expression of effective B cell responses to most antigens	
9–18 months	Maturity of B cell differentiation and homing patterns	Minimal or no B cell response to polysaccharide antigen
6–24 months	Effective B cell response most protein antigens[1]	
4–6 years	–	Fully mature B cell responses, acquisition of marginal zone B cells in lymph node

[1]Except measles.

It has been proposed that Th1 maturational delays may be genetically determined and significantly influenced by the environment. Temporal studies on neonatal immunocompetence carried out by Holt et al. [43–45] have suggested that non-atopic infants will under normal physiologic conditions exhibit increasing Th1 response as the child grows. However, atopic subjects conspicuously fail to exhibit such improvements in the Th1 response. Conversely, it appears that over time the Th2 responses decrease in normal non-atopic subjects, but the atopic subjects will continue to exhibit increasing Th2 responses [43–45].

External Environment: Impact on Immunologic Homeostasis

Geophagy: Eating Dirt

The relationship between the external environment and human health and disease must be as old as the evolutionary biology of mammalian life itself. In a fascinating report, Callahan [46] describes an interesting ritual of eating dirt from the earth at the conclusion of daily church services in a shrine in Esquipales, Guatemala; as well as in the Chapel of El Santuario de chomayo in the hills of Northern New Mexico.

Eating dirt is a common practice in virtually every mammalian species including primates [47]. What is, however, amazing is that geophagy is also

intentionally practiced by many human groups at different ages and in different parts of the world. Geophagy is considered normal, without any major adverse and sometimes even with some beneficial effects in most animals. On the other hand, this behavior is considered by many to be abnormal for humans, and referred to as soil pica. It has been reported that non-pathologic dirt eating is not an uncommon practice by pregnant women in sub-Saharan Africa, migrants from this culture in other parts of the world and by young children during their formative years worldwide. From a contemporary behavioral standpoint, it has been proposed that consumption of >50 g of soil/day should be considered a pathologic soil pica [46].

In today's human society, soils contaminated by sewage, animal wastes, industrial or human pollutants, poses considerable risk from infections, toxins, heavy metal poisoning and carcinogens. It should be taken into account that the type of soil unaffected by human waste, and other man-made environmental products enjoyed by our ancestors must have been quite different than the top crust of earth's soil today.

Since recorded history, it has been almost impossible to keep children away from dirt. The EPA estimates suggest that children in the USA consume on an average 200–800 mg of dirt/day and some children may in fact consume more [46]. Most common infections associated with soil pica are *Toxocara canis*, raccoon round worm, and ascaris infection [48]. However, all parasitic agents that infest soil do not uniformly infect humans who eat dirt, nor do all subjects who eat dirt contract disease routinely [46]. It has been estimated that there are about 4,600 species of prokaryotic microorganisms per gram of natural soil, and about 7,000 g of biomass per cubic meter of soil [49, 50].

Biomass and Microbial Flora of Mucosal Surfaces

Since the evolution of non-nucleated single cell life forms over 4 billion years ago, most microorganisms, especially the bacteria, have continued to develop a complex relationship with other life forms including man and other mammals. It has been estimated that more than 10^{29} bacteria live on the planet and as many as 10^{14} (or over 100 trillion), comprised of over 500 species of microorganisms live in or on each human being [46, 51]. This relationship begins shortly after birth and continues throughout a person's life span. At the same time, it is estimated that a fully developed human contains about 10 trillion 'human' cells derived from the original fertilized egg. Thus, we must accept the sobering fact that for every human cell, there may be as many as 10 or more bacterial cells living in constant symbiosis with the human cells in the human body [52]. In the human gut alone, the total weight of microflora is estimated to be 1 kg. Thus, the collective genome of our colonizing bacterial commensals must be incorporated in the comprehensive view of health and disease of all human and other mammalian life forms.

Microbial colonization of the human mucosal surface begins at birth after exposure to maternal microbial flora in the genital tract and gut. The neonate

is initially colonized by enterobacteria and may reach a microbial concentration of as much as 10^9/g of feces. By the beginning of the 2nd week of life bifidobacteria predominates in the breastfed infant, whereas enteric organisms remain the predominant organism in formula-fed infants [53, 54]. By 1 month of age, bifidobacteria predominates in both groups, although their proportion is about 10 times higher in breastfed infants. Furthermore formula-fed infants exhibit a more complex microflora with bacteroides, clostridia and streptococcal species contributing significantly to the resident population of organisms. The significance of the differences in the composition of mucosal microflora between breastfed and formula-fed infants are not fully understood. However, the rich diversity of immunologic products in the maternal milk, type of proteins ingested, availability of iron, presence of oligosaccharides and other products in the breastfeeds appear to play a very important role in determining the nature of microbial colonization and subsequent immune responses to environmental and dietary antigens [55].

Bacterial Flora and Outcome of Immune Response

There is now sufficient evidence to suggest that mucosal microflora directly influences the outcome of mucosal immune responses. Nonpathogenic salmonella infections in in vitro (tissue culture) settings have been shown to inhibit the development of mucosal inflammation by blocking NF-κB-induced activation of genes coding for inflammatory cytokine expression [56]. Similarly colonization by commensals such as thetaiotaomicron (a bacteroides species) in germ-free mice induces decay-accelerating factor. The decay-accelerating factor appears to inhibit cytotoxic damage from microbial activation by complement components, C-reactive protein, ductin, (a possible receptor for intestinal mucosal trefoil factors) and sprrza, a family of proline-rich proteins involved in barrier functions [57]. Finally there is strong evidence to suggest that the nature of commensal bacterial flora acquired during the neonatal and early postnatal period is necessary for the development of tolerance to dietary proteins. Development of tolerance to IgE production against ovalbumin in the intestine was found to require colonization with a single, or polymicrobial flora in the intestinal mucosa [58].

Microbial colonization also appears to affect expression of host genes that regulate postnatal maturation, nutrient uptake and metabolism, processing of xenobiotics and development of angionesis [57]. Increased expression of genes regulating carbohydrate absorption may also explain the increased need for increased caloric consumption (by over 30%) in germ-free mice to sustain body weight similar to conventional mice [58]. It is possible that similar differences in microbial flora in early infancy could affect the nutritional outcome in obese vs. lean individuals in later life. The nature of microbial colonization in different segments of human mucosal surfaces reflects very unique patterns. Studies reported by Alderberth et al. [59] have suggested that the microbial load in different body surfaces ranges from essentially no organisms in the

normal lung, to $<10^3$/ml in the stomach, about $10^{3–5}$/ml in small intestine, $10^{10–11}$/ml in large intestine (feces), and about $10^{8–10}$/ml in the nasopharynx and the lower urinary tract and the female genital tract. It appears that both microbial, as well as host-derived factors facilitate residence of different segments of human mucosal surfaces. These include inter- and intra-species communication and quorum sensing, and biofilm formations. Examples of such sophisticated arrangements between the host and microbial flora exist in other settings, such as dental plaques where the biofilm-commensal plaque is assembled through a complex process of adhesion of early arriving anaerobic streptococcal species to the dental pellicle (host-derived) on the surface of the tooth. This is subsequently followed by secondary interbacterial adhesion and intergenic communications with other organisms, with further bacterial adhesions, resulting in the formation of a distinct bacterial structure of organisms on a defined tissue site [60, 61]. In more recent studies carried out in infants delivered via cesarean section or after normal vaginal delivery, infants delivered by cesarean section were found over time to be colonized more often with klebsiella and enterobacter than infants delivered vaginally. Whereas bacteroides species colonized about 30% of vaginally delivered infants and the colonization rate increased slowly, significant colonization by such anaerobes was delayed by up to 1 year in cesarean section deliveries [62].

Immunologic Outcome of Host–Microbial Interventions

The information summarized in the preceding sections has established a framework for the impact of microbial flora and other macromolecules in the environment on host immunologic homeostasis, and of the host, on the microbial flora continuously entering the host's mucosal surfaces.

The potential impact of environmental factors on the development of T cell responses and disease association is summarized in table 7. In addition to these observations, it is felt that the prevalence of several possibly immunologically mediated diseases has drastically increased over the past century in most regions of the world as the socioeconomic conditions have changed [63, 64]. At the same time, it is felt that many tropical infectious diseases continue to benefit the host by their ability to confer protection against certain immunologically mediated diseases, while other infections may adversely contribute to the development of other allergic or immunologically mediated disorders.

Allergic Disorders: Protective Effects of Microflora

Certain environmental antigens, dietary proteins and other external or endogenous allergens are commonly associated with the development of such allergen-specific diseases as asthma, hay fever, eczema, anaphylactic shock, or allergic rhinitis. Such disease processes are thought to be initiated by the CD4+ Th2 lymphocyte subpopulation. These cells are responsible for producing

Table 7. Evolution of cytokine paradigms in the mucosal surfaces and their possible disease association

Increased Th1 cytokine profile	Increased Th2 cytokine profile
Environmental factors	
Normal commensal flora	Diet in developed world
Inflammatory cytokines	Processed foods
Breastfeeding	Introduction of cow's milk and formula products
Many infections[1]	Antibiotic use
	Infections
Disease association	
Autoimmune thyroiditis	Leishmaniasis
Experimental autoimmune uveo-retinitis	Mycobacterial infection (M. TB, M. leprae)
Crohn's disease	Candida
Other immunologically mediated disorders (EAE, MS, IDDM)	Toxoplasmosis
	HIV
	Asthma
	Atopic dermatitis
	Allergic rhinitis

EAE = Experimental allergic encephalitis; HIV = human immune deficiency virus; MS = multiple sclerosis, IDDM = insulin-dependent diabetes mellitus.

[1]Also see table 8.

IL-4, IL-5, IL-9, and IL-13. Th2 cytokines are also responsible for the regulation of IgE and other immunoglobulin-isotype production by B cell development and recruitment of eosinophils and other inflammatory cells, contractility of airway smooth muscle, and mucous production. Earlier epidemiologic studies and more recent in vivo and in vitro experiments have suggested that some infections may provide significant protection against the development of allergic diseases, despite the ubiquitous nature of environmental allergens (table 8). For example, the presence of a positive tuberculin skin test prior neonatal immunization with bacillus Calmette-Guérin (BCG) vaccine and/or infection with tuberculosis before 20 years of age are significant markers of protection against the development of allergic disorders later in life [64–67]. The anti-allergic effects of mycobacteria may be related to modulation of Th1 responses and by other Th1-independent mechanisms, involving IL-10, TGF-β and induction of CD11c cells [68].

In addition to mycobacteria, infections with cell-associated pathogens such as *Chlamydia trachomatis, Listeria monocytogenes*, and intradermal inoculation of BCG have been associated with significant improvement in the clinical scores of atopic dermatitis, but not the severity of asthma [69]. However, in one other study, replicating live BCG vaccine was found to result in a significant improvement in asthma scores in adults [70].

Table 8. Possible role of different infections in the mechanism of protection against or pathogenesis of allergic and autoimmune disorders

Microbial agents	Clinical disorders and role of infection			
	allergic		autoimmune	
	protective	pathogenic	protective	pathogenic
Bacteria				
Bordetella		+		+
Borrellia (Lyme disease)				+
Chlamydia		+		
Conventional microflora				+
Pathogen-free flora			+	
Lactobacilli, other probiotics	+		+	+
Mycobacteria, BCG	+		+	+
Salmonella	+		+	
Staphylococcus		+		
Streptococcus group A				+
Parasites				
Ascaris		+		
Fasciola		+		
Filaria			+	
Heligmosomoides	+		+	
Hookworm	+			
Hymenolepis			+	
Nippostrongyloides		+		
Schistostoma			+	
Strongyloides	+	+		
Toxicara		+		
Tricuris			+	
Viruses				
Coxsackie				+
Hepatitis A	+		+	
Influenza	+	+		
Measles				+
Metapneumovirus		+		
Rhinovirus		+		
RSV	+	+		
Rubella				+
Theiler's				+

+ = Positive association.

Considerable investigative effort with probiotics has generated interesting information about their usefulness in the control of allergic diseases. Studies of lactobacillus, bifidobacteria and other probiotics have suggested a strong correlation between maternal (prenatal use) or the use of infant-formula

containing *Lactobacillus rhamnosus* or *Bifidobacterium lactis* in the neonate and subsequent protection against atopic dermatitis [71]. However, such dietary intervention had little or no benefit in patients with established asthma or food allergies [72].

Infestations with common parasites in the tropics have also been associated with decreased incidence of allergic diseases. Infestations with hookworm, ascaris, toxicara, and schistosomes have been associated with reduced expression of atopy and of the atopic phenotypes [73, 74]. Other studies have shown that specific antiparasitic chemotherapy instituted for long-term intestinal helminths was associated with a significant increase in mite skin test reactivity in allergic subjects [75]. Recently, in vivo experimental studies in mice have shown that introduction of *Nippostrongylus brasiliensis* and *Strongyloides stercoralis* result in suppression of allergic responses in the lungs and a decline in allergen-induced eosinophilia and eotaxin production. These beneficial effects of parasitic infestations appear to be IL-10-dependent [76–79]. In addition to the bacterial and parasitic infestations listed above, epidemiologic observations have also suggested a protective role of viruses such as hepatitis A virus, respiratory syncytial virus (RSV) G protein and influenza A virus on the development of allergic diseases [80–83].

There is also evidence to suggest a strong role for bacterial–viral interactions in human mucosal surfaces, on the outcome of certain viral-induced inflammatory disease processes. Several investigators have observed protective effects of bifidobacteria, *B. bifidum* and lactobacilli on the course of murine and human rotavirus infection, gut endotoxin concentration, and human intestinal barrier functions [84–86]. Experimentally induced mucosal viral infections have also been shown to alter mucosal permeability and subsequent IgE responses to dietary antigen and other environmental allergens [87–89].

Allergic Disorders: Pathogenic Effects of Microflora

Although most infection processes in the tropical setting have been found to be protective against allergic diseases as outlined above, there is evidence to suggest that some infections may in fact facilitate or directly induce allergic disease manifestation (table 8). RSV, influenza, rhinoviruses and the recently identified metapneumovirus appear to be risk factors for the development for childhood asthma [90, 91]. Additional evidence has suggested that chlamydia and mycoplasma may also contribute to the development of childhood asthma. In other studies, *Staphylococcus aureus* and *Bordetella pertussis* have been associated with increased expression of allergic disorders by enhancement of inflammation of the skin and airways, respectively [92–95]. Many helminths have been implicated in the development of allergic disorders. These include natural or experimentally induced infestation with ascaris and toxicara [74], *Fasciola hepatica* [96], *N. brasiliensis* [74], and *Strongyloides venezuelensis* [97] (table 8).

Autoimmune Disorders: Protective Effects of Microflora

Unlike allergic diseases, autoimmune disorders are thought to result from tissue damage secondary to an abnormal immune response to infectious or infection-induced neoantigens in the host. As outlined in table 7, some infections seem to provide protection against the development of autoimmune diseases, while others may in fact contribute to their evolution in the human host and in animal models. It has been suggested that exposure to mycobacteria, salmonella, certain viruses and several helminths provide significant protection against the development of insulin-dependent diabetes mellitus in experimental murine models [63, 98–100]. Clinical studies have raised the possibility that certain infections within the first few years of life, decrease the risk of developing inflammatory bowel disease, multiple sclerosis and possibly insulin-dependent diabetes mellitus [101, 102]. Experimental studies with murine models have also provided some evidence of protection afforded by other parasitic agents. These include schistosomes in diabetes in nonobese diabetic mice [103], lactobacilli, or *Heligmosomoides polygyrus* in spontaneous colitis, or inflammatory bowel disease [104–106], *Trichuris suis* and filarial antigen E562 in Crohn's disease [107], and other autoimmune disorders [108].

Autoimmune Disorders: Pathogenic Effects of Microflora

While many parasitic infestations and several bacterial infections have been associated with varying degrees of protection against autoimmune diseases discussed above (table 8), it is interesting that some of the classic examples of autoimmune disease are those which occur shortly after certain infectious insults. These include post-infectious acute rheumatic fever, encephalitis, or glomerulonephritis after group A hemolytic streptococcal infection; infection with antibiotic-resistant Lyme arthritis; congenital rubella virus infection and development of insulin-dependent diabetes mellitus (IDDM), and chronic myocardial disease or IDDM after Coxsackie B virus infections [108–111]. Spontaneous development of experimental allergic encephalomyelitis is not seen in mice maintained in pathogen-free microflora, but is characteristic in mice kept with conventional microbial flora [108]. There is also evidence to suggest that direct intracerebral inoculation can result in the development of autoreactive T cells followed by the development of demyelinating encephalitis [112].

Microbial Mechanisms of Disease Production or Protection

Allergy

It is clear that microbial exposure under natural as well as controlled experimental situations can protect or reduce the risk of disease, or induce or

aggravate the clinical expression of allergic and autoimmune disease processes. Although the precise mechanism underlying the protective or pathogenic roles of different infections in these two disease states remains to be defined, several explanatory mechanisms have been proposed.

Currently, it is believed that increased Th2 CD4+ T cell responses to dietary proteins, environmental allergens, and other external or endogenous antigens, provoke the development of allergic diseases. Th2 CD4+ cells produce a number of cytokines and chemokines, including IL-4, IL-5, IL-9, IL-10, IL-13, and granulocyte-macrophage colony-stimulating factor, after exposure to specific peptides of different allergens presented by mucosal antigen-presenting cells. In addition, Th2 cytokines induce activation of B cells responsible for IgE, IgA and other isotype of antibody response. Such allergen-specific cellular products and antibody responses lead to many immunological events, including the recruitment and activation of eosinophils and mast cells and their degranulation, mucous production, and development of smooth muscle hyperactivity. Such Th2 effects are augmented by Th1 CD4+ T cell responses that lead to chronic inflammation. Other mechanisms involved in the development of allergic disorders appear to be associated with increased activation of IL-8, which favors recruitment of polymorphonuclear leukocytes [113] and IL-17, a potent stimulus for mucus production [114]. Other products which have been implicated in the development of allergic diseases include other chemokines, such as IL-13 [115], and other immunoregulatory cytokines [116].

The beneficial effects of infections on the outcome of allergic disease may be related to the following immunoregulatory events. (a) Increased induction of Th1 CD4+ T cell responses associated with increased IFN-γ production. Such responses have been shown to inhibit the Th2 response after infection with mycobacteria, influenza and RSV. (b) Increased production of TGF and IL-10 cytokines. Studies with certain mycobacterial species have shown that allergen-induced bronchial hyperactivity is significantly inhibited by increased IL-10 production and not by IFN-γ-producing Th1 cells [65, 117]. Similar mechanisms may exist for the effects of helminthic infestations and protection against allergy [78, 79]. (c) There is experimental evidence to suggest that certain mycobacterial infections induce the selective development of CD11c cell populations. These cells produce IL-10 and TGF-β [68].

Autoimmunity

Expression of autoimmune disorders as pointed out earlier is considered to be a reflection of immunologic response by the host to self (auto or neo) antigens, resulting in abnormal cytokine production and subsequent recruitment and activation of effector cell mechanisms and specific tissue damage. The development of infection-induced altered tissue antigens (neoantigens), exposure of otherwise hidden tissue antigens and the appearance of autoreactive T and B cells are normally seen but to a lesser extent in a healthy subject's immunologic repertoire. As mentioned earlier, infections with

mycobacteria, salmonella, several helminthic agents, and possible viral hepatitis A are strongly associated with decreased evidence of autoimmune diseases, especially for the occurrence of IDDM inflammatory bowel disease, and encephalitis. The possible mechanisms which mediate infection-induced protection against autoimmunity include the following. (a) Infections induce IL-10 and TGF-β. These cytokines may directly inhibit expression of autoimmunity. IL-10 production may also induce selective activation of Treg lymphocytes which have been shown to suppress autoimmune responses by inhibiting autoreactive T cell function largely by altering cell-to-cell contact, and by IL-10 and TGF-β production. (b) Helminthic and mycobacterial infections can induce activation of NK cells (NKT). Such cells induce cytotoxicity for autoreactive antigen-bearing cells and can thus inhibit the evolution of autoimmunity [103]. (c) Certain infectious agents, especially probiotic bacteria induce Th1 CD4+ responses and such a response can directly lead to reduced expression of autoimmunity. (d) Recently, it has also been suggested that a population of CD25+ T cells characterized by the expression of Foxp3 transcription factor may also be important in mediating protection against inflammatory bowel disease, independent of IL-10 production [105].

As pointed out earlier, while most helminthic and several bacterial infections appear to be protective against autoimmunity, many viral infections and some bacterial pathogens have been highly associated with the development of autoimmune disorders. Several explanations have been proposed to explain this association. (a) Direct cytolytic effects of some bacterial, viral and parasitic infection may lead to increased expression of infection-induced cellular self (autoreactive) neoantigens, or tissue determinants otherwise hidden in a normal setting. Such cytolytic processes can lead to increased presentation of autoantigens by DCs, macrophages and other antigen-presenting cells, resulting in autoreactive Th1, Th2, CD8 + CTL, NKT and/or B cell responses with subsequent expression of clinically manifest autoimmune disorders [64, 112]. (b) Bystander activation effects may occur during stimulation of the innate immune system by PAMPs. These include LPS, δsRNA, and bacterial lipoproteins which result in activation of the innate immunologic mechanisms with increased expression of a number of co-stimulatory molecules, cytokines and chemokines [118]. IFN-γ and IL-15 produced by such cells can result in activation or proliferation of CD8+ CD44hi+ effector or memory T cells. In addition, after antigen exposure, Th1 cells produce IFN-γ in response to IL-12 and IL-18. Human memory cells of the CD4+ phenotype have also been found to be activated by similar cytokine pools. All these events have been observed to occur independent of TCR signaling [119]. Such antigen-independent T cell activation has been proposed as a major mechanism in the evolution of tissue damage in rheumatoid arthritis [120], and possibly in other autoimmune disorders. It is possible that previously dormant, inactivated or tolerized autoreactive T or B lymphocytes are

activated by TCR-independent and immunoglobulin receptor-negative effects, which are mediated by several cytokines including IL-2, IL-12, IL-15, IL-18, IFN-γ, and IFN-β. (c) In addition to the antigen-independent activation of the T and B cell repertoire, there is evidence to suggest that pathogen-specific Th1, Th2, CD4+ T cells, B cells and CTL can also cross-react with self peptides and other autoantigens, to result in the development or aggravation of underlying autoimmune disease processes [68].

Concluding Remarks

It is evident from the information reviewed here that mammalian immunologic homeostasis is a function of a complex interplay of the external environment (consisting of a multitude of antigens, microbial organisms, other naturally acquired or induced macromolecules) with a mammalian host richly endowed with components of nonspecific barrier mechanisms, innate immune functions and components of adaptive immunity at mucosal surfaces and in different systemic tissues. The nature and the state of immunocompetence in a mature host is a reflection of continuing evolutionary developments driven to a large extent by the changing external environment. Of particular importance is the mucosal microflora and its influence on the regulation of systemic and mucosal immunologic reactivity. In contrast to the adult, the neonate exhibits certain unique immunologic characteristics including: low levels of innate immunity; reduced expression of proinflammatory cytokines; impaired neutrophil, macrophage, DC and other antigen-presenting cell function; altered cellular and T cell-dependent antibody responses, and a relative increase in eosinophils and enhanced mechanisms of apoptosis. This is particularly important because the normal neonate is delivered rather suddenly from an essentially microbiologically sterile uterine environment into the perinatal and postnatal environment loaded with a diverse spectrum of pathogenic and non-pathogenic microorganisms. Despite the immense exposure and the high potential for acquisition of and colonization by such organisms, the development of clinically apparent disease in a normal neonate is an exception rather than the rule. Nevertheless, some infections are unique to this age and may set the stage for subsequent chronic disease outcomes. One such example is the development of otitis media in early childhood. The development of infections with viruses, such as RSV, influenza and rhinoviruses appear to be important determinants of otitis in the first year of life. Such infections appear to set the stage for subsequent acute or chronic suppurative infections with *Streptococcus pneumoniae* and other bacterial pathogens. In a series of elegant investigations, it has been observed that the development of suppurative otitis media in young children up to 1 year of age was directly related to nasopharyngeal colonization with the same organisms well before the development of otitis. Increased rates of colonization in

nasopharynx with *S. pneumoniae*, *Moraxella catarrhalis* and non-typable *Haemophilus influenzae* were characteristically seen in subjects who subsequently developed suppurative otitis media with the same organism [121–123]. Other studies, reviewed in more extensive detail elsewhere at this symposium, have provided very impressive evidence to suggest increased mortality from infections later in life secondary to nutritional impairment or other environmental insults in early childhood. Such increases in mortality and morbidity may have been related to malnutrition-induced smaller thymic size, reduced TCR excision circles, a marker for thymic output of T cells, reduced thymic output, and possibly reduced IL-7 activity [124, 125]. Other investigators have suggested that impairment of antibody response to polysaccharide antigens in adult life may be related to prenatal and early childhood nutritional impairment [126]. Acquisition of normal or abnormal mucosal microflora in the early perinatal and neonatal period may be one of the most critical environmental factors underlying the later development of such immunologic abnormalities in the host.

It appears that available environmental microflora and its colonization of the mucosal surfaces is significantly influenced by the immunocompetence of the host and the level of community sanitation and personal hygiene. Such societal approaches can significantly alter the level of bacterial, viral and helminthic load required to generate balanced physiologic immune responses or expression of clinical disease. Reduced exposure to Th2-promoting helminthic infestations in the gut may account for Th1 hyperreactivity and related inflammatory bowel disease. On the other hand, reduced exposure to Th1-promoting commensal pathogens in the neonatal period and infancy, use of antibiotics, and lack of breastfeeding may result in increased Th2 responses in the respiratory tract with increased allergic diseases. Mucosal DCs and regulatory T cells may be critical in directing and controlling T cell responses in such altered states.

Recent animal experiments have shown that sustained exposure to helminths results in protection against experimentally induced colitis and other immunologically mediated diseases. Induced infestation with parasitic agents has also been employed to treat (with some success) ongoing inflammatory bowel diseases. Such infestation induces mucosal Th2 responses, with expression of IL-4, TGF-β and IL-10. Helminthic infestation also appears to induce regulatory T cells which limit effector T cell function. These effects seem to be related to the induction and functional activation of different TLRs (in particular TLR-4) in the mucosal tissues [127]. Why different TLRs are activated with different stimuli remains to be determined. However, a large number of environmental factors including the nature of microbial colonization in early life, genetic polymorphism of the TLR, and engagement of signaling intermediates during TLR activation may be related to such differential activation processes. Activation of interferon-regulating factor-3 has been shown to result in increased expression of IFN, while activation of the

NF-κB pathway results in increased expression of inflammatory cytokines and chemokines.

Based on the evidence available to date, it has been proposed that signaling through TLR preferentially favors development of Th1 (IFN-γ, α) responses necessary for protection against microbial pathogens. On the other hand, development of Th2 (IL-4, IL-5, IL-15) responses favor protection against parasitic agents. The decision to express Th1 or Th2 cytokines responses appears to be made by the host very early during the course of the immune response to an infectious agent. For example, in studies with *Mycobacterium leprae*, it has been observed that induction of the Th1 response is associated with the development of tuberculoid leprosy which is characterized by a low mycobacterial organism load and very few systemic manifestations. On the other hand, Th2 responses are typically observed in lepsomatous leprosy, which is associated with a very high mycobacterial load, and severe systemic disease [128].

The complex nature of TLR activation, its association with the type of mucosal microflora and the nature of the subsequent Th response (Th1 vs. Th2) may be the principle mechanism underlying the development of protection against or the pathogenesis of a disease process. Other important considerations which may influence the effects of infection include the temporal relationship of the infection to the development of immunologic disease; age at the time of infection; dose and severity (antigenic load) of the infection, and the possible route and sites of localization of infection [128].

Based on the information summarized in this report and in other reviews presented here, it is very likely that the interplay of commensal microflora, pathogenic microorganisms, helminths, prebiotic factors and other dietary immunomodalities during the unique immunologic window in neonates and early infancy, with the host's innate immune system, is critical in determining the subsequent nature of the adaptive immune responses, the development of protective immunity, or the induction of immunologically mediated (allergic vs. autoimmune) disease process.

These observations provide an immunologic basis to the 'hygiene hypothesis'. This concept, initially proposed by Strachan [129] in 1989, is based on careful observations of household size and local hygiene as risk factors for hay fever, and utilizes even earlier observations by Gerrard et al. [130] for serum IgE levels in two hygienic settings.

The implications of these and more recent observations are discussed in detail elsewhere at this symposium. However, it is interesting to note that during the past century, mankind has witnessed a dramatic reduction in the global burden of many infectious diseases and discernable improvements in the overall quality of life. These changes are in a large part due to the introduction of community sewage systems and sanitation, better nutrition and most significantly due to the introduction of childhood vaccines for available vaccine-preventable infectious diseases. Paradoxically, these successes have

also been accompanied by the increasing use of antibiotics and the evolution of large numbers of antibiotic-resistant organisms with severe or fatal disease outcomes in nosocomial and community-based settings, and the identification of new disease-producing agents or the emergence of infections with agents previously considered to be nonpathogenic, and the development of new immunologically mediated disease processes. These events have in turn fostered a worldwide preoccupation with extreme personal hygiene with the introduction of newer antiseptics, antimicrobials and other modalities designed to kill bacteria and other environmental microorganisms.

Regardless of the advantages and disadvantages the mammalian host has acquired over the past 3 billion years of evolutionary changes, it is difficult for man to turn the clock back and revert to the hygiene and sanitation prevalent in earlier decades when allergy and autoimmune diseases were not a major health hazard. However, it may be possible to develop newer modalities which may foster some respect for our environment and mucosal microflora and a reevaluation of the contemporary societal attitudes which have contributed to the development of abnormal or altered immunologic homeostasis.

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Discussion

Dr. Bier: You mentioned that allergic diseases are relatively recent in evolutionary history, and even though lots of new antigens have been introduced in the last several centuries, it seems to me that the great bulk of earth-like antigens have been present for vast periods of time; bacteria have been present for vast periods of evolutionary history. So my two related questions are firstly what is the evolutionary adaptive advantage of re-inventing the acquired immune system in every generation, and secondly, since does not persist, does this mean that those environmental agents like the bacteria, etc., have actually developed systems that we don't understand that prevent them from persisting?

Dr. Ogra: I don't know the real answer to the first question. It would seem that the innate immune system is the first line of defense with a rather limited repertoire for pathogen interactions, somewhere in the range of 100–1,000 receptor sites. The adaptive immune system must have evolved to provide more breadth to the process of immunologic interactions to the host in order to deal more effectively with the ever-changing exposure to a large environmental antigenic reservoir. This is supported by the fact that the receptor repertoire in the B and T lymphocytes is somewhere in the range of hundreds of billions of sites.

With regard to the second question, there is very clear specificity which has evolved through the evolutionary process for interactions between the host and the organisms. Under physiological conditions, the patterns of mucosal colonization in selected mucosal sites are highly restricted to certain species of organisms and seem to be directed both by the host as well as by microbial factors. For example, there are

proteases which are generated by certain organisms, and there are specific ligands generated by the host and designed for binding to such specific bacterial ligands. This relationship has evolved over millions of years.

It should be pointed out that no microbial agent is totally benign. For example, many probiotics are considered benign, but under certain nonphysiological conditions these 'benign' agents can be highly pathogenic and can produce very severe disease. The pathogenic nature of the microbial agents and their relationship to the host is a dynamic one, and often a compromise which favors the survival of the host as well as the microbe.

Dr. Björkstén: I agree that parasites may regulate immunity as you suggested. I will show similar data on tolerance induction in infancy from Estonia where there are no parasites. My suggestion is that the supposed effects of parasites may in fact be due to the very broad and diverse microbial spectrum that you have in Africa. Fifteen years ago Eastern Europe was in some respects Africa minus parasites. B The 'hygiene hypothesis' is a most unfortunate term because it suggests that poor hygiene would be good for your health, which nobody in his right mind would argue for. It has been suggested that vaccinations could be risk factors for the development of allergy. The fact is that vaccines are very good for what they were intended but they are neither good nor bad in relation to allergy. We studied *Bordetella pertussis* which is a well-known Th2 stimulant. There was no relationship with allergy development, except in the placebo group where unvaccinated children who caught whooping cough had an increased incidence of allergy.

Dr. Ogra: Let me respond to the issue of vaccines as a risk factor for disease. There are currently about 800 websites directed against the use of vaccines. There are many individuals who feel very strongly that we should not vaccinate any child at any time. One of the arguments put forth is that some naturally acquired infectious diseases are associated with or can cause the development of certain autoimmune processes or allergies. Such a rationale implies that using vaccines in very early childhood results in unnatural exposure to many infectious agents early in life. Such an exposure can subsequently produce autoimmune disorders or allergies later in life. However, the large number of epidemiologic studies and other experimental data available at this time strongly indicate that currently licensed vaccine antigens are not the cause of any autoimmune or allergy disease identified to date. Most vaccines are absolutely safe and not associated with any long-term deleterious effects.

Nevertheless, there are data to suggest the induction of subtle or overt immunologic alterations after immunization with some vaccines. Studies with repeated immunization with DPT have demonstrated increased IgE levels in the serum, but such increased IgE activity has not been clearly related to the development of allergic disease processes in such vaccines. Furthermore, other non-vaccine components or possible contaminants present in earlier vaccines have been associated with the development of some even serious side effects. These include alum, mercury, antibiotics, egg proteins and possibly other adventitious infectious agents and tissue culture components. However, in the currently available vaccine preparations, these potential sources of hazard have been partially or totally eliminated. It is abundantly clear that the introduction of vaccines has been the single most important success story of modern human technology. In the past century this approach has eliminated diseases such as smallpox and significantly reduced the disease burden of infections such as poliomyelitis, measles, mumps, diphtheria, tetanus, *Haemophilus influenzae* type B, and many other serious and sometimes fatal infections. The available data suggest that the increasing incidence of allergic or autoimmune disease is not a reflection of vaccine-induced control of these infections nor the direct result of vaccine products employed.

Dr. Björkstén: No, I think DPT is a good story. We developed a test and were able to show that IgE antibodies are in this product, but it is an immunological phenomenon that has nothing to do with the disease. There is only one pertussis vaccine, which is not on the market, that has a purified component and it really induced strong IgE antibodies.

Dr. Walker: This is a complex topic that has evolved very rapidly over the last few years. A lot of energy has been directed at the prevention of immune-mediated disease, and most of that has been directed towards the neonatal period. What do we know about dealing with disease once it has already expressed itself in an immunologic fashion? Are there ways that we can potentially reverse the process and turn the disease off?

Dr. Ogra: Excellent question. One approach recently employed has been to induce specific oral tolerance, even after expression of the clinical disease, employing the use of sublingual mucosal tissue sites for antigen production. Dr. Björkstén may have additional recent data to expand on this approach.

Dr. Björkstén: At least from the allergy field, various anti-IL-5 and IL-10 receptors have been tried and were largely failures because of the side effects. At that stage they had such broad ranges that it was not possible to interfere because then you are really interfering with the entire cascade. The idea is that interference must be early, and early at this stage, at least from the allergy immune responses, is probably within the first 3 years. We had a paper in *Nature Immunology* [1] on that; all these compounds may theoretically be very good very early, but they certainly are not useful at this stage in established disease.

Dr. Ogra: Some of you may recollect an elegant study done by Sulzberger and Chase several decades ago, in which they demonstrated the induction of tolerance to systemic immunization with a hapten by prior oral feeding of the hapten antigen. This is commonly referred to as the Sulzberger-Chase phenomenon, and is probably the first documentation of oral tolerance as we know it today. Whether such tolerance induction can prevent the development of disease or influence recovery from an established disease remains to be determined.

Dr. Prentice: I want to return to the question that Dr. Bier asked: what is the adaptive reason for not maintaining all those specific memory responses in the host? Dr. Hanson has always told me that it would simply be impossible to do, that it would need an absolutely enormous genome and the cost of maintaining it would be phenomenal, and even if we had a single T cell which could recognize all the possible antigens, then that would require greater than the entire body mass of our whole body. So I think evolution is a compromise. We can only maintain a few of the innate protective mechanisms and it is impossible to maintain all the memory ones.

Dr. Ogra: It seems that memory is not life long. Re-exposure to the antigen via the environment is important to keep on reinforcing the processes of down- and/or upregulation of specific immunologic activity. It is not known whether memory is a function of persistence of the antigen-reactive cells, recruitment of a new cell population after antigen re-exposure, or to persistence of the antigen itself.

Dr. Hanson: As to the remarkable evolution of the immune system as you get old: it may be that we keep what is useful, simply by being continuously exposed and boosted with the antigens around. We do away with the rest, not affording it, not needing it, doing quite well most of the time. Is that what happens?

Dr. Ogra: That's a wonderful question, and I don't know the answer. I think there is something which nature has learned over millions of years of evolutionary biology, and that is senescence. Every life form has to die eventually. We all die as a collective organism because the individual cells die. What may regulate the programmed senescence or apoptosis of the lymphoid tissue as we get old may be a function of the environment to a large extent. Dr. Bier, do you have any additional thoughts on this issue?

Dr. Bier: I have no idea because it is not my field. I would like to comment on two things, one is Dr. Prentice's remark which I think is a highly plausible approach. I would argue that there are a vast number of antigens, common things, that one could probably develop a relatively generic repertoire to deal wit. Perhaps we don't need to deal with the genome, we may need to deal with some other ways of preserving genomic memory, such as epigenetic things that are turned on and off, methylation, I don't know. It just seems to me that there are possibilities there. The other is the issue of the hygiene hypothesis, the cleaner environment. We are just in a somewhat less dirty environment, we are not in a clean environment, and that is the problem I have with that particular approach.

Dr. Wilson: As you showed on one of your early slides, evolution really doesn't seem to give a darn about anyone over about 25 – it never has. Evolution is driven by reproductive fitness and is thus focused on having people survive and be fit through their years of greatest reproductive capacity. There is one possible caveat to this notion – if post-reproductive age caregivers help to keep their grandchildren alive, there might be some minor evolutionary pressure for fitness at an older age, particularly for women. The second point relates to Dr. Bier's comment. If you look at evolution and ask how this informs your question, it is only in bony fish and higher vertebrates where one sees an adaptive (antigen-specific) immune system in its traditional sense. Pancer et al. [2] have shown that jawless fish have generated an adaptive immune system through an alternative strategy (the use of variable receptors formed from leucine-rich repeat domains rather than immunoglobulin domains as in higher vertebrates), a form of convergent evolution. Lower phyla, some members of which are long-lived, have only innate immunity, but this form of host defense seems to be sufficient for them to thrive. What then accounts for the necessity of adding a highly complex adaptive immune system, which carries with it the risk of autoimmunity and cancer, and which also requires that you need to develop and ramp-up a specific adaptive immune response each time you encounter a new type of pathogen? This issue is a matter of ongoing speculation.

Dr. Barker: I would like to comment on the hygiene hypothesis and on Dr. Bier's remark that the changes in hygiene have been really quite slight. I am guilty of inventing the term 'hygiene hypothesis' as an explanation of the epidemic of appendicitis which followed the introduction of running hot water into housing in Western countries. The reason for producing that hypothesis was as a dietary explanation for the huge rise in appendicitis, from being a nonexistent disease around 1900 to being the commonest cause of child death in rich people around 1920. There was a time when 1 in 5 people in Scotland had acute appendicitis during their lives. The reason of bringing it forward was because the dietary explanations for appendicitis had holes all over them. The changes in hygiene which followed the availability of running hot water in homes must have been quite considerable, and if they weren't then we must be very sensitive to small changes because it generated a massive epidemic which killed huge numbers of children.

Dr. Giovannini: What is your opinion on functional compounds having a major effect on human milk, especially with regard to formula with prebiotics and probiotics?

Dr. Ogra: That is an area of intense investigation at this time. I already mentioned that both Peyer's patches and cryptopatches can be activated by probiotics. Both probiotics and prebiotics are present in milk. Do they play any role in the control of allergies? Possibly, yes. We may hear more about it from Dr. Björkstén. There is already significant evidence to suggest that probiotics influence the outcome of allergic rhinitis and some gastrointestinal diseases, including infections with rotavirus. However, as I pointed out earlier, there is no specific organism which is always beneficial and safe for the host, and there is no specific agent which is always fatal to that species. Under

normal evolutionary adaptation, all hosts and their adapted microbial flora must live together in some symbiotic compromise. However, if the ecosystem changes rapidly and the host–pathogen balance is lost, organisms which are normally non-pathogens will re-acquire virulence and the potential to produce disease. The same concepts may hold true for today's 'probiotics'. It would seem prudent to exercise caution and not to flood the mucosal ecosystem and replace the flora with any probiotics exclusively, at the cost of other existing normal mucosal flora.

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Induction of Antigen-Specific Immunity in Human Neonates and Infants

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Abstract

The first months of life represent a period of heightened susceptibility to infection, but the immunological differences involved are as yet incompletely understood. T cell-independent B cell (antibody) responses are markedly compromised in the first year of life. T cell-dependent antibody responses mature much earlier, but neonates and infants may require multiple immunizations to achieve or sustain titers comparable to those in older individuals. Neonates can mount effective antigen-specific T cell responses, but CD4 T cell responses are often slower to develop, less readily sustained, and in general more easily biased towards a Th2 type response. The last observation likely reflects in part the less efficient capacity of neonatal dendritic cells to establish a milieu that favors a Th1 CD4 T cell response, but this limitation can be overcome given appropriate stimuli, as occurs in neonates immunized with bacillus Calmette-Guérin. We currently lack a clear mechanistic understanding of the molecular basis for these immunological differences between adults and neonates. The goal of ongoing and future studies is to generate the mechanistic insights needed to enable the rational design of vaccines and adjuvants for use in neonates and young infants, and thereby reduce the morbidity and mortality of infections early in life.

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Globally, infectious diseases continue to account for more than one half of deaths in the first 5 years of life. Even in the developing world, where the risk of infections has declined substantially through improvements in hygiene, nutrition and immunization, infection continues to be a major cause of morbidity and mortality in early life [1]. Further progress in reducing this burden will require the development of vaccines against globally important infectious diseases that are effective yet safe when given in the first days to weeks of

life. Such progress will require that we understand more fully the immunological basis for the increased susceptibility of neonates and infants to infection and delayed or diminished responses to many vaccines [2–4]. At present, it is unclear whether these differences in outcome result from immunological differences from adults that are intrinsic to the neonate's T cells and B cells, antigen-presenting cells (APCs), or other aspects of the innate immune system that bridge innate and antigen-specific immunity.

Antigen-Specific B Cell and Antibody Responses

In their native context, polysaccharide antigens, such as the capsular antigens of *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae*, induce T cell-independent (TI) antibody responses. TI antibody responses are mediated predominantly by marginal zone B cells that are activated when these multivalent antigens bind to and cross-link their antigen receptors, which consist of cell surface immunoglobulin (Ig) molecules. In addition to Ig cross-linking by antigen, other signals are required for these B cells to proliferate and differentiate into antibody-secreting plasma cells. These signals are provided by cytokines, including BAFF and APRIL [5], produced by APCs in response to stimulation by microbial molecules, commonly referred to as pathogen-associated molecular patterns (PAMPs) or microbe-induced endogenous 'danger signals' [6]. TI antibody responses do not efficiently induce affinity maturation, Ig class switch recombination and long-term memory. Moreover, TI responses are undetectable in humans until at least 3 months of postnatal age, are markedly reduced until ~18 months and only reach adult competence beyond 4–5 years of age [2, 3] (fig. 1A). These age-related changes parallel the accrual of marginal zone B cells, but other factors may also contribute.

Antibody responses to protein antigens or to antigens that are covalently linked to proteins, e.g., polysaccharide-protein conjugate vaccines, induce T cell-dependent (TD) antibody responses (fig. 1B). TD antibody responses are mediated predominantly by follicular B cells. Binding of antigen to surface Ig provides one signal required for activation of these B cells, while a second obligatory signal is provided by engagement of CD40 on the B cell surface by CD40 ligand on the surface of an activated CD4 follicular helper T cell (Th). Activation of the Th cell, and thus provision of the second signal needed to activate the B cell, is dependent on the B cell internalizing through surface Ig antigenic proteins; the B cell generates and presents peptides from these proteins on its surface as complexes of antigenic peptides with class II major histocompatibility molecules (MHC; also known as HLA in humans). Together these two signals allow B cells to proliferate and migrate to germinal centers where they undergo affinity maturation, Ig class switch recombination, and differentiation into long-lived antibody-secreting plasma cells, which then home to the bone marrow. The specific Ig class to which a B cell switches is

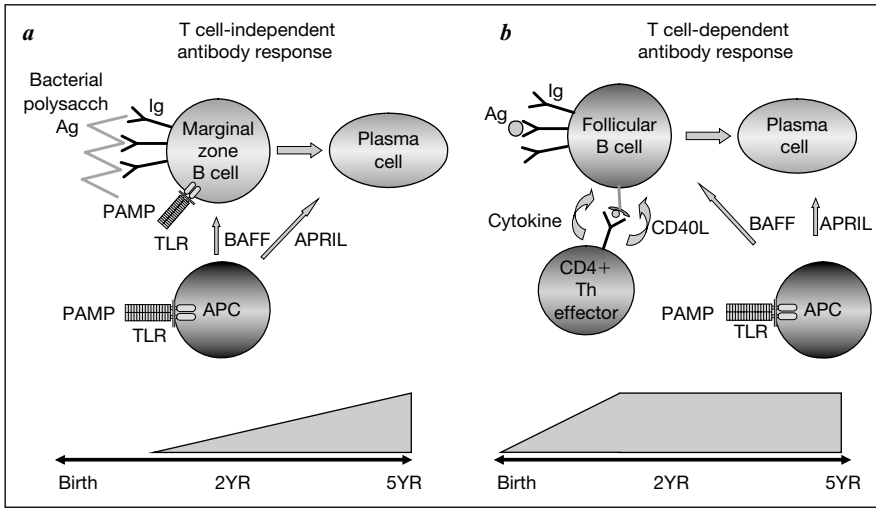

Fig. 1. Ontogeny of T cell-independent (***a***) and T cell-dependent (***b***) antigen-specific antibody responses in humans. (***a***) T cell-independent antibody responses are mediated by marginal zone B cells in response to cross-linking of surface Ig by multivalent antigen along with the cytokines BAFF and APRIL secreted by antigen-presenting cells (APCs) in response to binding of PAMPs to TLRs on these APCs. (***b***). T cell-dependent antibody responses are mediated by follicular B cells in response to engagement of surface Ig (shown as a Y shape) by antigen in concert with an obligatory second signal provided by CD40 ligand on follicular helper (Th) CD4 T cells, which have been activated by peptide antigen-MHC complexes displayed on the B cell; cytokines produced by T cells and BAFF and APRIL produced by APCs influence the Ig isotype and facilitate TD antibody responses, respectively. Age-dependent maturation of responses is illustrated by the timelines at the bottom.

also influenced by cytokines produced by Th cells. While not obligatory, BAFF and APRIL produced by APCs in response to PAMPs facilitate TD responses and are required for plasma cell survival.

In contrast to TI responses, TD antibody responses mature at an earlier age and can be induced in response to infection in utero. Consistent with these observations, the relative numbers of follicular B cells and their Ig variable region diversity are similar to adults at birth [2, 3], although V region diversification through somatic hypermutation is somewhat diminished in the first 6 months of life and may limit affinity maturation in some contexts. Immunization in the first days of life with some TD vaccines, including hepatitis B, diphtheria-tetanus toxoid, Hib-tetanus toxoid conjugate, and oral polio, induces little to no detectable antibody but does prime for greater antibody responses after subsequent immunization. Nonetheless, multiple immunizations in the first year of life are commonly required to induce substantial antibody titers. Even so, antibody titers are often not sustained at levels as

great as in older individuals, thus necessitating booster doses in the 2nd year of life [3]. Similarly, antibody responses to measles vaccine are absent before 6 months of postnatal age and reduced before 1 year of age even when passively acquired maternal antibody is absent [7]. Thus, while TD responses can be induced in utero in the latter part of gestation, these responses only reach full maturity in the 2nd year of life.

Antigen-Specific T Cell Responses

The major subsets of T cells are defined by the presence of CD4 or CD8 on their surface. Unlike B cells which are activated by antigens binding to surface Ig, T cells are activated by antigenic peptide–MHC complexes on the surface of cells. CD4 T cells have antigen-specific T cell receptors (TCRs) that recognize antigenic peptides bound to class II MHC, while CD8 T cells have TCRs that recognize peptides bound to class I MHC. Prior to the initial encounter with antigens (i.e., initial infection or immunization), T cells are poised to respond but are 'naïve'. The initial activation of naïve T cells is mediated by and dependent on specialized APCs known as dendritic cells (DCs), which take-up antigens in the tissues and transport them to secondary lymphoid organs where they are scanned by naïve T cells to determine if they have antigenic peptide–MHC complexes to which their TCRs can bind. If they do, the interaction of peptide–MHC with the TCR provides one of three signals needed to activate a naïve T cell. The other two signals are provided by costimulatory molecules, in particular CD80 and CD86, and cytokines, such as IL-6, IL-12 and type I interferons (IFNs), which are produced by DCs and other APCs in response to PAMPs. Together these signals induce naïve T cells to produce IL-2 and to express high-affinity IL-2 receptors, driving their proliferation and differentiation into effector and memory T cells. Effector T cells help to eliminate active infection, after which some persist as memory T cells poised to respond more rapidly and effectively to subsequent challenge.

CD4 T cells differentiate into several different types of effector and memory cells depending on the nature of the infection and, thus, the type of response needed to provide protection [8]. Th2 cells provide protection against multicellular parasites by producing the cytokines IL-4, IL-5 and IL-13; Th17 cells produce IL-17 and IL-22 and protect against extracellular bacterial pathogens, particularly in the gut; Th1 cells protect against viruses and intracellular bacteria (e.g., mycobacteria) and protozoans (e.g., *Toxoplasma gondii*) through the production of IFN-γ and IL-2. CD8 T cells collaborate with Th1 cells, producing IFN-γ and killing infected cells before intracellular pathogens can reproduce. In contrast, regulatory T cells protect us from our own immune system by maintaining self-tolerance.

Neonatal T cells are almost uniformly naïve and thus their activation is dependent on the presentation of antigenic peptides by DCs [2]. Neonates

can generate IFN-γ-producing Th1 CD4 and CD8 T cell responses but may do so less efficiently than adults [2–4]. For example, human neonates infected with herpes simplex virus (HSV) at parturition developed detectable HSV antigen-specific CD4 T cell responses 4–6 weeks later than do adults experiencing primary HSV infection, but once antigen-specific T cells were detected they proliferated and produced the Th1 cytokine IFN-γ in amounts similar to adult T cells [9]. Similarly, CD4 T cell responses to cytomegalovirus (CMV) infection acquired in utero or in infancy are slow to develop and may only become detectable when shedding of infectious virus ceases months to years later, but once detected CMV-specific CD4 T cells produce Th1 but not Th2 cytokines [10, 11]. Such a delay was not apparent in infants infected shortly after birth with *Bordetella pertussis*, in whom Th1 not Th2 CD4 T cell responses were detected approximately 2 weeks after the onset of infection [12]. Unlike CD4 T cell responses, CD8 T cell responses to CMV were evident on initial evaluation in infected infants, have been detected in infected fetuses as early as 28 weeks of gestation, and were similar to adult CD8 T cell responses in diversity and function [13]. This is also the case for CD8 T cell responses of neonates infected in utero with *Trypanosoma cruzi* [14]. However, compared to adults, the CD8 T cell response to HIV in infected infants is reduced, particularly in the first 6 months of life and even in those in whom concomitant CD8 T cell responses to co-infection with CMV are readily detected [15]; the basis for this discordance is not known. Thus, in response to infection, the late-term fetus and young infant appear to mount CD8 T cell responses more efficiently than CD4 T cell responses, but Th1 CD4 T cell responses can be mounted albeit often at a slower tempo.

In contrast to infection-induced responses, responses to some vaccines are relatively Th2-biased in infants compared to adults. When administered to infants shortly after birth and at 2 and 4 months of life, hepatitis B and oral polio vaccines induced stronger antibody responses, similar but more persistent Th2 CD4 T cell responses, but diminished IFN-γ-producing Th1 CD4 T cell responses compared to immunized adults [16, 17]. Similarly, diphtheria-tetanus-acellular pertussis (DTaP) vaccine induced both Th1 and Th2 cytokine-producing T cells in infants, but the Th2 response was more sustained than the Th1 response [18]. And while measles-mumps-rubella vaccine induced similar numbers of measles-specific CD4 T cells that expressed CD40 ligand (and are thus capable of facilitating antibody production by B cells) when given to 6-, 9- and 12-month-old infants and adults, IFN-γ-producing CD4 T cell responses were lower at 6 vs. 12 months of age and both were lower than in adults [7]. In contrast to these vaccines, diphtheria-tetanus-whole cell pertussis (DTwP; containing killed *B. pertussis*) induced Th1 responses in infants, which were comparable to those induced by acute pertussis infection in infants and in children >5 years of age [12]. Similarly, bacillus Calmette-Guérin (BCG) immunization shortly after birth or at 2 or 4 months of life induced antigen-specific Th1 CD4 T cell responses that were at

least as strong as responses by adults [19]. BCG given at birth also induced antigen-specific CD8 T cell responses, but for ethical reasons these studies did not contain a comparison group immunized at an older age [20]. Moreover, when co-administered with hepatitis B vaccine in the first days of life, BCG acted as an adjuvant to enhance antibody and CD4 T cell responses to hepatitis B vaccine, although it did not alter the relative Th2 bias of the infant's response to hepatitis B [19]. These findings indicate that neonates can mount effective T cell and TD B cell responses, and can mount Th1 responses to infection or immunization in certain contexts, while in other contexts Th1 responses are weaker or are not as sustained as Th2 responses. These findings have led to the suggestion that the context-dependent differences in T cell responses reflect the efficiency with which specific infectious agents or vaccines activate neonatal DCs to provide the signals needed for the neonate to mount or sustain Th1 CD4 T cell responses [2–4].

APCs Link Innate and Antigen-Specific Immunity

As noted above, DCs play a unique and essential role in the initiation of antigen-specific T cell and TD B cell responses, and activation of DCs by microbial PAMPs plays a critical role in this process. DCs utilize invariant innate immune receptors, which include toll-like receptors (TLRs), to detect PAMPS or microbe-induced endogenous 'danger signals' on the cell surface and in endosomes and NOD-like proteins, RIG-I and MDA-5 for detection inside the cell [6, 21]. Signaling through these innate immune receptors induces DCs to migrate to sites where T cells are found in the secondary lymphoid tissues. At the same time, DCs upregulate expression of antigenic peptide–MHC and co-stimulatory molecules, i.e., CD80 (B7–1) and CD86 (B7–2), and produce cytokines, which together provide the three signals needed to activate naïve T cells. Thus, DC activation by PAMPs through TLRs and other innate immune receptors plays a critical role in the initiation of antigen-specific T cell responses (fig. 2).

There are two major DC subsets. Myeloid DCs (mDCs) are the principle cells involved in T cell activation, while plasmacytoid DCs (pDCs) are major producers of type I IFNs. Both DC subsets are present at birth in humans in numbers that are not greatly different from adults [22, 23]. Expression of MHC and co-stimulatory molecules on resting neonatal and adult blood mDCs and pDCs is similar [22, 24]. However, following stimulation of whole blood with two PAMPs – lipopolysaccharide (LPS) and poly I:C (ligands for TLR4 and TLR3, respectively) – expression of CD40 and CD80 increased less, whereas expression of class II MHC and CD86 increased to a similar extent on neonatal and adult mDCs [24]. Similarly, after stimulation of pDCs with CpG DNA (a ligand for TLR9), class II MHC expression increased to a similar extent on adult and neonatal pDCs, but CD40, CD80 and CD86 increased less

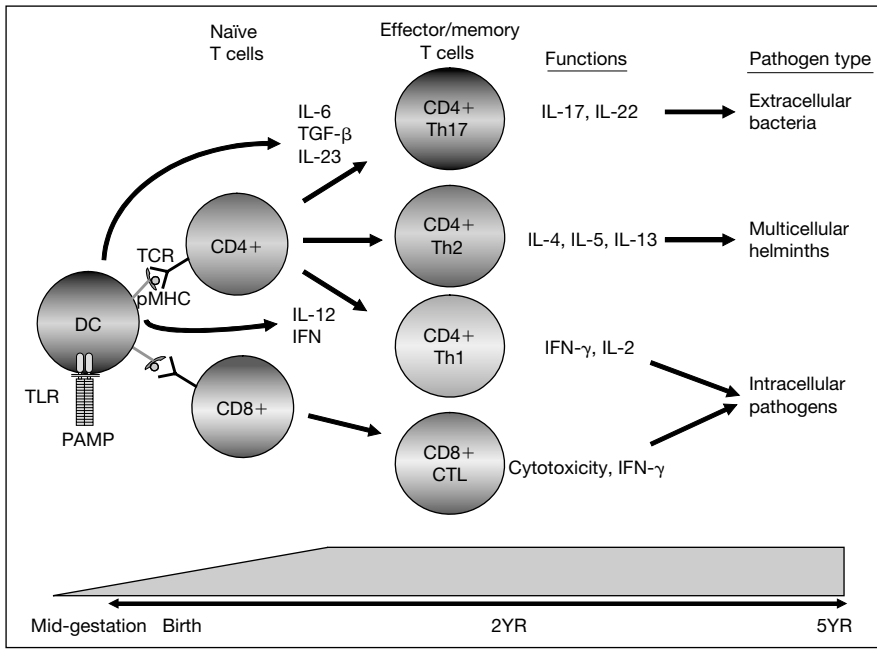

Fig. 2. Ontogeny of antigen-specific T cell responses in humans. Naïve CD4 and CD8 T cells are activated by binding of their TCR (shown as a Y shape) to peptide-antigen-MHC complexes displayed on dendritic cells (DCs). DCs are activated in response to binding of PAMPs to their TLRs, causing them to express CD80 and CD86 on their surface that bind to CD28 on the naïve T cell (not shown in this drawing) and to secrete cytokines, thus providing essential second and third signals, respectively, needed to stimulate naïve T cells to proliferate and differentiate into effector cells. CD4 T cells can differentiate into specialized Th17, Th2 or Th1 effector and memory cells depending on the cytokines secreted by DCs, while CD8 T cells differentiate preferentially into cytotoxic CTLs, which produce IFN-γ. Age-dependent maturation of responses is illustrated by the timeline at the bottom.

in neonatal pDCs. Thus, PAMP-induced upregulation by neonatal DCs of two of the three signals required for activation of naïve T cells appears to be less robust than by adult DCs.

Cytokines produced by DCs provide the third signal needed for activation of naïve T cells, and also shape the quality of the CD4 T cell response depending on the pattern of cytokines DCs produce [8] (fig. 2): IL-6 alone favors the differentiation of naïve CD4 T cells into IL-4-producing Th2 cells; the combination of IL-6, transforming growth factor-β (TGF-β), and IL-23 favors the generation of IL-17-producing Th17 cells (at least in mice); IL-12 and/or type I IFNs favor the generation of IFN-γ-producing Th1 cells, due both to the

direct action of these cytokines on CD4 T cells and, in the case of IL-12, by stimulation of natural killer cells to produce IFN-γ, which in turn acts on CD4 T cells [2, 8]. Consequently, ligands for TLRs, that are efficient inducers of IL-12 and type I IFNs, including TLRs 3, 4, 7/8 and 9, favor the induction of Th1 or mixed Th1/Th2 responses. Other ligands, such as TLR2 ligands, that are less efficient inducers of these cytokines, may favor Th2 responses.

Unfortunately, our knowledge regarding cytokine production by neonatal DCs is rather limited, since nearly all studies have been done by assessment of cytokines in culture supernatants of stimulated whole blood or blood mononuclear cells, which largely reflects cytokines secreted by monocytes not DCs. Using flow cytometric analysis to specifically measure the response to LPS of mDCs in whole blood, one group reported comparable IL-1α but an ~50% reduction in TNF production (and percent TNF-producing cells) by neonatal vs. adult mDCs [23]. Two groups found that purified neonatal pDCs produce only 20–50% as much type I IFNs as adult pDCs in response to CpG DNA [22, 25]. Though not shown directly, it is also likely that decreased type I IFN production by neonatal blood cells in response to poly I:C reflects decreased production by mDCs [24], while decreased type I IFN production in response to HSV likely reflects decreased production by pDCs [26]. The only other studies that, to our knowledge, have directly evaluated cytokine production by DCs used DCs generated by culturing monocytes in granulocyte-macrophage colony-stimulating factor plus IL-4 as a surrogate for mDCs. These studies produced discordant results: two showed a marked reduction in the production of IL-12p70 in response to LPS or poly I:C [27, 28], and the other showed no difference in response to LPS [29].

Implications for Vaccine Development

Existing information regarding neonatal DCs suggest that they may function less efficiently than adult DCs, particularly in the production of cytokines that favor the development of Th1 responses. However, the current data set is limited and inconclusive and does not provide sufficient insights as to why some vaccines, i.e., BCG and DTwP, efficiently induce antigen-specific Th1 immunity and act as potent adjuvants for co-administered vaccines when given at birth, while others do not. Moreover, there is little or no information regarding the production by neonatal DCs of cytokines that induce or support the survival and function of regulatory T cells or Th17 cells in human neonates. Contemporary tools are available to address these unanswered questions and should help to facilitate the rationale design of vaccines for use in early life.

In this regard, the responses to Hib conjugate vaccines given at birth provide a cautionary note and illustrate the need for mechanistic insights. Three different Hib conjugate vaccines are in use in the United States. These vaccines differ in the protein to which Hib polysaccharide is conjugated – either

tetanus toxoid (TT), a mutant diphtheria toxin (Crm) or meningococcal outer membrane complex (OMPC). OMPC is rich in lipoproteins and a potent TLR2 agonist [30]. Each of the Hib conjugate vaccines induces TD B cell responses and protective immunity when given at 2, 4 and 6 months of age, but only Hib-OMPC induces protective antibody titers after a single dose at 2 months of age [31], apparently reflecting the adjuvant activity of OMPC. However, when Hib-OMPC was given to neonates, not only was antibody not induced but little or no antibody was induced by booster immunizations in the first 6 months of life [32, 33]. Although Hib-OMPC recipients in one of these studies [33] subsequently mounted responses to Hib-Crm vaccine given at ≥15 months of age, these studies suggest markedly different effects of the TLR2 ligand OMPC at 2 months and beyond than at birth. In contrast, immunization of neonates with Hib-TT vaccine neither enhanced nor inhibited the response to subsequent immunizations [34]. Similar but less consistent evidence suggests that whole cell pertussis vaccines may also induce partial tolerance when given in the first days of life [2]. The basis for the induction of persistent unresponsiveness/tolerance by Hib-OMPC is unknown, but could be related to the apparent propensity for TLR2 ligands to induce the immunosuppressive cytokine IL-10 or to promote Th2 responses. We must unravel the basis for these untoward outcomes if we are to develop safe and effective vaccine adjuvants for use in newborns and young infants.

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Discussion

Dr. Ogra: In terms of mouse–human TLR interaction, when you put a human TLR in a mouse cell, is the responsiveness to LPS the same as a mouse TLR in a mouse cell or a human TLR in a human cell?

Dr. Wilson: The cytoplasmic domains of TLRs are highly conserved, whereas the extracellular domains are much more variable. The differences in responsiveness to different forms of LPS between humans and mice are due to sequence differences in the extracellular domain of TLR4 and differences in the MD-2 protein, which interacts with the extracellular domain of TLR4 to form the LPS receptor complex. So the answer to your question is that when we put the human receptor complex into a mouse cell in vitro or in vivo, the responses observed are similar to human TLR4/MD-2 in a human cell rather than mouse TLR4/MD-2 in a mouse cell. With regard to TLR9, we know that the human TLR9 BAC transgenic mouse expresses human TLR9 in a pattern similar to that found in humans rather than the pattern typical of mice, but these findings are preliminary at the present time.

Dr. Ogra: My second question relates to the delayed CD4 response in HSV infection. Is it the result of HSV infection or because of the underlying CD4 immaturity? Is it an antigen- rather than a CD4 cell-driven process? I am trying to understand the possible mechanism for the delayed CD4 response in these babies.

Dr. Wilson: You are asking whether the delay is unique or is it a general phenomenon. Delayed development of antigen-specific CD4 T cell responses has been observed in infants with neonatal HSV and congenital and perinatal CMV, and there is some evidence that the development of tuberculin reactivity after BCG immunization occurs somewhat more slowly when this vaccine is given at birth. By contrast, CD8 responses appear to develop more readily in neonates and even in utero in response to CMV infection. This is not a definite answer, but there appear to be several situations in which the CD4 response is delayed in the newborn infant.

Dr. Ogra: It would be important to find out whether this is a marker for immunity or disease. For BCG immunization or infection with *Mycobacterium tuberculosis*, is expression of IFN-γ a marker for induction of immunity or expression of clinical disease?

Dr. Wilson: IFN-γ is essential for TB immunity in the mouse and the evidence in humans is also incontrovertible based on the data of Casanova and Abel [1]. They have shown that individuals with a genetic deficiency of the IFN-γ receptor, IL-12 or IL-12 receptors are profoundly susceptible to mycobacterial disease, including disease produced by *Mycobacterium tuberculosis*. Thus, I think it is clear that IFN-γ is key for protective immunity in humans, although it is less clear in humans than in mice exactly how it acts. Thus, it is a marker for protective immunity, but is very clearly not the only thing that is required for protection.

Dr. Ogra: So you don't see IFN-γ during the active disease process in patients with tuberculosis?

Dr. Wilson: Of course we do. In individuals with active tuberculosis disease, you may not be able to detect IFN-γ-producing cells in the blood, but you can find them in the infected tissues. You already gave the example of lepromatous versus tuberculoid

leprosy, in which those with lepromatous leprosy – who have high numbers of bacteria and fail to control the infection – have T cells that are not making IFN-γ but rather are making IL-4 and IL-13.

Dr. Walker: An infant is born with the Th2 bias, and presumably that exists to prevent the infant from being prematurely delivered or rejected in utero. What are the data to support that or is that theoretic?

Dr. Wilson: I would say it is largely theoretical in humans. The bulk of the data showing fetal loss related to immunological mechanisms in humans relates to premature labor associated with innate immune/inflammatory processes. In fact there is evidence in the mouse that NK cells, which produce IFN-γ, actually facilitate pregnancy in its earlier stages, as you probably know. Regarding the importance of the Th2 bias for pregnancy success, I think the data of Lin et al. [2] in the mouse are probably the most compelling and gave rise to this notion. They found that a Th2 cytokine is demonstrable in the placenta in normal mid to late gestation in the mouse and that premature parturition is associated with the loss of that bias. More recent consideration of this notion suggests that it is overtly simplistic [3]. So in my opinion, this concept is biologically plausible and highly appealing, not completely proved but reasonable.

Dr. Walker: The data on cord blood suggest that IFN-γ production is a productive factor against the expression of allergy; so it looks as if Th1 responses can occur. Something has confused me. You pointed out that Th2 cells can be used to combat parasitic infection and the IgE response was a protective response to parasites. That makes sense, but the IgE response to allergy is really not something that is wanted. Is there a difference in the IgE responsiveness from an immunologic perspective in allergic responses versus parasitic responses, or is it just a genetic change and an ability to react to non-harmful antigens?

Dr. Wilson: The clearest evidence is largely derived from murine models, which suggests that the pathways by which one gets to Th2 and IgE are similar in allergy and helminth infections. The pathogen-associated molecular patterns on parasites that induce Th2 responses are not well-characterized. I think we are getting closer by finding cytokines like thymic stromal lymphopoietin (TSLP) that can push the response in the Th2 direction. However, exactly how TSLP and Th2 responses are induced is not clear. Reese et al. [4] have data suggesting that to generate a Th2 CD4 T cell response you only need IL-4 production by the CD4 T cells themselves. Thus it is possible that the CD4 T cell response defaults to Th2 in the absence of factors that favor Th1 or Th17 responses. However, the extent to which this default occurs may be strongly influenced by the genetics of the individual.

Dr. Malka: I am a little confused about the DPT response to the Th1-protective response, and that acellular DPT, which is being used clinically, does not have a good response to Th1. Was that in mice?

Dr. Wilson: No, it is true in humans as well. I think the sense is that the DPT whole cell vaccine was abandoned in the developed world not because it wasn't protective but because it was more toxic. Thus, we went to the less reactogenic vaccine because it still provides adequate protection. However, if you look at the T cell response, there is a clear difference between the two vaccines. The acellular DTaP vaccine has no innate immune activating microbial components; the only adjuvant is alum. Alum is a very good Th2 cytokine inducer and is good at facilitating antibody responses. That is probably just fine for this vaccine because it works and it is much better accepted in the developed world. In the developing world, the WHO still promotes the whole cell vaccine because it is considered less expensive.

Dr. Björkstén: I have a comment regarding what Dr. Walker said and a question for you. The comment is really that those children who develop allergy have a slight delay

in the induction of IFN-γ, but the main issue is that they overshoot both on Th1 and Th2 cytokine production. They make more IFN-γ and they make more IL-4 and IL-5 than those who do not develop allergy. This seems also to be the case for infants who are at risk of autoimmune disease. Thus, the Th1 Th2 concept is too simplistic, rather it seems to be a question of induction of T regulatory cells. In newborn mice there are no dendritic cells or antigen-presenting cells on the mucosal surface of the respiratory airways, and they seem to develop roughly by the time of weaning, which is an explanation for some of your conclusions. Have similar studies been done in infants who died neonatally?

Dr. Wilson: Are you asking what we know about dendritic cells in mucosal surfaces?

Dr. Björkstén: Yes, it would be a rather straightforward study to reproduce the findings in mice in which you can see the dendritic cell network like a spider web on the mucosa in rodent airways. I have been looking for similar data in humans but have not been able to find any.

Dr. Wilson: I think you are correct. There are quite a lot of data on Langerhan's cells and on dendritic cells in the gut indicating their presence in early life. But I am not aware of high quality studies indicating how many dendritic cells are present in respiratory mucosal sites at this age.

Dr. Ogra: There are data both for the respiratory tract and gut. The sublingual area seems to have a lot of dendritic cells which look more like Langerhans cells of the skin in terms of their function but the sublingual area does not appear to have an underlying lymphoid tissue, so it may be the the manner in which antigens are handled and presented to the lymphoid tissue at different mucosal sites that determines the degree of production of IgE and subsequent development of allergies in the respiratory tract and protection against parasitic disease in the gut. Here again the differences in the development of immune response may be to a large extent host-driven rather than bacterial or parasitic antigen-driven.

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Growth and Host–Pathogen Interactions

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Abstract

Differing trajectories of infant and child growth are associated with different patterns of disease and mortality in adulthood. Since postnatal growth patterns are partially modifiable by diet, these associations raise fresh questions about what constitutes an optimal growth rate. We use data from contemporary societies that still suffer poor nutrition and high burdens of infectious disease to illustrate early growth patterns that have likely been typical of our evolutionary past. Pathogenic assault is a major suppressor of growth; populations frequently average −1.0 to −1.5 z scores (standard deviations relative to standard growth curves) for height, and −2.0 to −2.5 z scores for weight, body mass index and head circumference. Many infections are symptomatic (e.g. diarrhea, malaria, pneumonia, HIV), but others are subclinical (e.g. hepatitis B, cytomegalovirus, Epstein-Barr virus, herpes, *Helicobacter pylori*). The great majority of young children become infected by multiple pathogens which initiate a downward cycle of infection→suppressed appetite and malabsorption→reduced growth→lowered immunity→repeated infection. Examination of the evolutionary 'norm' for early growth, and the external environmental factors that influenced it, may provide clues towards identifying the current day optimum for growth.

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Introduction

There is now a substantial body of evidence linking the growth trajectories of infants and young children with later chronic disease [e.g. 1–5]. Nutritional interventions in premature babies suggest that an excess (forced) early growth has detrimental long-term sequelae [6], but most retrospective epidemiological studies indicate that poor growth in infancy is detrimental [1–5], especially if followed by positive centile crossing in later childhood [3]. Some studies suggest that the effects of these differing postnatal trajectories are independent of birthweight, and others reveal evidence of an interaction.

Postnatal growth is arguably more amenable to nutritional manipulation than fetal growth because the latter is buffered by maternal and placental physiological mechanisms. This raises new questions about defining the optimal rate and shape of growth in infancy and childhood. Over recent decades there has been a transition in thinking from the 'biggest is best' view held in the 1950–1970s (i.e. that the very high growth rates of infants fed high-solute, high-protein infant formulas were optimal), towards a more moderate view that holds the breastfed infant as the optimal model [7] and suggests that previous recommended intakes for energy represented a recipe overfeeding [8]. Further progress towards identifying optimal growth will require interrogation of longitudinal data from both observational and intervention studies. But perhaps we can also draw useful inferences from an understanding of the likely evolutionary norm of human growth, and the factors that impaired or stimulated growth.

Archeological data can inform us about final attained body size in ancient populations from which we can interpolate that child growth was usually very poor, but cannot contribute to a detailed knowledge of the trajectory of ancient growth rates. The best proxy for such information can probably be gleaned by studying contemporary populations in developing countries that are still affected by poor diet and a heavy burden of infections.

The struggle between the human host and pathogenic organisms, both enteric and systemic, has been the norm over most of evolutionary time and remains a major challenge for the majority of the world's infants raised, as they are, in unhygienic conditions. This article uses our research from a rural population in The Gambia as a basis for describing the effects of such host–pathogen interactions on early child growth.

The Burden of Infection in Poor Populations

Using analysis of genetic variation, Linz et al. [9] have recently shown that the ubiquitous stomach pathogen *Helicobacter pylori*, spread from East Africa at the same time as humans around 58,000 years ago. Their results show that anatomically modern humans were already infected by *H. pylori* before their migrations from Africa and that the bacteria has remained associated with its human populations ever since, thus providing a graphic reminder of the longevity and intimacy of relationships between the human host and its multitudinous pathogens.

Table 1 shows data from a cohort of almost two hundred 21st century Gambian infants followed longitudinally from birth. It shows that by 9 months of age over two thirds have abnormally raised levels of the acute-phase marker, α1-acid glycoprotein. α1-Acid glycoprotein is marker of infection with a half-life of several days. Nasopharyngeal swabs taken from the infants and cultured for the presence of pneumococci indicate a very sharp rise from

Table 1. Indicators of postnatal infections in Gambian infants (% affected)

Marker of infection	Age, months				
	Birth	2	5	9	12
Raised α1-acid glycoprotein	0	18	43	68	62
Nasopharyngeal pneumococcal carriage	2	78	88	79	NM
Helicobacter pylori infection	NM	42	32	54	56
Chronic environmental enteropathy	NM	11	13	32	46

NM = Not measured. Normal cutoff for α1-acid glycoprotein ≤1 g/l. *H. pylori* infection assessed by the ^{14}C-urea breath test. Chronic environmental enteropathy determined as abnormal values for the lactulose:mannitol ratio in the dual-sugar permeability test. Data from 197 infants participating in a randomized controlled trial high vs. low dose vitamin A [Darboe et al., unpublished data].

almost zero carriage soon after birth to 78% carriage at 2 months and 88% at 5 months. Carriage indicates exposure rather than infection, but indicates that the infants are having to defend their respiratory tract from these potentially fatal organisms. *H. pylori* infection, assessed by the ^{14}C-urea breath test, affected over half the infants by 9 months of age; a figure that is lower than previous studies in these communities [10].

Figure 1 shows unpublished data, gathered from a number of separate studies in rural Gambia, on antibody positivity against a variety of other pathogens: hepatitis B virus, cytomegalovirus, Epstein-Barr virus and *Herpes simplex*. By their 3rd year, 100% of children have turned antibody positive to cytomegalovirus and Epstein-Barr virus, and 70–80% are positive to herpes and hepatitis B. Except in rare cases all of the acute infections leading to these immunological conversions will have passed clinically unrecognized, yet the children will have been expending their nutritional resources on mounting the humoral and cellular responses necessary to control the infections. These data provide a powerful reminder of the intensity of the battle between host and pathogen; one that is underestimated using simple clinical screening.

Growth Failure

The pattern of infant growth in rural Gambia is illustrated in figure 2. Babies are typically born small but then show rapid catch-up growth during the first 3 months of life when fully breastfed and generally free from infections. This catch-up is so successful that at 3 months of age the population average is close to Western growth norms. Thereafter there is a precipitate deterioration in growth so that by the end of infancy the population average

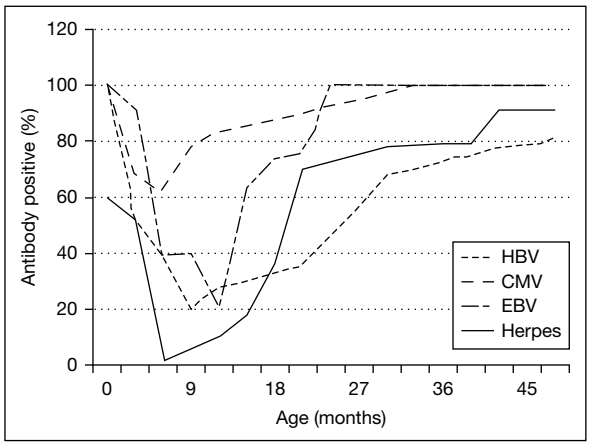

Fig. 1. High prevalence of early infections in Gambian children. Results show antibody positivity. HBV = Hepatitis B virus; CMV = cytomegalovirus; EBV = Epstein-Barr virus. Early high levels represent transplacental acquisition of maternal antibody that wanes during the first year. The subsequent rise represents postnatal exposure. Previously unpublished data kindly provided by Prof H.C. Whittle, MRC Laboratories, the Gambia.

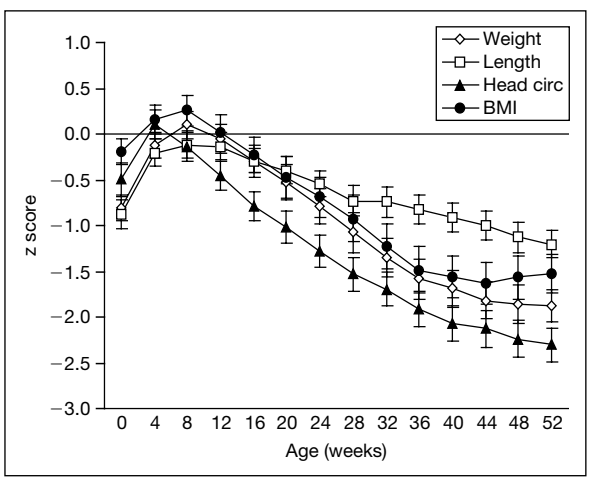

Fig. 2. Early growth faltering in Gambian infants. Data from 138 Gambian infants assessed longitudinally and expressed as z scores relative to the UK 1990 standards. Reproduced with permission from Collinson et al. [11].

z score is close to −2.0 for weight, −1.2 for length and −2.3 for head circumference. This represents the average growth pattern across all calendar months and is strongly modulated by seasonal variation [12]. During the rainy season (July–October) growth virtually stops in many infants as a consequence of a sharp increase in infectious diseases including diarrhea [13], and a deterioration in maternal care practices due to the fact that mothers have to work long hours in the fields and frequently leave their infants with a young nursemaid or a grandmother who is no longer able to work. An audit of the number of severely malnourished infants referred by pediatricians to the local therapeutic feeding center revealed an average of 8 admissions per month in May to 29 per month in August.

The 'Weanling's Dilemma'

Weaning foods in sub-Saharan Africa typically have a very low energy and nutrient density, and frequently have high levels of bacterial contamination [14]. The issue of when to start introducing complementary foods has been described as the 'weanling's dilemma' by Rowland et al. [15]. The dilemma is that if mothers introduce weaning foods too early they risk causing diarrhea and inhibiting their own lactational performance, but if they introduce them too late their infant's energy needs may have started to exceed their milk energy supply. This dilemma is faced by all mothers but is much more acute in poor communities with few facilities for hygienic food preparation where weaning foods are likely to be contaminated. The hazards associated with this transition frequently result in the initiation of a downward cycle of infection → suppressed appetite and malabsorption → reduced growth → lowered immunity → repeated infection.

Quantitative Effects of Infections on Growth

The quantitative effects on early child growth of high levels of infectious disease in developing countries were first described in the late 1960s and early 1970s in Guatemala and The Gambia [13, 16–18]. These studies used clinical monitoring of the frequency and duration of infections in order to establish the levels of exposure together with stool analysis to search for enteric pathogens and blood films for malaria. Three decades later, and in spite of a 10-fold reduction in infant mortality, we find that infants in The Gambia still display these very high levels of infection (table 1; fig. 1).

Diarrheal disease has for many years been considered the chief cause of growth faltering in young children. Early work in Guatemala focused intensively on identifying links between diarrhea and growth. Mata et al. [17, 19] were among the first to review the detailed host alterations seen with specific

enteric infections that lead to malnutrition. These include: mucosal dysfunction; cytokine-mediated systemic metabolic responses; impaired intake, digestion and absorption; nutrient losses; altered immune responses, and ultimately, impaired growth and development. A little later a quantitative regression analysis performed by Rowland et al. [13] in The Gambia confirmed that gastroenteritis was the main infection suppressing growth. Malaria actually had a larger effect per day of infection, but this was offset by the much higher prevalence of diarrhea with children suffering for up to 20% of the time in the wet seasons. These results were accepted for many years and still have currency, but there are some contradictory pieces of evidence in the literature. Briend [20] has questioned the direction of causality of the link between malnutrition and diarrhea and concludes that the evidence is strongest in the direction of malnutrition predisposing to diarrhea. In the same Gambian population in which Rowland et al. [13] first established the quantitative association, Poskitt et al. [21] later showed that a pronounced year-by-year reduction in the diagnoses of gastroenteritis in clinic records had not been accompanied by a secular improvement in growth.

Gastrointestinal Infections

Various bacterial and viral pathogens have been implicated as etiologic agents for diarrhea in The Gambia [22–24]. Some have been found to be significantly associated with diarrhea while others have been seen to be equally prevalent in asymptomatic children. Bacterial contamination of the jejunum was predominant in a small series of malnourished children with diarrhea [25].

In a longitudinal community study on giardiasis (measured by serology) and weight gain of rural Gambian infants, elevated titers of *Giardia*-specific IgM antibodies were associated with decreased weight gain in the 2-week period prior to serological conversion [26]. High *Giardia*-specific IgM was also associated with elevated intestinal permeability values and decreased mannitol absorption [27]. However, the mean IgM titers per child over the entire study period did not predict differences in long-term growth or intestinal permeability. In a community-based study it was also shown that although intestinal inflammation (as measured by fecal neopterin) was inversely associated with growth, the presence of giardiasis was neither associated with poor growth nor poor intestinal permeability [27]. It appears that in the Gambian setting, giardiasis is more prevalent in chronic diarrhea and malnutrition, but its role in modulating the acute growth of infants seems to be less clear.

An early study of *H. pylori* infection in severely malnourished Gambian children showed that close to half the children aged between 40 and 60 months had serologic evidence of infection [28]. Half of the children with chronic diarrhea and malnutrition were positive as compared to a quarter of

the healthy controls and undernourished children. In a later study using the ^{13}C-urea breath test, it was shown that acquisition of *H. pylori* infection may occur before 3 months of age as 20% of the 3-month-old infants were positive [29]. An analysis of longitudinal growth data and serial breath tests demonstrated that children who acquired *H. pylori* earlier ended up shorter, lighter and thinner than their uninfected peers [10]. It has been proposed that early *H. pylori* causes a transient hypochlorhydria and thereby increases the likelihood of enteric infection thus compromising intestinal function and nutrition. *H. pylori* infection may serve to reduce the mucosal defenses and allow further colonization of the small intestine with pathogens [10].

Chronic Environmental Enteropathy as a Contributor to Growth Failure

Persistent gastroenteropathy, as characterized histologically by small-intestine mucosal villous shortening and broadening, crypt hyperplasia, increased crypt depth, and lymphocyte infiltration into the lamina propria and epithelium, is displayed by many Gambian children [30, 31]. Early research established this inflammatory condition to be strongly associated with growth failure. First described in 1962, persistent enteropathy was found to affect individuals throughout the tropics, in Africa, Asia, South America and the Caribbean. For this reason it acquired the name 'tropical enteropathy'. The condition was particularly observed in those living in less developed, or more contaminated, environments of the tropics. It was later shown that people living in temperate areas may develop similar histological and functional changes if living in environments with similarly high levels of microbiological pathogens. For these reasons the expression 'chronic environmental enteropathy' is now accepted as a more accurate description of the condition than 'tropical enteropathy'.

Associated functional changes include subclinical malabsorption of fat and an increased mucosal permeability. The latter is demonstrated by markedly and consistently raised lactulose:mannitol ratios in the dual-sugar permeability test towards later infancy (table 1). Raised lactulose:mannitol ratios have also been described in children in several other parts of the developing world. The dual-sugar permeability test assesses both gut integrity and absorptive capacity, and has been used in numerous studies characterizing the etiology of growth failure in The Gambia [32] and elsewhere [33, 34].

Two sets of immunohistologic studies have been performed in The Gambia. These are consistent with past biopsy studies in marasmus and kwashiorkor done in other developing countries over the past four decades; in particular they describe a wide spectrum of crypt hyperplasia and villous atrophy across cases. In addition, immunohistology revealed intraepithelial lymphocyte infiltrates in the surface villi and crypt [30]. The most recent biopsies done in

The Gambia [31, 35] have demonstrated a generalized cellular hyper-responsiveness and a cytokine profile biased towards proinflammatory cytokines. These immunohistologic studies contradict the commonly held belief that malnutrition is associated with an immunosuppressed state, and instead suggest that both lymphocyte activation and ineffective enterocyte development play a significant role in chronic environmental enteropathy and malnutrition. These findings support the view that malnutrition is not necessarily accompanied by severe T lymphocyte deficiency, and that T lymphocyte dysregulation may be present [36].

Catch-Up Growth Following Acute Infections

It is well recognized that there is a strong innate drive towards catch-up growth in the period immediately after acute weight loss caused by infections [37, 38]. To achieve its full potential the child must be cleared from infections and then provided with a regular supply of energy and nutrient-dense feeds. Under such conditions children can achieve short-term growth rates many times above the norm. It is sometimes stated that there is a window within which this catch-up must be achieved and that failure to do so will reset the growth trajectory, but appropriate studies to test this thesis have not been done, and there is considerable counterevidence.

Possible Implications for the Developmental Origins of Disease Theory

We have described above some of the interactions between infections and growth in poor children in developing country settings on the principle that these probably represent close to the norm for early human growth over most of evolutionary time. Can these further inform our understanding of how early growth patterns influence later health? At one level the immunological responses illustrated by the antibody positivity shown in figure 1 represent a perfect example of the 'predictive adaptive response' proposed by Gluckman et al. [39], and we have shown that an apparent failure to mount such a response by babies born in the Gambian hungry season is a very strong predictor of later survival [40]. We believe that there may be, as yet undetected, links between early programming of the immune system and the chronic disease outcomes that have dominated the field of developmental origins of adult disease. The question of whether the growth repression caused by infections represents an intentional response with later adaptive value or whether it is simply an unavoidable result of the infection–malnutrition cycle seems to have a clearer resolution. Even though being a small adult in an

energy-restricted environment has survival advantages, the fact that there is a strong drive towards catch-up growth in children as soon as infections resolve would suggest that growth suppression is physiologically undesirable. This would support the bulk of the epidemiological evidence in Western populations that suggests that growth restriction in infancy represents an undesirable exposure that predisposes to later ill health.

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Discussion

Dr. Walker: That was a very provocative talk which allows a lot of room for thought about growth in general, as well as specifically in your setting. The period of nursing seems to be the most positive time period for these infants when they are born small, they grow well transiently, and then they fall off. Is it possible to control how long mothers nurse their babies; can they nurse them for a year for example?

Dr. Prentice: Mothers certainly cannot nurse them for a year. Of course the WHO now recommends exclusive breastfeeding to 6 months, which has been something that I, as many in this audience, have had to battle with because, physiologically speaking, I am not sure that the majority of mothers can really sustain that. In the early years of our work we brought all children in Keneba to a community supplementation center at 3 months of age where we were then able to randomize every alternate child and bring them back to that center at 6 months of age instead. We showed

very clearly, as Dewey et al. [1] have shown in Honduras, that milk production does increase substantially if alternative feeding is delayed, so mothers can produce more milk. Unfortunately the babies don't grow any better. They grow just as well but they don't grow any better, and I think that again reflects the high pathogenicity of the environment. We really have got to get rid of the bugs before we are going to really impact on that. But remember that there is a constant battle between the mother and her infant. The mother has to think about her total reproductive output. She is not so much interested in the individual child, she has to defend herself and her nutritional resources at some stage for future offspring, and that is the very important compromise.

Dr. Walker: The slide you showed of the intestine and the corresponding drawing suggest that you are dealing more with infection than with malnutrition for two reasons. One, there are a lot of lymphoid cells which frequently is not the case with severe malnutrition. But more importantly if that drawing is accurate, then there is a large proliferation of crypt cells which usually does not occur in malnutrition but does occur as a compensatory response to insult to the epithelium.

Dr. Prentice: Absolutely the case, and the other proof is that we have tried everything we can think of in terms of interventions: glutamine, zinc, protein, vitamin A, multiple micronutrients, etc., and they do not benefit growth. Incidentally, there was an enormous effect on survival in our population. The mortality rates are now less than one tenth of what they were when we started working there, but there is still no impact on growth failure.

Dr. Björkstén: Thank you for this beautiful presentation. Aaby et al. [2] have an intriguing and provocative story about certain vaccines in Guinea Bissau increasing childhood mortality. They suggest that it has to do with Th1 Th2 stimulatory vaccines and at what stage they are given. Do you have any comments in the light of your extensive studies?

Dr. Prentice: Yes and no. Earlier this morning I might have been tempted to give you my particular view on that. I think I would now prefer to hold back because there are people in the audience who are much greater experts than myself, and I imagine that you have your own answer and it would be much more informative than mine. But let me make the point that of course I am persuaded that there are windows of opportunity, particularly with regard to the data presented by Dr. Marchant with the timing of BCG. There are windows of opportunity which may be critical to directing and sign posting the immune system. I am well aware of the data of Aaby et al.; I am also very aware that it is highly controversial and that I should be cautious making other comments.

Dr. Haschke: I have a question related to the high z score. You showed very nice tracking, it is -1 from birth and they finally end up with -1, which contradicts a little bit what we discussed yesterday in non-tracking, but here it is very clear in this selected population. Did I understand you correctly when you interpret their genetic potential as starting at -1 and end up at -1, or is the -1 at birth already the modified genetic potential which is due to intrauterine factors, which we don't know, or even factors before conception?

Dr. Prentice: I think the latter is the case. I would imagine that the genetic potential is pretty much around zero. I think the genetic potential in Gambians is pretty much the same as anyone else, and it is intriguing that that has held itself within the genome in spite of the fact that for millions of years no one has been growing anywhere near that? We have evidence that there would be positive selection in terms of birthweight. What we know from birthweight is that the optimal survival birthweight is always to the right hand side of the mean birthweight of any population that has been studied. So we know that there is always a selective favor in terms of bigger

birthweight. I am quoting some rather old data so I am not sure that it would necessarily still be true now that we have probably got pretty much towards maximal types of birthweights, but in the old days certainly it was true that optimal birthweight was always bigger than mean birthweight in any population. It may be true that optimal survival is slightly bigger than the mean size of children. Certainly that would be true in The Gambia, bigger children are going to survive better. So something is holding it in the genome, but I think it is intriguing in terms of our thoughts as to what is the optimal.

Dr. Scholl: I want to ask you about preterm birth or shortened gestation and thymic size because I recall from some of your publications that during the hungry season, when women lose weight, their gestation duration is shortened. When they have these babies that are born before term or preterm perhaps, are their thymuses smaller?

Dr. Prentice: We don't know that from The Gambia because we haven't got a large enough data set. But from the huge data set in Bangladesh we are starting to tease that out, and particularly because we also have serial ultrasound measurements in pregnancy and last menstrual period for all those 1,700–1,800 children, so that will give us excellent data.

Dr. Hanson: What do you know about the milk content of fat components when comparing mothers during the hungry and the non-hungry season? Also would it matter if they had been born during the hungry season or not with regard to the fat content or energy content of the milk?

Dr. Prentice: It is a struggle to look at that because seasonality is both a wonderful opportunity and a hazard. Any particular child, dependent on its day of conception, is going to go through a completely different pattern of exposure and remarkably they seem to cancel each other out. In the survival curve I showed at the beginning, the children were really remarkably similar in the early years of life, so they tend to cancel each other out in terms of many of these phenomena and in fact survival, so that the latent mortality effect was one of the first things that we were really able to show very strongly. Of course we are chasing this down in terms of other effects, in terms of other immune effects, we are trying to look at it specifically in terms of milk. What we can show is that there are some changes in the composition of the milk throughout the seasons of the year and, as I described, changes in immunological factors and also the recent IL-7 work of Ngom et al. [3] show that there are certainly seasonal changes which we think have effects on the infants.

Dr. Boev: With regard to Dr. Walker's comment that he thinks that small bowel atrophy has mainly nutritional and infectious causation, do you think this is direct damage by infection or is it an immunological response to infection, and secondly, what kind of infection, generalized systemic infection or localized gut infection? Is there any evidence of a particular widespread organism causing this?

Dr. Prentice: We would love to know the answers to your questions which clearly have been going through our research for some 20 years, and I am ashamed to say that we still haven't got the bottom of it. One line of research was that this is perhaps a food-related antigen that is creating the problem. I stress straight away that I am not a gastroenterologist, so my comments are perhaps rather naïve. We do think it is infectious in origin, and we have tried to look at particular infectious agents both viral and bacteriological. As you know the general story there is, of course, that children get many diarrheal episodes where you can't even find the pathogenic organism; perhaps we find the specific pathogen in 30% of the cases, those are a very mixed bag of organisms (apart from the rotavirus epidemics that occur in January in the dry season). But one thing that does seem to be coming out pretty strongly is *H. pylori*, and Dr. Solon will soon be publishing data showing that even if you take biopsy tissue from the small

intestine then those do respond to *H. pylori* CAG and VAC antigens, and so it would seem that perhaps the *H. pylori* infection is having a particular aggravatory effect further down the intestine but we are not sure yet.

Dr. R. Bergmann: As I understood, one of the great hazards is weaning food. Why is it not possible to teach people to prepare the supplement from the dry and the fluid ingredient (or the water) just before feeding?

Dr. Prentice: Yes, but it is so very difficult. We have gone down all sorts of lines, for instance fermented weaning foods, acidification, all sorts of lines. The basic problem is the conditions. The quality of the water is not good and it is full of pathogens. One of the problems is that when the mothers cook the weaning foods, they then have to leave it with the nursemaid. A powdered corn flour acts as a thickening agent so if too much of this is used and it is brought to the boil, it creates a glutinous gruel which the children can't eat. So two things are done. One they don't put very much powder in (resulting in a very low nutrient density), and secondly they don't bring it to the boil, and that combination is a real problem. We have been doing a lot in terms of education.

Dr. Ogra: That was a very elegant look at nature, perhaps at its best or its worst, depending on how we look at it. The last picture which you showed was of these beautiful children: the picture of perfect health. Perhaps we should think of this as the positive outcome of benign neglect in nature. These children are from the same environment, yet they have grown, they have survived the whole environment and have become very 'healthy' children. Perhaps this is how nature fosters survival relative to the environment.

In the context of your elegant studies in this setting, is there any relationship between any of the functional alterations you see in the children related to the size of the thymus? Are there any direct correlations between pathogens and weaning foods, the development of asymptomatic infections and mortality or morbidity in the long run?

Dr. Prentice: Let me start with the last question because I can quickly say that I can't contribute any answer to that. It is an intriguing question but we simply don't have the data which would allow us to address it. Going back to your first point, you may be right, that would be one view. We are vaccinating children, we are trying to make optimal survival; in fact evolution has really come almost to an end because we are saving premature babies at 28 or well before 28 weeks, and we are aiding people through IVF to have children when they normally would not have them. That is the way we have chosen to go as a world. Under those conditions then Dr. Barker's theories are important because we do want to have optimal survival. Now it is a real conundrum and problem because, taking hemoglobin for instance, iron deficiency, is something we are really struggling with. In the West we want to optimize iron nutrition. Why do we want to do that? Because we believe that it affects cognitive function, motor development, etc., in children. But in the developing countries (I have to thread a very careful line here) I actually think that a little bit of mild anemia is protective, almost as you are suggesting in terms of growth. One interferes with that at one's own peril, and the recent data from the Pemba trial with an increased mortality in the groups receiving iron are really a manifestation of that concern. There are lots of times when growing small, having a low hemoglobin, etc., have been adaptive and protective, but we have changed the goalposts now and that is what the challenge is. Those children, as you say, look very healthy but if you measured them you would see that they look healthy because their weight for height is actually relatively good. If they are put side by side with a European child, you would see that they are profoundly smaller than the European child. That is what we are really struggling with: what is the optimal rate of growth in terms of optimizing these later outcomes.

Dr. Malka: About 83% of these malnutrition-related deaths were attributed to mild to moderate malnutrition and immune function. My question regards malnutrition among girls, does it affect them later in life like iodine deficiency, cretinism etc.?

Dr. Prentice: Of course it affects the girls. We are just doing some DEXA measurements of pelvic size in relation to total height and size in the girls, and we will shortly publish a paper showing that pelvic size is inappropriately small even given their height. So yes, it would have effects and we would imagine that it would have effects in terms of uterine constraint and other things which would be passed on to future generations; the affect of their early life growth. So that is precisely in line with the sorts of theories that we are addressing at this meeting.

Dr. Cameron: Just to add to Dr. Prentice's comments about the cost of poor growth. It is not just a structural cost, because you have been malnourished as an infant child, you are not just stunted for life. There is also work from Guatemala which clearly demonstrates that there is a functional cost of early malnutrition [4]. Individuals who were stunted between the ages of 3 and 6 showed in early adulthood, at 18 upwards, significantly worse scores on tests of functional ability, mental and physical functional ability. So there is a real cost involved, it is both structural and functional, and clearly that is why we need good growth.

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Neonatal Microbial Flora and Disease Outcome

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Abstract

The now outdated perception of microorganisms of the gastrointestinal tract as pathogens or at best commensals continues to undergo remodeling. It is now clear that the microbiome of the gut participates in many activities including: digestion, ecologic protection from pathogens, and an increasingly appreciated immunoregulatory role in vertebrates. Studies of the complex interactions of microbes and hosts point to a convergence of two well-supported (though imperfect) hypotheses: the 'hygiene hypothesis' and the 'fetal programming hypothesis' proposed by Strachan and Barker, respectively. Our current understanding is one in which factors that exist before conception, during gestation, or occur perinatally in the infant milieu, in addition to exposures to nutrients and microbes, have the potential for long-term effects in the development of healthy offsprings and adults. Epidemiology, basic science and clinical research in such previously diverse areas of study such as microbiology, allergy, gastroenterology, endocrinology, immunology, rheumatology, infectious disease, perinatology, and nutrition are providing evidence that appropriate development and tendency towards the development of certain diseases are directly affected by intestinal microbe–host interactions. It appears likely that perinatal colonization of the gastrointestinal tract is a particularly pivotal process in which microbe-host programming occurs. Intestinal microbes and hosts have co-evolved that, when in appropriate balance, they produce and propagate a life-long mutualism.

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Introduction

The 'hygiene hypothesis', originally proposed by Strachan, suggests that the relative sterilization of the modern world through health practices and improved sanitation has led to a decreased exposure to microbial antigens,

and that this in turn has had an adverse effect on the developing immune system with an increased tendency towards developing allergic conditions. It is increasingly clear that intestinal microbial flora plays an integral role in the relationship of microbial exposures, immunologic programming and when inappropriate, may lead to the development of allergy. Though in itself it is imperfect, there is a substantial support for the hygiene hypothesis and its fundamental prediction that host–microbe interactions that occur early in development have long-term consequence [for review see 1]. A caveat of the hypothesis is that the immune system can also be skewed towards an autoimmune predominant response by exposure to microbes. In multiple models and clinical conditions, exposure to viral or bacterial products promote the development of autoimmune and immune-mediated diseases such as the relationships of Coxsackie B4 virus to type 1 diabetes and *Campylobacter jejuni* to Guillain-Barré syndrome.

Barker [2] and colleagues are credited with the 'developmental origins of adult disease' hypothesis that predicts that events that occur in utero and conditions at birth have potential for long-term phenotypic effects in adulthood. Their initial epidemiologic observations of in utero and perinatal nutrition with sequelae leading to a predisposition for ischemic heart disease has been expanded to multiple other clinical entities including non-insulin-dependent diabetes, hypertension and stroke in adulthood [2]. It has become increasingly appreciated that the intestinal microbial community plays a major role in development of an appropriately balanced Th1/Th2 immune system. The most crude example being that animals born into germ-free conditions have a Th2 predominant immune response. Studies of the sequential (seemingly programmed) colonization of the gastrointestinal tract after birth, the relative stability of a host microbiome, and the influence of prebiotics and probiotics support the adaptation of the Barker hypothesis to a host-microbiome colonization corollary, e.g. that specific (though as yet incompletely defined) host–microbe interactions are necessary and interactions during gestation (with the mother's immune system and microbiome) and particularly with the neonate are essential and have implications into adulthood.

Though the etiologies of many noninfectious clinical entities have been postulated to be associated with microbe–host interactions (see below), one group that continues to become inexplicably prevalent is allergic disease. Allergic disease is the result of an immune system imbalance, therefore it is a useful entity for evaluating the relationship of immunologic programming to the development of disease. The recent increase in allergic disease in the developed world is possibly related to altered microbiologic exposure and immune system programming at a critical window in development (application of both the hygiene and Barker hypotheses). In addition to epidemiologic and basic science research supporting this concept, clinical interventions with probiotics impressively demonstrate that altering the flora of both the mother and newborn modifies the offspring's predisposition to disease [3].

Our understanding of factors that affect maternal health, gestation, programming of the immune system, perinatal microbial colonization, and microbial ecology, as a nexus of interrelated factors leading to the development of health and disease, increasingly point to the mutalistic relationship of the immune system and microbes within the gastrointestinal tract.

Gestation

Many variables including genetic, metabolic, nutritional, toxic and infectious factors affect maternal health and directly or indirectly the health of her offspring. Maternal health before conception can have a significant impact on the health of her offspring [2]. After conception, teratogenic programming can occur in utero either by exposure to a harmful factor (i.e. congenital cytomegalovirus infection) or by lack of exposure to an essential factor (i.e. the role of folate deficiency in the development of spina bifida). There is evidence that maternal immune health and exposure to microbial and dietary antigens play a role in the development of an appropriate immune response in her offspring. Ruiz et al. [4] have observed that maternal atopic dermatitis is a more significant risk factor than paternal atopy. The potential role of maternal consumption of dietary antigens and the risk of food allergy in her offspring has led to the recommendation by some to avoid and others specifically not to avoid certain foodstuffs during pregnancy. It is likely that the exchange of epigenetic programming, antigen and immunomodulatory molecules between a mother and her fetus contribute to appropriate immunologic development [5]. Lending support to this, as well as the likely role of the maternal immune system and microbiome, is the observation by Blumer et al. [6] that when a bacterial product (lipopolysaccharide) is administered to a pregnant mouse it modifies the immune response in her offspring. Together these and additional findings point to the likely role of the maternal microbiome in programming of the immune system during gestation. Figure 1 depicts several of the complex factors that determine the development of immune function and health through different life cycle stages.

Gastrointestinal Immunology

The gastrointestinal tract performs diverse functions including digestion, absorption, immunoregulation, and hormone production. The intestine plays a remarkable and essential (though as yet imperfectly understood) role in the development of immunologic tolerance. Multiple diverse cell populations inhabit the gastrointestinal tract and participate in host defense in addition to cells of the immune system. These include acid-producing parietal cells, mucus-producing goblet cells, defensin-producing Paneth cells, among oth-

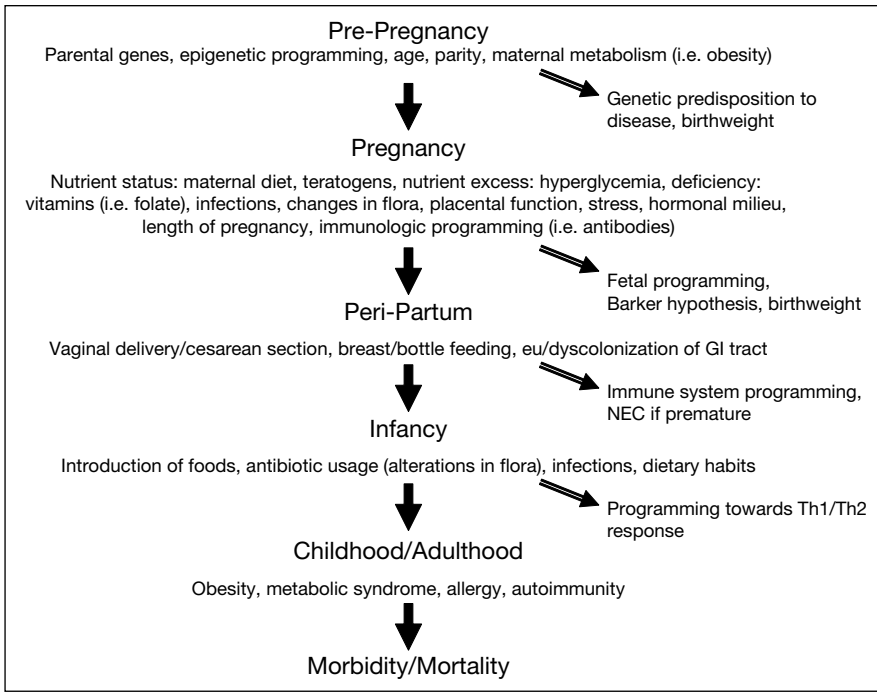

Fig. 1. Schema depicting positive and negative developmental factors which have implications for short- or long-term morbidity and/or mortality. Underlined items are known or hypothesized to affect/be affected by host–microbe interactions.

ers, thereby creating distinct environmental niche conditions and selective pressures, and generating diverse microbial communities in the gastrointestinal tract. From birth the mature human intestinal tract is fully cable of responding appropriately to an array of antigens and pathogens while tolerating the colonization of the intestinal lumen with a plethora of potentially pathogenic, commensal and symbiotic bacteria. The intestine is home to the largest collection of lymphoid tissues in the body. Some are highly organized such as in Peyer's patches and mesenteric lymph nodes while others are diffusely localized dendritic cells and lymphocytes of the intestinal lamina propria and epithelium [7].

The lumen of the gastrointestinal tract needs to be selective in the molecules and signals it transfers. Two highly evolutionarily conserved components of the innate immune system are families of pattern recognition-sensing molecules: Toll-like receptors (TLRs) and Nod-like receptors (NODs). These receptors are able to bind ligands that the host has never encountered and perform an integral role in the interaction of luminal microbes with host

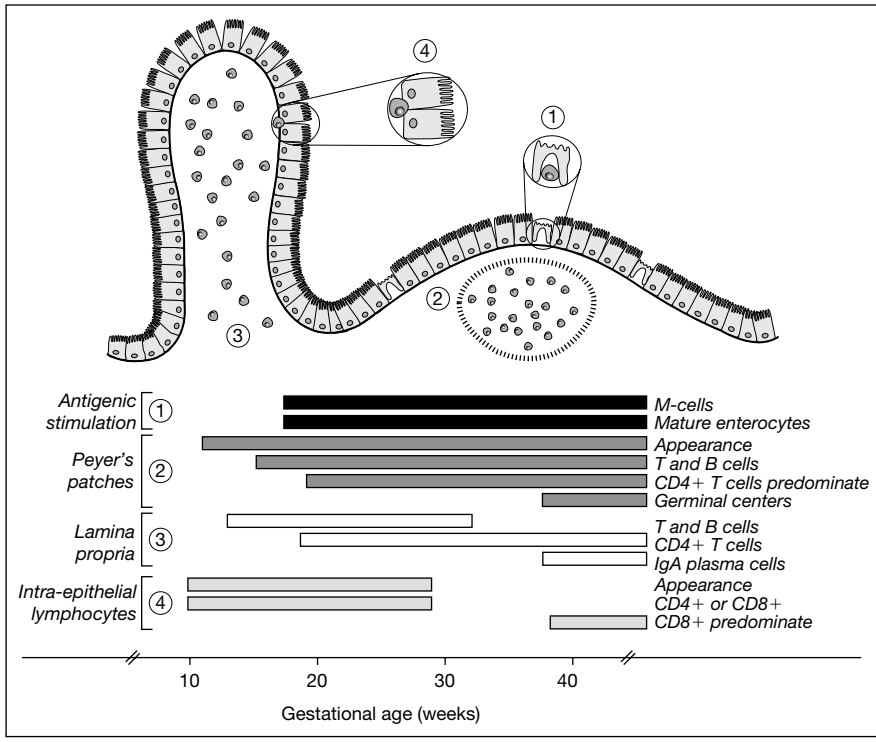

Fig. 2. Cartoon outlining the development of mucosal immunity. Each component (1–4) is affected after delivery and initial colonization, and continually thereafter by interactions with microorganisms.

immune system defense, immune cell recruitment, and inflammation [8]. Lending support to the symbiotic nature between certain microbes and hosts is that TLRs participate in the maintenance of intestinal homeostasis through interaction with specific intestinal commensal bacteria [9].

The interaction of microbes and immune cells in the gastrointestinal tract participates in maturation of the adaptive immune system. The homing of T cell populations to the intestine is associated with the developmental colonization with microbes perinatally (fig. 2). Hooper et al. [10] have shown that a specific product of the commensal bacterium *Bacteroides thetaiotaomicron* participates by directly stimulating host defense mechanisms. In addition the greatest exposure to antigens that the immune system encounters is through ingested antigens and bacterial products. Proper maturation of the immune system has been linked to the colonization of the gastrointestinal tract with specific flora [11]. It is becoming increasingly clear that bidirectional communication

promotes the symbiosis between the host and its intestinal flora. As an example, germ-free animals are known to have an impaired tolerance mechanism. Continuous immunologic vigilance permits and promotes symbiosis and peaceful coexistence with intraluminal microbial communities [12].

Colonization and the Peripartum Period

At birth, animals pass from a sterile environment to one filled with bacteria, fungi and viruses. It has been shown that the method of delivery, vaginal versus cesarean section, has a significant effect on the colonization of humans. It has also been observed that the progression of bacterial species during colonization is altered by the route of nutrition, either breast milk or formula [13]. Indicating that the relationship of gut maturity may need to be coordinated with microbiota, findings suggest that formula-fed infants acquire 'mature' colonization sooner than babies fed breast milk [14]. Immunoreactive factors that are transferred from mother to child in breast milk include immunocompetent cells, immunoglobulins, antimicrobial peptides, growth factors, cytokines, lysozyme, lactoferrin and complement. A recent stimulating observation is that a component in breast milk binds to a TLR [15].

The neonatal immune system is initially characterized by a Th2 response but, as the immune system develops and is exposed to microbes and greater antigenic diversity, this becomes more Th2/Th1 balanced. An additional association is that infants behave clinically as moderately immunocompromised until they reach an age associated with more adult gastrointestinal flora (personal observation). Gronlund et al. [16] have observed that the patterns of colonization of the newborn may affect the development of the naïve immune system.

Ultimately, the human gastrointestinal tract is colonized with greater than 500 species of bacteria. The communities of microorganisms differ along the length of the gastrointestinal tract and across the three dimensions of the lumen and mucous layer. It is calculated that the total number of genes possible by the microbial community colonizing a single human intestine exceeds by more than 100-fold that of a human genome, and only with the recent advance in large scale sequencing have investigators been able to generate profiles of many previously unidentifiable non-culturable species [17]. The known activities of the microbial factory in the intestinal lumen includes digestion, production of nutrients, detoxification/toxification, defense from pathogens, gut motility, angiogenesis, and immunomodulation. Turnbaugh et al. [18] have recently reported a transmissible phenotype of increased capacity for energy harvest and total body fat by inoculation with flora from obese mice to thin mice, thereby identifying a novel effect on physiology by the microbiome–host relationship to the area of energy metabolism. Though populated by a dynamic and complex (and currently ill-defined) ecosystem, it

appears that there is a relative stability of an individual's microbiome. This is borne out by multiple lines of evidence including that antibiotic use or probiotic use have significant but not permanent effects on gastrointestinal microbial ecology.

Immunologic Programming and Allergy

It has been hypothesized that appropriate stimulation by environmental antigens (microbial and dietary) is a stimulus for the neonate to shift from Th2 to Th1 predominant immunologic responses. It is not clear whether these effects occur in utero and/or perinatally. It is likely that the development of allergic disease (and likely some autoimmune disease) is secondary to inappropriate immunomodulation (or lack thereof) by microbial exposures during critical periods. Within the gastrointestinal tract, co-stimulatory signals and nutrition promote (or inhibit) the origin of an organismal tendency towards the allergic state. The study of allergic disease contributes to our understanding of the relationship of microbiology and immunologic dysregulation [19]. The role of exposure to environmental antigens in the etiology of immune system dysregulation has been supportive positive evidence of the hygiene hypothesis proposed by Strachan. Additional understanding is necessary to account for the fact that there has been a concomitant increase in Th1-associated autoimmune disease with the increase in allergic Th2-predominant allergic disease.

Multiple observations support the thesis of the pivotal role played by the colonization of the gastrointestinal tract and immunologic programming including: (1) intervention with probiotics prenatally and in infancy can have a prolonged effect on the prevention of allergic disease [3, 20]; (2) differences in the bacterial composition of the intestinal flora in infants precedes the manifestation of atopy [21]; (3) bacterial colonization in the neonatal period can promote immunologic tolerance [22]; (4) an association between atopy with polymorphisms in NOD genes; (5) microbial flora is necessary in the neonatal period for the development of tolerance [23], and (6) a ubiquitous bacterium promotes anti-inflammatory responses in the intestine [24].

Prebiotics and Probiotics

Probiotics have been well studied and have been demonstrated to have beneficial immunologic effects that influence both systemic and gut-associated immune responses; they likely function by having both direct and indirect (immune system) effects on the microbial community in the intestine [25]. Some of the strongest supportive data thus far for use of probiotics is that they are most effective during the development of the immune system and initial colonization. It is tempting to speculate that these findings support a

theory that these two processes are themselves linked. Clinical entities that have been shown to be ameliorated by the ingestion of probiotics include: childhood infectious gastroenteritis and antibiotic-associated diarrhea, with potential efficacy of probiotic strains in better growth of infants; atopic eczema; inflammatory bowel disease; *Helicobacter pylori* gastritis; neonatal necrotizing enterocolitis; prevention of Candidal colonization in very low birthweight infants, and as a substitute for inadequate initial neonatal colonization through as yet unclear mechanisms [25–27]. The immature intestine is particularly susceptible to the inflammatory entity of NEC which has substantial mortality and morbidity in primarily preterm newborns. The administration of probiotics to preterm infants has been shown to confer a degree of protection from the development of NEC [27]. We have identified an intriguing mechanism by which probiotics downregulate the intestinal innate immune response (unpublished data). In addition, ingestion of probiotics prenatally and in infancy has been shown to have immune effects beyond the mucosa such as skin immune homeostasis in a mouse model [28], as well as affect the transfer of antibiotic resistance genes in mice [29].

Prebiotics are indigestible (to the host) food ingredients that have a beneficial effect by selectively stimulating the growth, activity or both of one or a restricted number of bacteria in the colon [30]. The utilization of prebiotics in addition to probiotics in support of intestinal ecology and prevention of disease is showing positive results [30].

Conclusions

The gastrointestinal tract performs a multiplicity of functions and plays a central role in the development of the immune system as well as immunologic tolerance. The dynamics of microbiome–host interactions are just beginning to be elucidated. There appears to exist a 'founder' immunologic effect on the microbiota associated with colonization in the perinatal period and that microbes play a role in programming of the immune system even before birth. It is our understanding of the interaction of the host immune system with microbial communities pre- and perinatally that permits us to make the observation of the merging of the Barker and hygiene hypotheses. Many questions are yet to be answered including: why and how is an individual's microbiome maintained; are specific species or bacterial molecules associated with specific clinical conditions; how is the complex action (or inaction) of immune tolerance carried out, and why is there a simultaneous rise in autoimmune disease which is traditionally though of as Th1 with the increase in Th2 conditions in the developed world?

Therapeutic strategies are beginning to include our most current understanding of microbiome–host interactions such as the ingestion of polymicrobial probiotic cocktails and prebiotic molecules or immunostimulatory

molecules such as DNA or helminthes. Technological means now exist to begin to address the interactions of entire bacterial communities and their role in immune system function spanning from gestation through adulthood. Ultimately we will continue to generate new models and interventions of microbial–host interactions to promote health and prevent disease.

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Discussion

Dr. Malka: What do you personally suggest by the hygiene hypothesis? In the first year of life, do we have to live with 2 dogs, 2 cats, under dirty conditions?

Dr. Walker: It is a very good suggestion, I am not sure I could make a clinical recommendation at this point other than to encourage vaginal delivery with hopefully the standard development of colonization and to try to reduce the use of antibiotics during that period of time. I can give you a personal experience. In the last 4 years both my daughter-in-law and my daughter have delivered by cesarean section. I had them put their babies on lactobacillus GG for a period of time, just based on what I know about this. I can't say that I would recommend this to everyone, but it seems to me in the absence of appropriate colonization, they might have a greater risk of developing some of the diseases we talked about.

Dr. Wilson: On your last slide on epigenetic programming, unfortunately I didn't see which genes and which cells you were talking about and what the nature of the evidence was. Could you elaborate please?

Dr. Walker: That whole section is to provoke discussion, it's very provocative. I was just quoting the authors' suggestion that allergens in a fetal environment could through epigenetic mechanisms affect methylation of DNA, deacetylation of histone, etc., a persistence in genes that are normally downregulated as the infants are moving towards development. That is total speculation; just a hypothesis.

Dr. Ogra: I am not sure whether bringing together the hygiene hypothesis with fetal programming is a good marriage because there are issues which might not blend in the long run. One of them relates to the corum sensing in the gut in terms of microbial cross-talk and the lack of neonatal exposure. Perhaps it is not simply a lack of neonatal exposure but a lack of appropriate exposure to appropriate organisms regardless of how they are born.

Dr. Walker: I raised this not because I necessarily believe it but mostly because it is the topic of the conference and I would like to provoke some discussion. There is evidence that mothers who are allergic during pregnancy are more likely to produce allergic children, that allergen exposure is likely to produce an allergic reaction. Earlier we talked a little bit about cord cells and it has been shown that there must be some stimulus in utero for the cord cells to produce some of the cytokines they do. This is speculative and I only raised it as a possibility.

Dr. Ogra: Obviously there is fascinating evidence of the cross-talk between the microbes and the mucosa, but I think there is also cross-talk between the organisms themselves which is probably as important, a critical mass of appropriate organisms which might provide either protection or result in the development of disease.

Dr. Walker: You are absolutely right and that is really a topic for another symposium, that microorganisms can communicate with each other. That is one of the criticisms of a probiotic being given to change gut flora, because it is an artificial situation and it doesn't necessarily create a problem, except when it has been shown to have some effect. The other thing that we don't know very much about, and needs to be looked at, is what is going on with the mother during her pregnancy that influences the infant, not necessarily bacteria getting into the amniotic fluid but certain responses the mother might have to microorganisms that are transplacentally passed onto the infant that modulates the infant's response.

Dr. Ogra: Do you have any thoughts about the types or species of probiotics that will be more appropriate for the induction of oral tolerance? Not all probiotics are alike.

Dr. Walker: That is another very good point; probiotics have different functions. But the problem is that only a few probiotics have been looked at extensively. The probiotic, used by the Finnish group in mothers in late pregnancy to affect atopic dermatitis, was lactobacillus GG. Lactobacillus GG is the most studied probiotic. That doesn't necessarily mean that it is better than others, it is just the others haven't been studied.

Dr. Smith: Why don't hygiene and fetal programming explain everything on allergy? There is a report in the UK suggesting a 7-fold increase in acute admissions for food anaphylaxis in the last 12 years, and a 5-fold increase in food allergy admission to hospital [1]. Eczema is increasing in milk studies in the same population, same survey, over 500% in a 30-year period. There was a 500% increase in food allergy in Australia over 10 years [2]. The telephone survey on peanut allergy by Sicherer et al. [3] also showed a doubling within 5 years in North America between 1997 and 2002. We haven't got that much cleaner, we are not using so many antibiotics, and there is still a lot more that we need to know about this. Have you any thoughts where this goes beyond these two conditions?

Dr. Walker: I couldn't agree with you more. I am not saying that the answer to the entire question of increased allergy is because of the hygiene hypothesis. I think peanut allergy and anaphylaxis are probably a different category of allergy that probably represents the more genetically based response. Why there has been an extensive increase over the last few years, I don't know. I am not an allergist so I can't say. Perhaps Dr. Björkstén could talk a little bit to that effect.

Dr. Smith: If it is genetically based are you suggesting epigenetic causes because there is a massive increase? In Malaysia for example peanut allergy is not seen in the native Malay population. I know roasting increases the allergenicity but it might also be the timing at which it is presented. As you suggested timing of bacteria is incredibly important to the types of responses that we get. Perhaps we are controlling things a bit too much with the way that we eat.

Dr. Walker: It is hard to explain the striking increase in specific allergies in a very short period of time. Under those circumstances there should be some environmental

factors perhaps with some genetic predisposition as opposed to a finite genetic defect. It could be an epigenetic response but again this is all speculation.

Dr. Giovannini: There is problem regarding the difference in microflora between natural birth and the cesarean section. Do you have any data on what will happen in the future? We know very little about probiotic strains. We know about *Lactobacillus casein* and bifidus, and we know that there are many problems with probiotics. Why have only two been studied and hardly anything is known about the others?

Dr. Walker: Your point is extremely well taken. This is an area that needs to be looked at much more carefully. Coming back to what Dr. Ogra and Dr. Wilson have pointed out, the gut is filled with many organisms that are not only communicating with the host but communicating with each other, and understanding what exactly is happening by the reductionist approach of using a single probiotic makes it very difficult. It might be better to look at the indigenous flora, for example prebiotics stimulate indigenous bifidobacteria and lactobacilli, and that may be a way to more naturally look at this process in vivo.

Dr. Björkstén: I would like to call attention to microbial diversity. I think that we may be on a dangerous track if we limit our thoughts to a traditional reductionist approach, thinking that one bacteria or one molecule would solve all problems. Less microbial diversity seems to be the only consistent difference in microbial ecology between allergic and non-allergic children. You can have basically the same bacterial species and numbers, for example *E. coli* or lactobacilli, or whatever, but the diversity is larger in healthy children than in atopic children. I would like in particular to ask the immunologists whether perhaps the gut microbial stimulation is a question of having enough hits on the immune system. If you continuously change *E. coli* and other strains of the various species in the gut as a consequence of a continuous exchange with the environment, rather than retaining the same strains for a long time, you get many more events that stimulate the immune system. The search for a single probiotic would thus not solve any problems. The microbial diversity is larger in rural than in urban areas and in children with an anthroposophic compared to a conventional life style.

Dr. Walker: The problem clinically with this whole area is taking observations made in an in vitro situation to a clinical study. So few clinical studies have been done that it is very hard to come back to your suggestion with a clinical recommendation; we haven't enough information. We just need to work more carefully.

Dr. Barker: Can I comment on the rather modest increase in the hygiene hypothesis. The intellectual model for the hygiene hypothesis, when we were thinking about appendicitis in the 1980s, was the polio epidemics which came about as there were improvements in hygiene in Europe. The first country that was hit was Sweden in the 19th century, and then the epidemics spread to different European countries. I don't know of any opposition to the idea that these were triggered by delays in encounters with the polio virus which attended improved hygiene. There must have been quite modest improvements but my goodness they killed a lot of people. The appendicitis epidemics, if they followed the same general model, attended relatively modest standard improvements in hygiene. The polio epidemics came to an end because we introduced vaccines, but the appendicitis epidemics came to an end just spontaneously. By what process could the kind of things that you have been describing induce an epidemic which then goes away?

Dr. Walker: I can only speculate and would suggest, as you pointed out, that using hot water allows for different milieus in which the bacteria will proliferate, and that is probably what caused the inflammation of the appendix. It doesn't take much to disrupt the balance between huge numbers of bacteria, some of which are potential pathogens, some of which are protective, to cause that to occur. The term 'hygiene hypothesis', you are right, is a misnomer, but from the original observation, which was

published in the late 1980s, there has been a lot of revision. What I tried to point out is that, as research has moved from pathogens to commensal or symbiotic bacteria, we are beginning to recognize that just general communication with colonizing bacteria may be what prevents these things rather than a child having to have infections such as hepatitis or influenza or whatever.

Dr. Haschke: In response to Dr. Giovannini's question about whether children born by a cesarean section are more frequently sick; yes, there are data clearly indicating this. They have more upper respiratory tract infections and more asthma. The question I have is, when you look at the different microbiota in infants born by cesarean section or vaginal delivery, and in view of what we have heard from Dr. Hanson that material from bacteria might be transferred through breast milk, could it not be an effect of the later start of breastfeeding in infants born by cesarean section and not only the mode of delivery?

Dr. Walker: It could be and your point is well taken, but this is a very new area. Until a recent paper, which I think Dr. Hanson mentioned, suggesting the mechanisms by which probiotics can get into the breast milk, the feeling was that it was contamination from the skin of mothers who were breastfeeding. It is a possibility that this is also a factor, and I think it is a combination of a number of factors. Children born by cesarean section who lack the initial normal colonization need an additional protective function to prevent them from having allergies, infections, etc. Not all of them get that so obviously there are some factors, and breast milk could be one. But at this point based on what has been reported, I can't say that it is bacteria in breast milk. That has to studied further.

Dr. Maldonado: With reference to the issue of appearing and disappearing epidemics, from the microbiologic standpoint there are a couple of examples of modern phenomena which may be related to bacterial pathogenesis and sanitation. One is rheumatic fever which we know from years of careful observation increased over many decades and actually began to decline before the advent of the penicillin use. In fact we know that the organism itself mutated and became less trophic for heart tissue, and of course as we know now it is much more trophic for skin and soft tissue. Some of these events may actually be related to organisms in the environment as well, and have adapted to something spontaneously or to other influences in the environment. The other example is that neonatal infections seen over the last several decades have also changed in terms of the types of predominant organisms that affect newborns; going from gram-positive infections, then moving along to gram-negatives and changing back to gram-positives again. Those of course have been stimulated by the effects of antibiotic use. Is that perhaps something you have evidence for?

Dr. Walker: There is something that I should have brought up before and that is genetic polymorphisms. It is very likely that some of these are conditions we are seeing in children who have a polymorphism. For example a study was done on children raised in a farm community with exposure to endotoxin; they actually developed allergy and had a fairly high percentage of the TLR4 polymorphisms. I think that confuses the observations we are making in the context of specific association, colonization, no colonization, what type of organisms exist, and so on. This is going to have to be worked out very carefully.

Dr. Malka: The infant born and raised in an inner city like New York has a higher endotoxin level than in the non-inner city infant. In the inner city household the endotoxin level was associated with less atopic dermatitis. At the age of 2 years the non-inner city children started to wheeze, similar to the inner city, metropolitan cohort. How can that be explained?

Dr. Walker: I can't. The child born in New York, in an inner city, could have a high exposure because they live in a low socioeconomic, less clean state. Urban versus

rural is not the answer; it must be looked at very carefully. One also has to look individually at the patient to see what is happening. I don't believe we can make generalizations on these observations, and that is one of the downsides to epidemiologic studies where an observation of one thing is compared with another. In my view that does not tell the whole story.

Dr. Björkstén: I think endotoxin is a marker for something else. In Sweden, we can confirm what has been shown in other places that endotoxin levels are inversely related to skin prick test positivity. In Estonia, however, which has a different environment, the endotoxin levels are much higher and there is zero relationship with skin prick test reactivity or allergic disease. So it seems that the relationship is limited to Western affluent societies. My other comment is that inner city wheezing to a large extent is not IgE-mediated allergic disease but has other causes.

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Impact of Fetal and Neonatal Viral (and Parasitic) Infections on Later Development and Disease Outcome

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Abstract

It is estimated that there are 4 million neonatal deaths and an equal number of stillbirths annually, the majority in the developing world. Neonatal deaths account for one third of deaths in children less than 5 years of age, and at least one third of neonatal deaths are related to infections. Infections also account for 80% of deaths in the post-neonatal period through 5 years of age. There are several viral and parasitic infections which produce fetal and neonatal morbidity and mortality. Neonatal infections occur during one or more perinatal periods: in utero (congenital), intrapartum (during labor and delivery), and early or late postpartum. Here the term perinatal refers to all of these stages of fetal or neonatal infections. The mechanisms of perinatal viral and parasitic infections vary depending on the specific pathogen, however, all begin with maternal infection. Following maternal infection, organisms may produce indirect placental infection with or without fetal infection, direct fetal or neonatal infection, or primary maternal infection and subsequent perinatal sequelae without either placental or fetal infection. Some pathogens may produce infections by more than one mechanism. This brief report will provide an overview of the pathogenesis, general outcomes, and known pathogens associated with perinatal viral and parasitic infections.

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Introduction – The Global Impact of Fetal and Neonatal Infections

It is estimated that there are 4 million neonatal deaths and an equal number of stillbirths annually, the majority in the developing world [1]. Neonatal deaths account for one third of deaths in children under 5 years of age, and at least one third of neonatal deaths are related to infections (fig. 1). Infections also account for 80% of deaths in the post-neonatal period through 5 years of

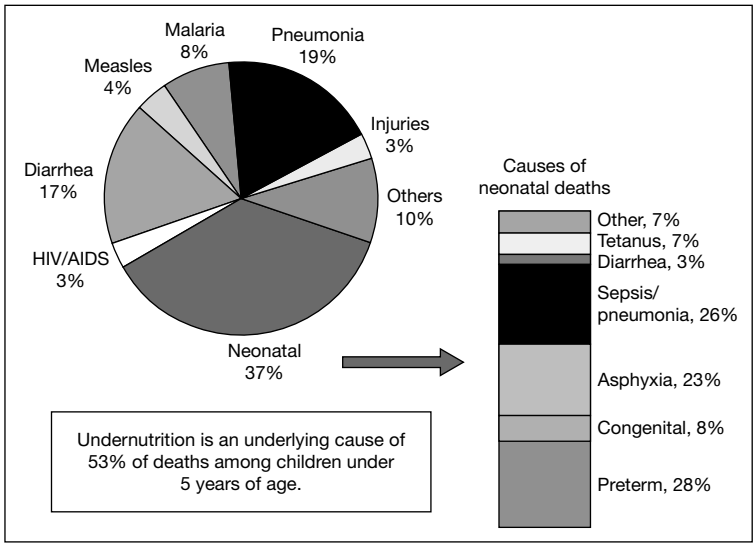

Fig. 1. Major causes of death among children under 5 years of age and neonates in the world, 2000–2003. Source: World Health Organization. Child and Adolescent Health and Development website. http://www.who.int/child-adolescent-health/OVERVIEW/CHILD_HEALTH/ map_00–03_world.jpg. Last accessed February 1, 2007.

age. There are several viral and parasitic infections which produce fetal and neonatal morbidity and mortality. This brief report will provide an overview of the pathogenesis, general outcomes, and known pathogens associated with perinatal viral and parasitic infections. It is beyond the scope of this text to discuss diagnosis and treatment.

Pathogenesis of Fetal and Neonatal Infections

Neonatal infections occur during one or more perinatal periods: in utero (congenital), intrapartum (during labor and delivery), and early or late postpartum. Here the term perinatal refers to all of these stages of fetal or neonatal infections. The mechanisms of perinatal viral and parasitic infections vary depending on the specific pathogen; however, all begin with maternal infection. Following maternal infection, organisms may produce indirect placental infection with or without fetal infection, direct fetal or neonatal infection, or primary maternal infection and subsequent perinatal sequelae without either placental or fetal infection (fig. 2) [2]. Some pathogens may produce infections by more than one mechanism. The most common viral and parasitic infections affecting the fetus and neonate are outlined in table 1.

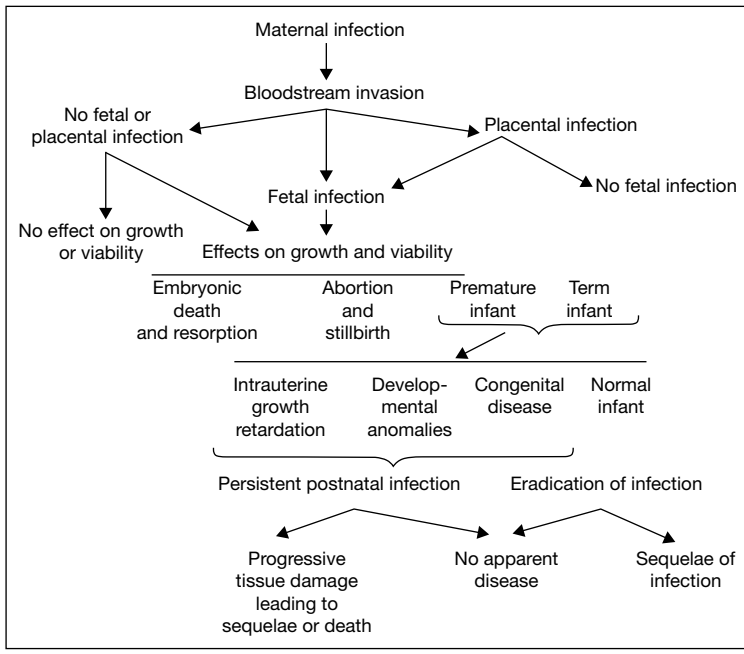

Fig. 2. Pathogenesis of maternal hematogenous transplacental infections. Reprinted from Remington et al. [2], permission pending.

The most common mechanism of fetal infection is transplacental passage of the organism after maternal infection and bloodstream invasion, with or without placental infection. Transplacental fetal infection is most commonly seen in congenital infections with cytomegalovirus (CMV), enterovirus, parvovirus, rubella and toxoplasmosis. Transplacental infections with herpes simplex virus (HSV) and varicella zoster virus (VZV) are rare. Intrapartum infections are most commonly seen with human immunodeficiency virus (HIV), HSV, human papillomavirus (HPV), and VZV, and early postpartum infections occur with HIV and are most common with CMV and hepatitis B. Some pathogens cause fetal or neonatal disease secondary only to maternal infection. Severe systemic maternal symptoms with these organisms may lead to abortion, stillbirth or preterm delivery. This is most likely to occur after maternal infections with malaria and [3, 4].

General Outcomes of Perinatal Viral and Parasitic Infections

Fetal and neonatal outcomes due to perinatal viral and parasitic infections range from asymptomatic disease to death. These outcomes include embryonic

Table 1. Viruses and parasites associated with perinatal infections

Viruses	Parasites
Cytomegalovirus	American trypanosomiasis (Chagas' disease)
Epstein-Barr virus	African trypanosomiasis (African sleeping sickness)
Enterovirus	Ascaris
Hepatitis B	*Entamoeba histolytica*
Human immunodeficiency virus	Giardiasis
Human herpes virus-6 and -7	Malaria
Human papillomavirus	Schistosomiasis (bilharziasis)
Herpes simplex virus	Toxoplasmosis
Influenza	Trichinosis
Lymphocytic choriomeningitis virus	
Mumps	
Parvovirus	
Respiratory syncytial virus	
Rubella	
Varicella zoster virus	
West Nile virus	

death and resorption, abortion or stillbirth, prematurity, intrauterine growth retardation, developmental anomalies and teratogenesis, congenital disease, persistent postnatal infection with progressive disease, or asymptomic infection. The range of outcomes is depicted in table 2.

Congenital TORCH Infections

There is a large body of literature regarding the congenital TORCH infections (**To**xoplasmosis, **R**ubella, **C**MV, **H**SV, **E**nterovirus) [5–9]. The overall clinical syndromes and outcomes are summarized in tables 3 and 4.

Congenital Infections with Other Viruses [10]

Hepatitis B

Hepatitis B is a preventable cause of intrapartum infection, leading to chronic hepatitis B infection in at least 90% of infected neonates [11]. No other congenital symptoms have been identified, although chronically infected individuals have a 25% lifetime likelihood of developing hepatocellular carcinoma. Among infants of hepatitis B-infected mothers, administration of hepatitis B vaccine in the first 12 h of life with concurrent administration of hepatitis B immunoglobulin, followed by additional doses of vaccine at 1–2, 4 and 6 months have 90–95% efficacy in preventing perinatal infection.

Table 2. Effects of transplacental viral and parasitic infection on the fetus and newborn infant

Organisms or disease	Prematurity	Intrauterine growth retardation/low birthweight	Developmental anomalies	Congenital disease	Persistent postnatal infection
Viruses					
Rubella	−	+	+	+	+
Cytomegalovirus	+	+	+	+	+
Herpes simplex	+	−	−	+	+
Varicella zoster	−	(+)	+	+	+
Mumps	−	−	−	(+)	−
Rubeola	+	−	−	+	−
Vaccinia	−	−	−	+	−
Coxsackievirus B	+	−	(+)	+	−
Echoviruses	−	−	−	−	−
Polioviruses	−	−	−	+	−
Influenza	−	−	−	−	−
Hepatitis B	+	−	−	+	+
Human immunodeficiency virus	(+)	(+)	(+)	+	+
Lymphocytic choriomeningitis virus	−	−	−	+	−
Parvovirus	−	−	−	+	−
Protozoa					
Toxoplasma gondii	+	+	−	+	+
Plasmodium	(+)	+	−	+	+
Trypanosoma cruzi	+	+	−	+	−

+ = Evidence for effect; – = no evidence for effect; (+) = association of effect with infection has been suggested and is under consideration.

Modified from Remington et al. [2], permission pending.

Human Immunodeficiency Virus

Perinatal HIV infection accounts for over half a million new perinatal infections and over 300,000 pediatric deaths per year [12]. Transmission may occur in utero, intrapartum, and postpartum via breastfeeding. Perinatal HIV infection is uniformly fatal due to progressive immunodeficiency and death secondary to opportunistic infections or organ dysfunction due to primary HIV infection. Approximately 25% of infants born to HIV-infected women will become infected in the absence of preventive antiretroviral therapy. In non-breastfeeding situations, less than 2% of infants will become infected if

Table 3. Clinical manifestations of neonatal 'TORCH' infections acquired in utero or at delivery

Clinical sign	Microorganism				
	rubella virus	cytomegalo-virus	*Toxoplasma gondii*	herpes simplex virus	entero-viruses
Hepatosplenomegaly	+	+	+	+	+
Jaundice	+	+	+	+	+
Adenopathy	+	−	−	+	
Pneumonitis	+	+	+	+	+
Lesions of skin or mucous membranes					
Petechia or purpura	+	+	+	+	+
Vesicles	−	−	−	++	−
Maculopapular exanthems	−	−	+	+	+
Lesions of nervous system					
Meningo encephalitis	+	+	+	+	+
Microcephaly	−	++	+	+	−
Hydrocephalus	+	+	++	+	−
Intracranial calcifications	−	++	++	−	−
Paralysis	−	−	−	−	++
Hearing deficits	+	+	−	−	−
Lesions of heart					
Myocarditis	+	−	+	+	++
Congenital defects	++	−	−	−	−
Bone lesions	++	−	+	−	−
Eye lesions					
Glaucoma	++	−	−	−	−
Chorioretinitis or retinopathy	++	+	++	+	−
Cataracts	++	−	+	+	−
Optic atrophy	−	+	+	−	−
Microphthalmia	+	−	+	−	−
Uveitis	−	−	+	−	−
Conjunctivitis or keratoconjunctivitis	−	−	−	++	+

− = Either not present or rare in infected infants; + = occurs in infants with infection; ++ = has special diagnostic significance for this infection. Modified from Remington et al. [2], permission pending.

prophylactic antiretroviral therapy is administered to infected pregnant women and their infants [13]. Among breastfeeding populations, at least 10% of infants will become infected despite prophylactic antiretroviral therapy [14]. Efforts to prevent breastfeeding transmission are being studied.

Table 4. Syndromes in the neonate caused by congenital TORCH infections

Microorganism	Signs
Toxoplasma gondii	Hydrocephalus, diffuse intracranial calcification, chorioretinitis
Rubella virus	Cardiac defects, sensorineural hearing loss, cataracts, microcephaly, 'blueberry muffin' skin, lesions, hepatomegaly, interstitial pneumonitis, myocarditis, disturbances in bone growth, intrauterine growth retardation
Cytomegalovirus	Microcephaly, periventricular calcifications, jaundice, petechiae or purpura, hepatosplenomegaly, intrauterine growth retardation
Herpes simplex virus	Skin vessels or scarring, eye scarring, microcephaly or hydranencephaly; vesicular skin rash, keratoconjunctivitis, meningoencephalitis, sepsis with hepatic failure

Modified from Remington et al. [2], permission pending.

Human Papillomavirus

HPV causes condyloma acuminatum (genital warts) and cervical condylomata. Infants born to a mother with HPV infection may rarely develop juvenile laryngeal papillomatosis, and possibly anogenital warts. Infection of the infant probably occurs by exposure to the virus at delivery, although papillomatosis has been described in infants delivered by cesarean section.

Despite the high prevalence of genital HPV infection, juvenile laryngeal papillomatosis remains a rare disease. The incidence of recurrent respiratory papillomatosis is approximately 3.96 per 100,000 in the pediatric population, with an incidence of 7 of every 1,000 children born to mothers with vaginal condyloma. Treatment of anogenital warts is not optimal, but podophyllum resin or podofilox are often used in older children and adults. Neither has been tested for safety or efficacy in children, and both are contraindicated for use in pregnancy. Laryngeal papillomas recur even after repeated surgical removal. Interferon has been used with some success for treatment of laryngeal papillomas.

Epstein-Barr Virus

Epstein-Barr virus (EBV) is a herpesvirus that causes infectious mononucleosis. Most women of childbearing age have been infected asymptomatically in childhood. Primary EBV infection during pregnancy is unusual because only 3.0–3.4% of pregnant women are susceptible. Early reports implicated EBV as a cause of congenital anomalies, particularly congenital heart disease. However, there is little evidence suggesting that natal transmission of EBV occurs. EBV

can be transmitted to newborns in the perinatal period by blood transfusion. There is no evidence at present that EBV causes congenital anomalies.

Human Herpesviruses

Human herpesvirus (HHV)-6 has been identified as a cause of exanthema subitum (roseola). HHV-6 is ubiquitous in the human population regardless of geographic area and infects more than 90% of infants during the first year of life. The usual route of transmission is perinatal or postnatal. No cases of symptomatic intrauterine HHV-6 infection have been confirmed since the agent was identified in 1986, although there is evidence of asymptomatic intrauterine infection. Evidence of re-infection after presumed congenital HHV-6 infection has also been demonstrated. As diagnostic assays become more widely available, congenital infections may be recognized. However, primary HHV-6 infection should be rare during pregnancy because almost all adult women have been infected in childhood.

In addition to roseola, postnatal HHV-6 infection may cause acute, nonspecific febrile illnesses in infants. Other associations among infants include fulminant hepatitis, a mononucleosis-like syndrome, and pneumonitis.

HHV-7 was discovered in 1990. It belongs to the Roseolovirus genus within the Betaherpesviririnae subfamily, along with HHV-6 and CMV. Like HHV-6, it is ubiquitous and causes primary infection during childhood. Symptomatic infection with HHV-7 appears to be less common than with HHV-6. The primary mechanism of transmission is from contact with saliva of infected individuals. Since HHV-7 DNA has been detected in breast milk, breastfeeding may be another source of infection. Pregnancy may be associated with reactivation of HHV-7. However, perinatal transmission from contact with infected maternal secretions is unknown, and neonatal infections with HHV-7 have not been reported. Clinical symptoms are rarely associated with HHV-7 infection, but include nonspecific fever, with or without rash. Clinically apparent HHV-7 infections appear to have a high rate of central nervous system involvement.

Influenza

Population-based epidemiologic studies have not demonstrated that influenza infections during pregnancy are associated with adverse perinatal outcomes. However, influenza infections during pregnancy are more likely to result in hospitalization than for nonpregnant adults. Intrauterine exposure to influenza virus does not cause a consistent syndrome. A number of studies have investigated the possible association between influenza infection in pregnant women and subsequent development of bipolar affective disorders or schizophrenia among their offspring, with mixed results. Infections acquired by infants in the neonatal period are not uncommon and may be fatal. Several outbreaks of influenza virus infection have occurred in neonatal intensive care units. In general, illness has been mild.

Pregnancy is not a contraindication for the administration of influenza vaccine.

Mumps

Congenital anomalies have not been associated with mumps infection during pregnancy, however, spontaneous abortion after mumps infection during the first trimester of pregnancy is increased. Mumps infection during pregnancy may be associated with development of endocardial fibroelastosis in offspring. Parotid swelling and pneumonia in perinatal mumps infection has been reported.

Parvovirus B19

Parvovirus B19, the cause of erythema infectiosum (fifth disease), is a known cause of congenital infection which may result in miscarriage, fetal hydrops and fetal anemia. The risk of transplacental fetal infection and fetal loss are 30 and 9%, respectively. Fetal loss occurs most often in the early second trimester. Parvovirus is associated with up to 20% of nonimmune fetal hydrops. Diagnosis of fetal infection can be based on the detection of virus in amniotic fluid and placental tissue. No treatment or vaccine is available, but intrauterine blood transfusion may prevent fetal loss.

Respiratory Syncytial Virus

There is no evidence that respiratory syncytial virus (RSV) causes intrauterine infection. Maternal infection has no known adverse effect on the fetus.

RSV infections are frequently acquired by infants and are associated with a high mortality rate. Two thirds of all infants will be infected with RSV in the first year of life, one third will develop lower respiratory tract symptoms, 2.5% will be hospitalized, and 1 in 1,000 infants will die [15]. Infection with RSV in infants younger than 4 weeks may be asymptomatic, consist of an afebrile upper respiratory syndrome, or be accompanied by fever, bronchiolitis or pneumonia, and apnea. Deaths occur most frequently in infants with underlying cardiac or respiratory conditions. Premature infants with bronchopulmonary dysplasia are especially likely to develop severe infections.

There is a lack of consensus regarding the use of aerosolized ribavirin in infants with RSV infection. No clear improvement in clinical outcomes is consistent across studies of both ventilated and nonventilated infants with RSV infection. However, there is clear evidence for the benefit of prophylaxis against RSV infection in infants at high risk for complications. A humanized anti-RSV monoclonal antibody preparation, palivizumab, is the preferred method of RSV prophylaxis.

Lymphocytic Choriomeningitis Virus

Lymphocytic choriomeningitis virus (LCV) is spread from animals, primarily rodents, to humans. Person-to-person spread has not been described.

Mice and hamsters are most often implicated as the source of human infections.

LCV infections during pregnancy may be underdiagnosed as causes of congenital infections, and are associated with abortion, intrauterine infection, and perinatal infection. Intrauterine infection of the fetus results in congenital hydrocephalus and chorioretinitis. Other problems include severe hyperbilirubinemia and myopia.

Because apparently healthy mice and hamsters may shed LCV chronically, pregnant women should avoid direct contact with these animals as well as with aerosolized excreta. LCV causes spontaneous abortions. Hydrocephalus and chorioretinitis are common in infants who survive intrauterine infection. Women who acquire an LCV infection during the weeks immediately before delivery may transmit the virus to their infants. Although the total number of intrauterine and perinatal infections from LCV is not large, the incidence of serious sequelae in the infant appears to be high.

West Nile Virus

West Nile virus (WNV) is a mosquito-borne flavivirus that has caused epidemic infections in the United States since its introduction in 1999. Since then, 3 cases of intrauterine and breastfeeding transmission have been reported. While spontaneous abortion and stillbirth have been associated with flavivirus infections, these viruses have not previously been reported to be teratogenic. During 2002, the Centers for Disease Control and Prevention investigated 3 other cases of maternal WNV infection in which the infants were all born at full term with no evidence of WNV infection or congenital sequelae.

Varicella Zoster Virus

VZV is a rare but serious cause of congenital infection associated with fetal death or severe embryopathy [16]. The risk of congenital infection is 1–2%, occurring almost exclusively in the first 20 weeks of gestation. VZV is more commonly associated with intrapartum or early postpartum infection which may produce severe or fatal disseminated disease in the neonate. These infants should be treated with intravenous immunoglobulin (IVIG) or VariZIG if that is available, in addition to intravenous acyclovir. Infants with perinatal VZV infection are at risk for early development of zoster.

Congenital Infections with Other Parasites [17]

Parasitic infections are highly prevalent in most of the world. The placenta serves as an effective barrier, even in infections with malaria and schistosomiasis in which systemic involvement and hematogenous spread are common. Although transplacental infections of the fetus are uncommon, in developing

countries the prevalence of parasitic infections among infants younger than 1 month of age is high, primarily through transmission during or shortly after birth.

Ascaris lumbricoides

Ascaris lumbricoides is the most prevalent parasitic infection worldwide, affecting up to 1 billion people. Because Ascaris may migrate to many organs, worms are occasionally found in the uterus and the fallopian tubes. Fetuses are apparently able to mount an immune response to maternal Ascaris infection, but congenital infections are extremely rare and appear to be benign. Investigators have reported fetal evidence of Ascaris infection in infants as early as 1 week of age and in one infant with failure to thrive and bloody diarrhea at 3 weeks of age who responded to levamisole therapy.

Giardia lamblia

Giardia lamblia causes a localized intestinal infection, with no systemic involvement. Hence, *G. lamblia* infection in pregnancy is not associated with fetal infection. Severe maternal infection that compromises nutrition could affect fetal growth, but such severe illness is rare. Neonatal *G. lamblia* infection can result from fecal contamination at birth. Infected infants are usually asymptomatic. Treatment of pregnant women with giardiasis is generally deferred until after the first trimester.

Trypanosoma cruzi

Millions of people in Central and South America are infected by *Trypanosoma cruzi* (American trypanosomiasis, Chagas' disease). Because of the chronicity of these infections, they have a significant impact on public health.

T. cruzi is transmitted by the bite of an infected vector, the cone-nosed bug. Infections can also be acquired by blood transfusion and transplacentally. Most congenital infections occur in infants born to women with the chronic form of the disease despite the fact that the mother is asymptomatic. Congenital infections occur in 1–4% of women with serologic evidence of Chagas' disease. Congenitally infected infants may develop symptoms at birth or during the first few weeks of life. Early-onset jaundice, anemia, and petechiae are common. Infected infants may have hepatosplenomegaly, cardiomegaly, and congestive heart failure, as well as involvement of the esophagus leading to dysphagia, regurgitation, and megaesophagus. Some infants have myxedematous edema. Pneumonitis has been associated with infection of the amnionic epithelium. Congenitally infected infants can be born with encephalitis or develop it postnatally. The cerebrospinal fluid shows mild lymphocytic pleocytosis. Cataracts and opacification of the media of the eye have been observed. Less than half of congenitally infected infants survive past 2 years of age. Of those who survive for 2 years or longer, 74% have no

serious clinical symptoms despite continued parasitemia. However, subclinical abnormalities may persist. Congenital infections can recur during subsequent pregnancies. The same mother, however, often has healthy children both before and after the affected one.

Trypanosoma brucei gambiense *and* T. brucei rhodesiense

Few cases of congenital infection with *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* (African trypanosomiasis – African sleeping sickness) have been reported. However, congenital infection is most likely underreported. Humans are infected by the bite of an infected tsetse fly. The parasite can be transmitted transplacentally. Transplacental infection can cause prematurity, abortion, and stillbirth. Central nervous system involvement is common in congenital infection and in some infants may be slowly progressive.

The diagnosis should be suspected in an infant with unexplained fever, anemia, hepatosplenomegaly, or progressive neurologic symptoms whose mother is from an endemic area. The parasite can be identified in thick smears from peripheral blood or in the cerebrospinal fluid. In infants, treatment with suramin or melarsoprol has been reported with good results.

Entamoeba histolytica

Entamoeba histolytica infection during pregnancy may be more severe and have a higher fatality rate than in nonpregnant women. Amebiasis has been reported in infants as young as 3–6 weeks of age. In most instances, person-to-person transmission is likely with the mother as the probable source of the infant's infection. Perinatal infections have occurred in countries such as the United States in which the disease is rare.

Most infants reported with amebiasis had severe illness, with bloody diarrhea, sometimes followed by development of hepatomegaly and hepatic abscess, rectal abscess, and gangrene of the appendix and colon with perforation and peritonitis. Maternal amebiasis has also been associated with low birthweight. Infants have been successfully treated with oral metronidazole. Critically ill children should receive intravenous therapy with dehydroemetine or metronidazole.

Malaria

Malaria is a major global health problem, and its impact on pregnancy and infant mortality has been underestimated. Up to 40% of the world's pregnant women are infected with malaria during pregnancy, and it is estimated that annually 75,000–200,000 infant deaths are associated with malaria infection in pregnancy. Those with little or no preexisting malaria immunity have an increased risk of maternal and perinatal mortality. Fetal and perinatal loss may be as high as 60–70% in nonimmune women with malaria. Both the density and the prevalence of parasitemia are increased in pregnant women

compared with women who are not pregnant. The prevalence as well as the density of the parasitemia decreases with increasing parity. Malaria infects the placenta as well as the fetus. Low birthweight is more common when the placenta is infected by parasites than when the mother is infected but the placenta is not. Both maternal anemia and placental insufficiency affect the fetus. Infants who have parasites demonstrable in their cord blood appear to be more severely affected than those who do not have parasitemia at the time of delivery. Studies of the effect of malaria on anemia, low birthweight, and infant mortality in malaria-endemic areas reveal that 3–15% of anemia, 8–14% of low birthweight, 8–36% of preterm low birthweight, 13–70% of intrauterine growth retardation and low birthweight, and 3–8% of infant mortality are attributable to malaria. Maternal anemia is associated with low birthweight, and fetal anemia is associated with increased infant mortality. Malaria therefore contributes to fetal loss, stillbirth, prematurity, and neonatal death.

Common clinical findings in congenital malaria are fever, anemia, and splenomegaly, present in more than 80% of cases. Anemia is associated with reticulocytosis in about half the cases. Jaundice and hyperbilirubinemia are found in about a third of cases. Either direct or indirect bilirubin may be elevated, depending on whether liver dysfunction or hemolysis is the most important process in an individual case. Hepatomegaly may also be present but is less common than splenomegaly. Nonspecific findings include failure to thrive, poor feeding, regurgitation, and loose stools. In developing countries, when malaria occurs during the first few months of life, it is frequently complicated by other illness, such as pneumonia, septicemia, and diarrhea.

Chloroquine alone has been beneficial when used as prophylaxis during pregnancy. Despite widespread use of weekly doses of chloroquine in pregnant women, teratogenic effects have not been confirmed in controlled trials.

Schistosomiasis

Schistosomiasis contributes to infertility by causing sclerosis of the fallopian tubes or cervix. It is estimated that 9–13 million women may be afflicted by genital schistosomiasis in Africa alone. The placenta usually does not become infected until the 3rd month of pregnancy or thereafter. Placental infection is as high as 25% in endemic areas, but the infestations are mild, and there is little evidence that the size or weight of the infant is affected. Placental bilharziasis is not an important cause of intrauterine growth retardation or prematurity.

Trichinosis

Prenatal transmission of trichinosis from mother to infant is rare. Despite this, *Trichinella spiralis* has been found in the placenta and the milk of nursing women as well as in mammary gland tissue, and can be passed to the infant via breast milk.

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Discussion

Dr. Ogra: You have very elegantly outlined the spectrum of infectious agents to which the fetus and the neonate are constantly exposed. It may not be a small number of organisms, and at the moment we may only be looking at the tip of the iceberg. Yet very few of them seem to produce significant disease in the fetal and newborn period. Do you have any thoughts on how important the placenta may be as a determinant of transmission of the infectious agents from the mother to the child? The ability of the fetus to mount an immune response at a given time may also determine the outcome of these fetal infections. Is there any information relative to the tropism of fetal tissues

to different organisms to produce specific disease? We know, for example, that poliomyelitis is not seen in fetal life as can be seen with other infections such as CMV, HSV, rubella, etc. Why don't we find fetal disease with all agents to which a susceptible mother may be exposed?

Are there any specific windows of programming for the development of subsequent disease outcome? I am not talking about hearing loss with CMV, but about something like the development of cancer with chronic hepatitis or the development of neurological disease with toxoplasmosis or heart disease with chlamydia. Are there any specific windows which we might be able to identify for this age group which could be focused on for future investigative effort?

Dr. Maldonado: Dr. Wilson and Dr. Arvin in my group have looked at HSV and CMV over the years, specifically at maternal but less at fetal responses. Clearly there is evidence that the fetus can to some degree mount immune responses to these organisms. However, the vast majority of the information really points to the maternal response as being the primary mechanism of prevention of disease in the infant, and most of the studies are epidemiologic as well as directly case-based. In the cohorts of women who are infected with particularly CMV or HSV, for which the group at Seattle is quite well known, the epidemiologic studies clearly show that women with predisposing immunity are much less likely to have infants with infection. We do know, of course, that the placenta plays quite an important role, and again as far as I can tell there haven't been studies comparing the roles of placental involvement versus maternal immunity in quite the depth that I would like, but maternal immunity plays quite an important role for the majority of these. Now having said that, there are organisms such as toxoplasmosis, enterovirus, some of the parasitic infections, malaria in particular, where immunity is important but does not guarantee the absence of infection in subsequent pregnancies, whereas that tends not to be the case for herpes or CMV disease. So clearly there are differential responses. The argument for placental disease and placental protection is an important one which is very difficult to assess. There are very poor data looking at that and at this point most of those are hypothetically based on animal studies. One of the prime examples of how the placenta can be effective is the absence of the placental involvement with HIV disease. With HIV infection there may be cell-free HIV that crosses the placenta, but primarily it is related to CD4-infected cells which cross the placenta, and the fact that the immune system is impaired really leads one to understand that those cells, in the absence of HIV and other infections, do not produce disease whereas since the target, the affector cell, is also the infected cell, the effect of losing the placental barrier results in about 20–25% affect of transmission.

Your final question about windows of opportunities; I think this will really vary depending on the disease. For instance, there have been efforts to develop vaccines which were quite unsuccessful in the past; in fact currently a circumsporozoite vaccine for malaria is being studied at a number of sites, and HSV and CMV vaccines have also been studied. Clearly the time to intervene would be before the reproductive age. HPV vaccine is for other reasons, not because of fetal and neonatal infection, best given in the pre-reproductive time. Rubella vaccine is another example of a vaccine that can be given at a time when immunity really prevents infection that would occur 15–20 years down the line. So the windows of opportunities for vaccinations are quite important. However, possibly because of latency and possibly because of poor efforts, and the lack of effect of immunogenicity on disease outcome, these viruses have been quite difficult.

Dr. Scholl: I have a question about breastfeeding by HIV-positive women. There is quite large transmission even when the mother is using antiretroviral therapy. Has anyone tried simple interventions such as heating the breast milk?

Dr. Maldonado: First, there are a number of studies that clearly demonstrate very low HIV transmission rates if pregnant women are treated with a highly active three drug regimen. Regarding heating of breast milk, yes, that has been done. Super heating is one of the areas that has been looked at. In fact it was part of the NIH master plan and we tried to prioritize what kind of strategies we can use outside the USA, primarily because in the USA most women don't need to breastfeed if they are infected. There is an easy way to super heat the milk even in the home, but the problem is that it is very difficult to even provide formula in many situations. So yes it is possible to heat breast milk, but in the developing world that it is not something that is easy to get into the community. The other issue is stigma; if a woman doesn't breastfeed people know why she isn't breastfeeding The WHO in their statement on changing breastfeeding practices through 6 months gave a very important signal to these women that it is alright to actually stop after 6 months. So that is a very good point that may not be able to be translated into reality.

Dr. Prentice: In relation to this issue there is a very exciting paper in the Lancet by Coovadia et al. [1] from Kwazulu Natal which really shows some very different numbers and is obviously going to reignite this whole debate, and I think it is already doing so. The 3- to 6-month risk for exclusive breastfeeding was actually only 4% and that was increased 11-fold by the introduction of replacement feeding. The mortality of exclusively breastfed children was 6% compared to the 15% of those receiving replacement feeds. So it is very much in the same direction of the other data but somewhat stronger.

Dr. Maldonado: We were talking about what to do next and it is really unclear. The issue is that the data strongly suggest that replacement feeding is not a panacea; after 6 months the risk of mortality among non-breastfed, non-HIV-infected babies is low, but it is not zero. The issue is that in the most recently done trials the risks are higher than anticipated and the transmission rates are still high. The other issue is that only about 30% of women around the world really exclusively breastfeed even in the first 3–4 weeks of life, and it is very difficult to promote exclusive breastfeeding all the way through 6 months. Although not proven, studies have suggested that exclusive breastfeeding may be better and actually the transmission rates of HIV may be higher among mixed breast-feeding babies, especially when solids are introduced before the first 6 months of life. At this point we are rethinking ways to come up with a replacement feeding which may help. Of course micronutrient supplementation does not seem to make a difference either.

Dr. Walker: As we all know the first 2 months of breastfeeding is probably the most important time because the most protective factors are delivered then. If breast milk is super heated, then nearly all the protective factors are killed. How extensively has antiretroviral treatment been studied in conjunction with breastfeeding compared to non-breastfeeding in terms of morbidity? Has that been looked at because this is a fairly new phenomenon? We don't really know whether antiretroviral treatment during breastfeeding is less or more dangerous than if the baby didn't get the breast milk.

Dr. Maldonado: It took us about 5 years to go from our phase 1 trial of infant antiretroviral therapy plus breast feeding to our phase 3 trial because of FDA issues and getting the study approved in the USA. We did our phase 1 trial in 75 mother–infant pairs in Zimbabwe several years ago. The numbers were quite small and the regimen was very safe, and there was some transmission but again statistically not significant. There were 2 infected infants out of 75 who were breastfed and received nevirapine. The current study of 1,500 mother–infant pairs is powered to look at about a 25% reduction in transmission. Other alternatives would be to try to vaccinate the infants with the canary pox vaccine which is undergoing phase 1 trials now in Uganda.

Dr. Martinez Cabruja: In the tropics there is an endemic viral infection called dengue which is transmitted by a mosquito. Do you have any data about the effect of dengue infection on the fetus during the first trimester of pregnancy?

Dr. Maldonado: There is not a lot of prospective data but the suggestion is that severe dengue actually works much more like malaria, like measles than influenza. The effect is primarily on the maternal side and will lead frequently to stillbirth or to early embryonic resorption. To my knowledge there is no evidence of PCR-based identification of the virus on the fetal side.

Dr. Abdelmoez: What is the mechanism of neonatal bilharzial infection, schistosomiasis?

Dr. Maldonado: Schistosomiasis affects the placenta. There is infection of the placenta and it affects the fetus in that respect. Actually schistosomes can be found in placental pathology in the small studies that have been done. Primarily there can be placentitis and then secondarily schistosomes will be identified in the fetal liver There are very few studies looking at the pathology in the fetus.

Dr. Kumara: Your data show that the incidence of HIV, using AZT and 3TC, is 14.9% compared to breastfeeding which is only 1.9%. This causes me some concern because we use AZT and 3TC according to the WHO recommendations but without breastfeeding. What is your opinion?

Dr. Maldonado: The issue is about the effect of antiretroviral therapy on HIV transmission and unfortunately there are different standards of therapy depending on what part of the world you live in. The WHO is very clear that the preferred regimen worldwide is AZT plus a single dose of nevirapine to the mother and the infant within the first 72 h of life. However, in the USA and the developed world the standard is the zidovudine treatment which begins in the second trimester of life as oral therapy, intravenous therapy during labor and delivery, and then 6 months of oral therapy to the infant after delivery. That is the recommended regimen, but in practice in the developed world most infected women, if they have access to care, will be receiving the triple therapy which in fact resulted in that 1.6% transmission rate. Using the AZT regimen only results in a two-thirds reduction. Although that is quite large it reduces the transmission from 25 to 8%; using the triple therapy in the women along with the AZT backbone results in an about 1–2% transmission rate, and using the WHO recommendations will probably result in something in the order an 8–10% transmission rate without breastfeeding, with breastfeeding you might add another 5% to that.

Dr. Kumara: Are your data without or with breastfeeding?

Dr. Maldonado: Without breast feeding, no breast feeding.

Dr. Wilson: The bottom line is that nevirapine is certainly not the ideal form of therapy for the trial you are looking at; it is very clear that the decision to use nevirapine is based on reasons other than efficacy. Now the real world reality is the real world reality, but this begins to smack of the problem that the WHO created with multidrug-resistant, now extremely drug-resistant TB, where they stuck with recommending a regimen that we knew was likely to fail. If in fact the goal is to drop the HIV prevalence in the world to as low as we possibly can in order to try to do something real against this epidemic, why are we doing things that we know are inadequate?

Dr. Maldonado: We have been trying to start this study for 6 years instead of doing the simpler generic triple study earlier on. The point is that originally when we first began these trials outside the USA, AZT treatment first had an unacceptable reduction of two thirds and we knew that HAART (triple antiretroviral therapy) therapy could reduce even more. But at the time triple therapy number one was quite expensive, it involved 3 different very expensive drugs that are given individually. As we know today those 3 drugs are now available in one pill, and more importantly as of 3 or 4 years ago those 3 drugs could be bought in a generic form for much less.

Unfortunately through the NIH system we were not allowed to use generic drugs in any of our trials. It was only about 3 years ago that we were allowed to do that, which is why we have been able to go forward with this trial. Even though the drug was being provided free of charge to us, the idea that we would move forward with the generic treatment was unacceptable. So we went through many proposed regimens before we set down one that would be acceptable to the funding agencies.

Dr. Wilson: But I think the question was right on: why is the WHO recommending a regimen that we know is inferior?

Dr. Maldonado: Another major concern is about long-term toxicity, primarily in the form of resistance in the mother who will receive one dose but may receive more than one dose if she has multiple infants, and potentially in the infant too. We are trying now to alter these trials. In fact there is presently a trial in South Africa using triple therapy but it is funded by different mechanisms and probably not powered the same way to answer the question properly. Probably the best practices will lag behind what is available by about 3–5 years at least. So currently if we were able to, we would be doing a triple therapy generic trial.

Dr. Kumara: I have additional questions that I think also need Dr. Walker's comments. What possibly happens with the microbiota of the neonates born vaginally to mothers with those infections?

Dr. Maldonado: What happens to the infants of the women who die? We don't know. That is again an operational question, and most of us who are involved with these studies actually spend a lot of the time not doing scientific trials but setting up community-based programs to sustain our programming for the women rather than doing clinical interventions. Many of us bring in funding to provide prenatal care, to find homes for the children, and those are additional costs that we seek funding for. We do a lot of community-based work, and governments and NGOs have been very useful for us in that situation. In sub-Saharan Africa the foreign donors have been quite helpful. In Asia the NGOs have received quite a bit of funding, not enough, but funding to support the care of those infants.

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Environmental Influences on the Development of the Immune System: Consequences for Disease Outcome

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Abstract

Early T cell responses to external antigens and autoantigens are subject to a variety of regulatory mechanisms. A unifying link between the increase in both Th1-dependent autoimmune disease and Th2-linked atopic allergy would be a disturbed immune regulation involving T regulatory cells. There is a strong global correlation between childhood wheezing and diabetes. It is increasingly recognized that microbial colonization of the gastrointestinal tract, linked with lifestyle and/or geographic factors, may be important determinants of the heterogeneity in disease prevalence throughout the world. These suggestions are supported by observations that germ-free mice do not develop tolerance in the absence of a gut flora. The potential effects of environmental stimuli on immune function is greatest in early life including fetal life when systems and responses are developing, and the maternal influences during fetal life could be particularly important for the development of immune regulation and tolerance induction. In recent years, focus has switched from searching for environmental risk factors towards an interest in factors that could induce and maintain immune regulation and tolerance to allergens and autoantigens. Currently evaluated strategies include the use of immunomodulatory factors, such as probiotics, prebiotics, and dietary nutrients, although data are still insufficient to make specific recommendations.

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Introduction

The prevalence of allergies, diabetes, inflammatory bowel disease and other 'immunologically mediated diseases of affluence' has increased progressively, particularly over the last 50 years. Two distinct, but rapidly converging, areas of research, i.e. the hygiene hypothesis and the study of probiotic/prebiotic

effects, have emphasized the need to understand, and ultimately to manipulate, our physiological interactions with commensal microbiota. The story began with allergic disorders but now type 1 diabetes and inflammatory bowel disease are increasingly involved.

Here the influence of environmental factors on the development of immunological mechanisms governing host responses to allergens and autoantigens will be discussed. Considerably more is known about the environmental impact early in life on allergy than about the interaction between the environment and the immune system in relation to the development of autoimmune disease. The reason is that the much higher incidence of allergies appearing early in life has made it possible to conduct prospective observational and intervention studies during the first years of life and thus to better understand how immune regulation to allergens develop. The focus in this presentation will therefore be on allergy. It is reasonable though that the major lines indicated here are also relevant for the understanding of why diabetes, inflammatory bowel disease and some other diseases are increasing in many parts of the world.

Immunological Background

It is clear that T cells responsive to both dietary and inhalant allergens, as measured by lymphoproliferation and cytokine secretion, are present in cord blood from virtually all subjects [1, 2]. Additionally, T cell cloning and subsequent genotyping studies indicate that the responsive cells are of fetal origin and exhibit a Th2-polarized and/or Th0 cytokine profile. It has been suggested that these T cells may have been primed by processed antigen crossing the placenta, perhaps bound to maternal IgG. Evidence showing the presence of detectable levels of allergen in complex with IgG antibodies in cord blood supports this suggestion [3]. However, it is also feasible that these T cell responses may be directed against cross-reacting antigens or anti-idiotypic antibodies.

These early T cell responses are subject to a variety of regulatory mechanisms postnatally, which are driven by exposure of the infant immune system to environmental antigen. A broad range of regulatory mechanisms are involved, which are dictated by the concentration, frequency and route(s) of antigen (allergen) exposure and developmental status of the individual at the time of exposure. The relevant immunoregulatory mechanisms involved are likely to span the full range from classical low zone tolerance to high zone tolerance phenomena (anergy and/or deletion via apoptosis), and will include contributions from subsets of T regulatory cells.

Cross-sectional and prospective studies indicate that, in atopic children, consolidation of Th2-polarized immunity against inhalant allergens is initiated in early infancy [1, 4] and may be completed by the end of the preschool years in children who do not develop clinically manifest allergy [4], or even earlier

[1]. In contrast, in infants who develop allergic manifestations, low level Th1 responses are established. Prospective studies from Estonia with a low and Sweden with a high prevalence of allergy indicate that the regulatory mechanisms are established more rapidly in Estonia [1]. It is possible that a traditional life style is associated with an early induction of a general regulation of T cell immunity. This notion is supported by the close correlation globally between the prevalence of wheezing and type 1 diabetes [5]. Thus, a unifying link between the increase in both Th1-dependent autoimmune disease and Th2-linked atopic allergy would be a disturbed immune regulation involving T regulatory cells, rather than merely either Th1 or Th2 immunity.

It is recognized that interaction with the normal microbial flora of the gastrointestinal tract is the principal environmental signal for postnatal maturation of T cell function (in particular the Th1 component) [6, 7]. Recognition of these signals is mediated by a series of Toll-like receptors (TLRs) expressed on cells of the innate immune system, and other receptors such as CD14, and it is noteworthy that a polymorphism in the CD14 gene has been associated with high IgE levels [8].

Microbial colonization of the gastrointestinal tract, linked with lifestyle and/or geographic factors, may be important determinants of the heterogeneity in disease prevalence throughout the world [6] and ongoing cohort studies are focusing in detail on this complex question. These suggestions are supported by observations that germ-free mice do not develop tolerance in the absence of a gut flora [9, 10] and by the demonstration of differences in the composition of the gut flora between infants living in countries with a high and a low prevalence of allergy and between healthy and allergic infants [for a summary see, 6].

The Prenatal Environment

The frequent appearance of allergic symptoms in the first months of life suggests that disease pathways are initiated very early in life, possibly even before birth. This has lead to interest in the role of environmental exposures in pregnancy. Although there is growing evidence that maternal exposures, including microbial products [11], smoking [12] and dietary factors [13], can influence infant immune development, experience of prevention strategies are still limited in pregnancy.

The adverse effects of maternal smoking in pregnancy on infant lung development are well recognized. There are also strong associations between maternal smoking in pregnancy and reduced lung function in later childhood [14]. The adverse effect of antenatal smoke exposure on lung function was much greater than subsequent postnatal effects. More recent studies also suggest that maternal smoking could have additional immune effects, which could contribute to allergic risk [12].

Very recent studies with probiotics suggest that the maternal influences may be more pronounced in tolerance induction than previously appreciated. There are now at least three studies trying to prevent food allergy and infantile eczema with lactobacilli. In the study with a negative outcome [15], the bacteria were given only to the babies, while in the two studies with some protective effect [16–18] they were also given to the mothers during the last month of gestation.

There is growing interest in potential proinflammatory changes in Western diets, including the specific role of dietary components with recognized immunomodulatory effects such as antioxidants and polyunsaturated fatty acids. As discussed in recent comprehensive reviews, the potential effects on immune function could be greatest in early life, including fetal life, when systems and responses are developing. Maternal dietary antioxidant intakes (vitamin E) have been associated with neonatal immune responses to allergens [19], justifying further studies on the effects of antioxidants on early immune function. So far, there has only been one intervention study in pregnancy to examine the effects of dietary nutrients on immune function. This study demonstrated that maternal n-3 polyunsaturated fatty acid (fish oil) supplementation had effects on neonatal immune function [13].

Postnatal Environmental Influences

There is consensus that breastfeeding has multiple health benefits and should be encouraged. This is particularly true in developing countries where the protection against infections may be a matter of life or death. Human milk affects the host defense and immunity of the infant in several ways [for review see 20, 21]. It provides passive protection against infections through numerous components of innate immunity and IgA antibodies, but it also provides the baby with components that enhance the development of the immune system. It is well established that human milk often contains food antigens that may induce IgE antibody formation. Less is known regarding the immunologic consequences of introducing foreign antigens while the infant is still breastfeeding. As indicated by studies on immunity to infectious agents, it is possible that this represents a mechanism by which immune responses are modulated. In the early 1990s, there was a pronounced increase in the incidence of celiac disease among Swedish infants [22]. Prior to the increase in celiac disease, gluten was gradually introduced while the baby was still being breastfed. Then, gluten was avoided for the first 6 months and then more or less abruptly introduced in large amounts. When the national recommendations were changed back to a gradual introduction of gluten while the babies were still partly being breastfed, the incidence of celiac disease dropped rapidly.

It is intriguing that microbial stimulation, in particular via the gastrointestinal tract, has also been implicated as an etiologic factor in respiratory

allergic diseases. This suggests that microbial stimuli from the gut exert effects beyond the mucosal tissue microenvironments adjacent to sites of exposure, and presumably can influence systemic precursor compartments such as bone marrow and thymus. The underlying mechanism(s) are likely to include stimulation of functional maturation of cells within the innate and adaptive immune systems during the early postnatal period, a process which may ultimately determine the overall efficiency of immune/tolerance induction during early life, with major flow-on effects into adulthood. A full understanding of the underlying mechanisms may open new venues for prevention by the modification of gut microflora, not only of local disease manifestations, such as food allergy, but also conceivably of diseases with manifestations at distant sites, such as diabetes and respiratory allergies.

While sensitization is a strong risk factor for persistent asthma, wheeze and bronchial hyperactivity, the relationship between early allergen exposure and the development of clinical symptoms has been much harder to confirm. The hypothesis that allergen avoidance early in life would prevent asthma is based on two independent observations, i.e. that exposure to high levels of inhaled allergen is associated with an increased likelihood of sensitization and that asthmatic children are often sensitized in early childhood. No studies have confirmed that the two observations are related to each other, however.

Bacteria are the most powerful immunostimulants in the normal environment, activating the immune system through a range of 'pattern recognition receptors' (TLRs). Although TLRs are found principally on cells of the innate immune system (including granulocytes, monocytes, and natural killer cells), they are also present on cells involved in programming and regulating 'adaptive' immune responses (such as antigen-presenting cells and regulatory T cells). It has been proposed that early microbial activation of both antigen-presenting cells and regulatory T cells may promote Th1 maturation and play an important role in reducing the risk of Th2-mediated allergic responses [23]. This is supported by animal studies demonstrating that bacterial lipopolysaccharide endotoxin exposure can prevent allergic sensitization if given before allergic responses are established [24]. These effects may be of greater significance in genetically susceptible individuals who appear to have weaker Th1 responses in the perinatal period [4]. Genetic studies also support a role for the CD14 /lipopolysaccharide [8] and TLR [25] pathways in the development of allergic disease.

Intestinal microbiota are arguably the most abundant source of early immune stimulation, and contribute significantly to the 'microbial burden' in early life. A number of studies have suggested differences in colonization patterns of infants who go on to develop allergy [for review see 26]. These differences were already apparent at 1 week of age, suggesting that early colonization can influence subsequent patterns of immune development. Studies in germ-free animals confirm that a microbial gut flora is essential for the development of oral tolerance and for the induction of normal immune

regulation [10]. The controversy regarding the role of gut bacteria in allergy development thus lies in the clinical consequences of these findings and not as much to what extent they affect the immune system.

Studies investigating the relationship between early childhood infection and atopy risk have been inconsistent or difficult to interpret. The immunological effects of microbial agents differ with the type of infectious agent and the site of infection [27]. Differences are also seen in the responses to vaccine antigens compared to the wild-type infections they prevent. Furthermore, nonpathogenic colonizing organisms are also likely to play a central role in immune development [26]. A recent large Danish national cohort study including 24,341 mother–child pairs found that early infections do not protect from atopic dermatitis [28]. However, they observed that other environmental factors, sometimes taken for indirect markers of microbial exposure (such as early daycare attendance, having 3 or more siblings, farm residence, and pet keeping), were protective. It is possible though that these protective factors are due to factors other than microbial exposure. For example, the inverse relationship between the number of older siblings and allergy risk may be due to altered maternal immunity as a consequence of repeated pregnancies, and exposure to animals could possibly be explained by high zone tolerance induction. This highlights the emerging concept that overall 'microbial burden' rather than specific infections may be more relevant in early life [29].

The growing awareness of the potential importance of early microbial exposure for early immune development has prompted speculation about the role of antibiotics and other antimicrobials in the first year of life. Several authors have subsequently assessed the possibility that antibiotics may be a risk factor for the development of asthma and other allergies and the results are slightly conflicting. It seems reasonable to conclude, however, that usage of broad-spectrum antibiotics but not penicillin in the first year of life is associated with an increased risk of allergic disease, although the data are conflicting.

It is logical to explore the benefits of probiotics earlier when immune responses are still developing, and there are now a number of studies addressing the role or probiotics in primary allergy and diabetes prevention (in Australia, Finland, New Zealand, Singapore, Sweden and the United Kingdom), examining the effects of various probiotic strains using direct infant supplementation. Some are still in progress.

As it appears increasingly unlikely that supplementation with a single probiotic strain will be sufficient to overcome the high environmental pressure to develop allergic disease, there has been a shift in interest to dietary substrates that could potentially have a more global effect on gut flora, namely *pre*biotics. Prebiotics are non-digestible but fermentable oligosaccharides (food starches) which specifically stimulate the growth of bifidobacteria and lactobacilli species. Altering the intake of foods containing these products can directly influence the composition and activity of intestinal microflora. This could

explain some of the protective effects of grains and cereals that have been seen in epidemiologic studies [30]. At this stage there are still very little data to directly confirm the immunological or therapeutic effects of prebiotic supplements, although one recent study has reported encouraging results [31].

Potential for Prevention

Most previous approaches to prevention were based on avoiding candidate factors which could be implicated in the development of disease. These studies have not been successful. In recent years, focus has switched from searching environmental risk factors towards an interest in factors that could induce and maintain immune regulation and tolerance to allergens and autoantigens. Thus, current research is more directed towards an understanding of how immune regulation develops and how tolerance could be induced early in life. More recent strategies include the use of immunomodulatory factors such as probiotics, prebiotics, and dietary nutrients (such as n-3 polyunsaturated fatty acids) although data are still limited and there is still insufficient evidence to make specific recommendations.

Until the 1970s textbooks in pediatrics did not discuss prevention strategies, or only mentioned them in passing. The increasing awareness of environmental pollution and the continuing increase in the prevalence of allergies, diabetes and other immunologically mediated diseases brought public attention to environmental factors that could explain the increase. The lesson learnt from 20 years of epidemiological analyses, observational studies and recent intervention studies, has so far not been successful in developing strategies for prevention.

Recommended preventive measures should be based on scientific documentation that is evaluated equally as strictly as for medical treatment, because even seemingly innocent advice may have a profound impact on a family. The World Health Organization has defined certain principles for decisions on preventive measures. First, the disease should be common and have potentially serious consequences. Second, the causes should be known and measures should be effective, safe and acceptable. There should also be resources for implementing the measures. Finally, the health economic consequences of the measures should be known.

It could be argued that it is never harmful to give health-promoting advice regarding, for example, the value of breastfeeding, ‘good’ nutrition, and the harm of tobacco smoking. Even seemingly innocent advice may have negative consequences, however, e.g. parental guilt feelings that not enough was done if the child develops disease. Advice on diet may interfere with optimal nutrition, customs and family economy; cleaning procedures may be taken so far that they interfere with daily life; ‘good’ ventilation may come in conflict with energy conservation, and ‘no air pollution’ may prevent a family from painting

the house. Advice on pet avoidance may profoundly affect a family with a loved pet, and visits to grandparents who keep pets and may even force people to move from a farm. Thus, the consequences of advising preventive measures should always be considered, including how advice may be interpreted by those receiving it.

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Discussion

Dr. Walker: You did a great job for the last lecture since everything has previously, presumably at least, been mentioned. The concern I have is that your study used a different probiotic than was used in the Finnish study; it is like comparing apples and oranges. Probiotics may function differently in different situations.

Dr. Björkstén: Yes, theoretically. Going through the literature, however, it seems that all probiotic lactobacilli actually work in the treatment of infantile gastroenteritis. They also seem to reduce lactose intolerance and prevent diarrhea induced by broad-spectrum antibiotics. The effects have been similar for several strains, provided that you give reasonable amounts of live bacteria. But you are quite right and as I showed, there are differences in the capacity of different lactobacilli to induce T regulation. Three allergy prevention studies show some effect. The only negative study was the one in which treatment started after birth.

Dr. Wilson: I too would like to thank you for an informative and entertaining talk, highly useful at this time of the day. I have a comment that may be relevant to the lactobacilli studies you and others have been doing. This is a gram-positive bacterium. Thus, the major TLRs through which it will act are 2+1 or 2+6. Soon to be published are studies showing allelic differences in TLR1, both alleles have a frequency of approximately 30–35% in North American populations of European ancestry. One of these two alleles results in hyporesponsiveness to TLR2+1 ligands. You will need to control for that kind of variability if you are using an organism that is principally going to be acting through those TLRs.

Dr. Björkstén: You are right. I would expect different outcomes in different populations for the reasons you have given. We do not even know if gram-positive bacteria should be used, as there are several reasons why the gram-negative flora actually could have more of an impact on immune regulation.

Dr. Wilson: I think that the state-of-the-art of looking at the diversity of microbial flora is really sequencing – essentially sequencing is the only way to determine what the true diversity of microbes is, as Dr. Gordon has shown. I wonder whether any of the samples that you or other studies have accrued are stored so that such an analysis could be done retrospectively? You pointed out extremely clearly that it is likely that some of the bacteria you are finding are surrogates for a more complex situation. Given where we are now technologically, I would think that that kind of information is obtainable.

Dr. Björkstén: That is a valid comment and I am happy to say that if you have the methods, I have the samples stored, as this is precisely what we hoped to do. Crude bacterial cultures were the only available methods when we started 12 years ago. We collected consecutive samples in the freezers and we had an 11-year follow-up of the first birth cohort. We could go back and look at diversity with modern technology of samples stored from Estonian and Swedish children who are either allergic or not by age 11 years. I also want to make it clear to the audience that I never said clostridia are bad, nor that lactobacilli are good. Our observations could be surrogate markers for other microbes. The fact that we have shown differences between groups in prospective studies does not prove that these bacteria are directly involved. I am only suggesting that the internal environment is more interesting than shooting dogs and strangling cats for allergy prevention.

Dr. Bier: As a person who doesn't have any stake in this, it is very hard to convince me that any of these things have meaning until they are molecularly typed. I don't know what it means if half the bacteria is thrown away. I think all of the data that have been collected over time really have to be redone in the context of us knowing what the true bacterial population is. In relation to the genotypes, alleles and polymorphisms that may exist in the regulatory genes, or in Estonia compared to Sweden, and until they are known, it is also very hard to understand whether these are differences due to environment or they are differences due to genotype.

Dr. Björkstén: These are ethnically quite similar populations so, at the population level, there is no reason to believe that there would be differences. Obviously there would be individual differences. There has not been a shift in our genetic set up over a 50-year period therefore the increase has to be due to environmental factors. I previously cautioned during the discussions that there may be a problem with a traditional reductionist molecular approach.

Dr. Ogra: Has anyone looked at idiotypes and idiotypic regulation of immune responses in these settings? Is there anything which we can identify at the regulatory level before we see Th1 and Th2 responses which might explain the differences between Estonians and the Finnish? For example, alcohol may be a very important modulator of the flora between the two populations. Furthermore there are many other variables which might affect the responses in these two population settings.

Dr. Björkstén: I don't want to be interpreted as saying that molecular studies should not be done, obviously they should. What I am saying is that we have also to adapt a more holistic approach when asking our research questions. In a way Estonia in the 1990s was a time capsule of Scandinavian life some decades back. The country is ethnically and culturally Finnish or Swedish. The lifestyle when I started to work there in the early 1990s really threw me back to my childhood after the Second World War. The diet comprised locally produced foods, no vegetables coming from the southern hemisphere in the winter. Fermented cabbage, carrots and beets were the main vegetables. Apples were available in autumn. In Sweden in spring I can buy an apple in a supermarket that has been transported from Tasmania. The low allergy prevalence cannot be explained by infections, breastfeeding, exposure to allergens, pollution, or tobacco smoking. All those external environmental factors have been

controlled for. What we obviously cannot say is whether differences in the diet would explain the differences in allergy prevalence between Estonia and Sweden.

Dr. Ogra: So how does it then fit with your observation that allergy is in fact increasing, and yet the old type of farm life was not protective but modern farm life is protective, although it is more sanitized?

Dr. Björkstén: This is the end of the day, and we may be allowed to speculate. The differences between urban and rural populations in a country are not only pollution and the presence of pets, but also a question of tradition. For example, people in rural Sweden eat differently than people in the cities these days. Whatever is now still prevailing in rural areas was also common in urban areas 50 years ago. I have no idea what it is, I have already given you what is known, so your guess is as good as mine at this stage.

Dr. Walker: Let me answer the concerns raised by Dr. Wilson and Dr. Bier; there is in fact a lot of cloning being done on the common probiotics on a mechanistic level, it is just that there are different types of studies going on. There is a lot of evidence that there is a slight 1 or 2 log difference in one organism versus another in allergy, inflammatory bowel disease and so forth. In an environment of a billion cells, how can a 1 log difference be so large in terms of expression of disease?

Dr. Björkstén: That is actually what I am trying to say and why I am cautioning against believing in one single miracle probiotic strain. Usually we are giving something like 100 million up to possibly one billion bacteria which is still 10^{14} less than the entire gut microbiota. What could be a reasonable explanation is that it actually modifies the composition of the microbiota. That is why I am careful not to say that what we see is strain specific.

Dr. Smith: How might a single probiotic continuously stimulate the immune system? In a natural environment, when a patient might be infected by many different bacteria, if an attempt is made to induce tolerance and stimulate the immune system, a single antigen might not provide that.

Dr. Björkstén: I agree. Thank you for giving me the chance to reiterate. Again we know from the germ-free animals that normal gut microbiota are essential not only for downregulation of IgE formation, but also for oral tolerance and the development of T regulatory cells. Several clinical studies do, however, indicate clinical benefit in the treatment of eczema in infants and also in prevention. There are three meta-analyses showing the shorter duration of infectious diarrhea in infants. We cannot explain how the limited number of bacteria given have an effect.

Dr. Smith: Do you think that a probiotic of the month would be needed? In the children who are on this trial, to replroduce what is in nature, they may need to be exposed to many different types. Do you think that might be better?

Dr. Björkstén: This is a reasonable suggestion from what I have said; I am not quite happy with the thought of it though. As doctors we were trained to prefer one drug for one disease rather than cocktails of compounds, but that is an obvious way to consider.

Dr. Giovannini: Speaking about lactobacillus, you said that some are effective and others not. All lactobacilli break down protein or milk protein, especially casein; in some patients it is effective but another patient may have protein milk allergy. If they are effective in some pathologies, milk should not be given because otherwise milk allergy may occur.

Dr. Björkstén: Lactobacilli have nothing to do with milk. They are predominantly present in any fresh food and we have lived with them through the evolution of man. All mammals seem to harbor lactobacilli. Anything that is fermented, whether it is vegetables or meat that has started to ferment, contains lactobacilli.

Dr. Giovannini: Is there a difference between rural and urban areas? In urban areas perhaps the lack of parasites may be a reason for the high rate of atopic manifestations also in breastfed babies.

Dr. Björkstén: We do not have parasites in our cold climate. The development of immune regulation in Estonian infants is similar to what has been shown in Africa and is suggested to be connected with parasites there. So I am suggesting that the broad microbial spectrum, possibly caused by the traditional lifestyle, has an effect that is similar to what has been reported for parasites. We looked for the possibility and the only parasites we would find in Estonia were ascaris and trichuris.

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Concluding Remarks

This symposium focused on the window of opportunity for nutritional interventions to prevent chronic disease. Following a recommendation by the UN Standing Committee on Nutrition, 2006, the window of opportunity was defined as the period from conception to 2 years after birth. We discussed what is known and what needs to be known about (a) growth during this window, (b) critical periods of development, (c) the effects of nutrition, and (d) possible interventions to improve nutrition.

Growth

What Is Known. The window of opportunity is characterized by rapid growth. The brain and lymphatic tissue grow especially fast. Growth is episodic rather than continuous. Growth at this time may alter the path of later growth; becoming thin at 2 years, for example, may induce the adiposity rebound at an early age in childhood and thereby lead to childhood obesity. Chronic diseases, including cardiovascular disease and type 2 diabetes, are related to the path and tempo of growth during the window. The same disease may originate through more than one path of growth.

What Needs to Be Known. We need to define the optimal paths of growth between 6 months and 2 years. We know little about the costs of rapid growth during and after the critical window. We do not know how the growth of girls at this time affects the development and health of the next generation.

Critical Periods of Development

What Is Known. Sensitive, or critical, periods of development are those when a system or organ is plastic and can be changed by the environment. For most systems and organs the critical period occurs before birth: the brain, liver and immune system remain plastic after birth. An organ may have more than one critical period: the heart, for example, has critical periods in both early

and late gestation. During a critical period different environmental stimuli may have different effects on the same organ: for example, either the growth of the heart or its maturation may be affected. Gene expression is determined during critical periods: studies of osteoporosis and type 2 diabetes have shown that the effects of genes interact with those of early body size. Alternatively genes may be silenced during critical periods.

What Needs to Be Known. We need to know in more detail when the critical periods for each organ and major system in the body occur.

Nutrition

What Is Known. Good nutrition is most effective if it begins before conception. The long-term consequences of the Dutch famine on people who were in utero at the time provide the clearest demonstration of the importance of nutrition during pregnancy. The baby is also nourished by the turnover of protein and fat in the mother's body. It is influenced by the mother's metabolism, as the effects of maternal obesity clearly demonstrate. Fetal IGF-1 concentrations are sensitive to nutrition and affect multiple organs and systems. Breast milk protects growth. Early initiation of breastfeeding protects against infections.

What Needs to Be Known. We do not know how early nutrition sets appetite or determines food preferences in later life. We know little about the differing needs of different babies. We need to know more about the role of the gut flora and the role of the microbes in breast milk.

Interventions

What Is Known. There is an ongoing debate about the relative merits of single nutrient 'magic bullet' interventions, for example a single vitamin as opposed to interventions with foods. Interventions may need to be targeted to vulnerable people, for example, in Western societies those with low educational attainment. In the future interventions could be targeted to systems, for example to epigenetic systems.

What Needs to Be Known. We need more biomarkers to demonstrate the effects of interventions. We need to know more about ways of changing peoples food choices, especially those made by girls and young women.

What Could Be Achieved

The Helsinki Birth Cohort is the best source of data in which growth during the critical window can be linked to chronic disease in later life. It has been estimated that if, within the cohort, each person had been in the highest third of

body size at birth (weight, length or ponderal index (birthweight/length^3)) and had not increased their standard deviation scores for body mass index between 2 and 11 years, chronic disease would have been reduced as follows: (1) coronary heart disease by 25% in men and 63% in women; (2) type 2 diabetes by 57% in men and women, and (3) hypertension by 25% in men and women.

These estimates take no account of the additional effects of optimizing growth from birth to 2 years of age.

David J.P. Barker

This session provided a comprehensive overview on the interaction between nutrition and growth during critical periods of early development.

Theresa Scholl demonstrated how specific nutrients may link maternal nutrition before and during pregnancy with the growth outcome of the fetus. During famine and starvation a low glucose stream from the mother to the fetus gives rise to a smaller size at birth. But even under normal conditions, a diet with a low glycemic index can alter maternal glucose production and consequently reduce fetal growth. Although a single micronutrient deficiency rarely occurs isolated in humans, it could be shown that iron deficiency anemia, even before conception, increases the risks of low birthweight and preterm delivery. A nutritionally or metabolically caused deficiency of circulating folate before and during pregnancy interferes with normal fetal growth and development. Many studies have demonstrated that micronutrient supplements before and during pregnancy improved pregnancy outcome.

Andreas Plagemann elaborated a fundamental concept for the fetal origins of obesity, diabetes mellitus, the metabolic syndrome, and subsequent cardiovascular diseases, launched by pre- and perinatal nutritional conditions and mediated by hormones. Hormones are organizers of the developing neuroendocrine-immune network. When present at nonphysiological concentrations during critical developmental periods, they can act as teratogens of the endocrine network, resulting in persistent functional and somatic alterations. Experimental results, clinical and observational studies demonstrated that elevated insulin concentrations in the fetus and newborn, induced by maternal diabetes and overweight, may program obesity and diabetes in the offspring. Screening and therapy of all types of diabetes during pregnancy, and avoidance of early postnatal overfeeding are recommended for a genuine primary prevention.

Renate Bergmann pointed out that it is problematic to use birth size for the assessment of poor fetal growth. The prevalence rates of intrauterine growth restriction are estimated to be highest in India and other Asian countries. The major determinant in developing countries is malnutrition before and during pregnancy, but in developed countries it is maternal cigarette smoking. While smoking cessation even before becoming pregnant is a sound

recommendation, balanced nutritional supplements during pregnancy may come too late. The development and education of young women should be supported as early as possible. An epigenetic modification can already occur in the periconceptional period. A hormonal network regulates fetal growth and development in accordance with signals from the intrauterine environment. The fetus 'predictively adapts' to an unfavorable environment and develops a 'thrifty phenotype'. Follow-up studies suggest that lean body mass rather than fat mass is programmed by intrauterine undernutrition. Catch-up growth is the consequence of intrauterine growth restriction. While catch-up in length is a favorable sign, an overshooting weight gain, mainly comprising adipose tissue, is accompanied by insulin resistance, a characteristic of the thrifty phenotype. Growth restriction in newborns therefore should be identified to avoid overfeeding. Increasing the muscle mass, e.g. by physical activity, may help to restrain this unfavorable development.

Lars Hanson described the role of human milk for growth, development and immune response of the young infant. Infants exclusively breastfed for the first 6 months are considered to demonstrate optimal growth. The new WHO growth standards are based on their growth data, which deviate from those of formula-fed infants even in later life. Breast milk contains growth-regulating hormones. Of importance is the maternal diet, e.g. in regard to LCPUFA, especially a balanced intake of n-3 and n-6 fatty acids. The immune defense provided by human milk includes the innate defense, e.g. by enzymes which additionally have nutritional functions, and components that can modulate toll-like receptors (TLRs) according to the microbial exposure of the newborn at delivery, and according to his own gut microflora. The dual function, i.e. protecting the infants from infection and promoting growth, is a feature of many nutrients and constituents of human milk, making it a unique food in infancy.

Dennis Bier demonstrated that postnatal growth is under different hormonal control than fetal growth. There is only a weak correlation between birth size and adult size, but the correlation between length at 3 years and adult height reaches 0.8. Genetic factors predominantly influence linear growth during the first 3 years, determining the growth velocity in order to compensate for intrauterine growth deviations. In early childhood, ponderal growth is different from linear growth. Although growth velocity and especially head growth is particularly rapid during the first 3 years, energy costs for growth decrease from 40% at 1 month to 3% and less at 1 year and later on. But the glucose consumption rates of the brain increase until about 4 years of age. Weight gain after a phase of slow growth, e.g. as an early adiposity rebound, is a risk for later obesity and its comorbidities. Cells and organs may remember their nutritional, and environmental experiences (e.g. maternal care) by permanent anatomic changes, epigenetic DNA imprinting, clonal selection, neuronal pruning or stable gut microbes.

Renate L. Bergmann

This session provided an in-depth discussion of the developmental aspects of Immune system in the neonate, nutrition and neonatal mucosal microflora relative to the impact of their interaction on subsequent disease outcome.

The introductory talk by *Pearay Ogra* suggested that immune response in the neonatal mucosal surfaces is a complex interplay of innate and adaptive immunity and available external environment, including the microflora. Changes in the mucosal microflora significantly influence the development and regulation of neonatal immune responses. Expression of autoimmune or immunologically mediated disease processes may be a reflection of a lack of immunological tolerance or altered mucosal immune responses. It was proposed that the underlying neonatal immune status may program for subsequent disease outcome later in life. *Christopher Wilson* discussed key features of antigen-specific immunity in the neonatal period. It is now clear that T-cell-independent antibody responses to polysaccharide antigens are absent and not amenable to intervention in the early neonatal period. However, T-cell-dependent antibody responses are present even prior to birth. Interestingly, the slow pace with which such responses develop during the neonatal period may allow microbes to induce infection before the expression of effective T-cell responses can contain such infections. It was also proposed that, in the absence of effective microbial TLR agonists, protective Th1 and other T-cell-dependent responses to vaccines reach protective levels somewhat slowly. It should however be emphasized that the extent to which these deficits are T-cell intrinsic or result from impaired neonatal dendritic cell response to TLR agonists is not known. Field studies on the relation of nutrition-immunity and mucosal homeostasis discussed by *Andrew Prentice* have shown that infections with pathogenic microorganisms are a major suppressor of growth. Of these, infectious gastroenteritis was considered to be an important cause of growth retardation in Africa. Catch-up growth seems to occur during recovery or convalescence from such infections. These studies have also suggested that unique windows of opportunity, if missed, can result in permanent growth suppression until puberty, at which time there may be another opportunity for growth catch-up. *Allan Walker* discussed the role of perinatal microflora and mucosal disease. As pointed out earlier, initial mucosal colonization appears to be very important to the nature and development of neonatal host defenses. Furthermore, mucosal colonization with probiotics and other commensal agents can affect disease expression. *Yvonne Maldonado* provided an extensive overview of microbial infections in the global setting and their role in disease outcome. It is clear that global mortality related to infectious diseases continues to remain very high especially in neonates and young children, with over 12 million deaths per year. The pathogenesis of perinatal viral infections appears to be related to primary maternal infections with common organisms. Postnatal and possibly breastfeeding-related HIV infections have become a major challenge in global perinatal HIV prevention. On the other hand, It is interesting to note that with the exception of malaria, most perinatal parasitic infections are generally

benign and may even contribute to the disease outcome especially in the prevention of allergic and/or autoimmune diseases. This concept of the hygiene hypothesis was discussed in detail by *Bengt Bjorksten*. His field studies have shown that allergy as a disease entity is on the increase in affluent societies especially in the young. However, rural farm life, and possibly hepatitis virus infection appear to be associated with protection. Recent investigations carried out in Estonia have suggested that infants are born with lower Th1 and Th2 responses, rapid development of secretory IgA responses, and a lower frequency of positive allergic skin tests. These changes may be related to significant changes in the composition of neonatal mucosal flora, especially for probiotics, other commensal and other anaerobes (clostridium) in the mucosa, and between allergic and nonallergic subjects. Nonallergic children seemed to be selectively colonized with lactobacilli and other bifidobacteria, while allergic subjects were more often colonized with clostridia. Other studies have shown that certain lactobacilli improve infantile eczema especially if given early. It was also suggested that mucosal microflora is essential for the postnatal maturation of the immune system and a prerequisite for the development of oral tolerance, the principal immunologic mechanism underlying the concept of the hygiene hypothesis.

It is clear that mammalian hosts especially the human neonate exhibit a delicate and a complex interaction with the mucosal environment (mucosal flora, nutrition), and the developing innate and adaptive immune functions. From a evolutionary perspective, it is important to recognize that mammalian host–microbial interactions have evolved over billions of years of coexistence. Virtually all human microbial agents, both pathogens and commensal, have been acquired from other animal species and all microbes must be considered pathogenic. However, some organisms over millions of years of cohabitation with human and other life forms have been attenuated by pathogenesis and continue to live in a delicate balance with the host, often favoring the host. However, that balance can shift in favor of the microorganism to render it pathogenic. Factors which can shift the balance include a variety of host as well as microbial functions. There is strong interest and some evidence for a protective role of some probiotic organisms in prevention and/or treatment of infectious, allergic or autoimmune disease processes. However, additional information must be acquired about the dynamics of the natural microbial ecosystem before these agents can be used for large scale therapeutic purposes.

To date, the single most important success story in the history of mankind has been the prevention of childhood infection through the use of vaccines. There is no evidence to suggest that the global introduction of vaccines against infectious diseases has adversely affected the development of the human immune system or the acquisition of mucosal or external environmental microflora.

Pearay L. Ogra

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