

A joint meeting of the Nutrition Society with the British Society for Immunology Developmental Immunology Group was held at the International Centre, Harrogate on 9 December 2005

## Symposium on ‘Nutritional influences on developmental immunology’

### Early immunological development and mortality from infectious disease in later life

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In rural Gambia the risk of mainly infection-related mortality is 10-fold higher for adults born in the nutritionally-debilitating ‘hungry’ season, suggesting that immune function may be compromised by events early in life. The current programme of research focuses on the biological mechanisms underlying this hypothesis, exploring early-life environmental influences on immune development and the long-term functional consequences these influences may have. Results obtained to date show that thymus development during infancy is critically sensitive to environmental exposures, with smaller thymuses observed in the hungry season. Measurement of the frequency of T-cell receptor excision circles indicate that thymus function is also sensitive to seasonal influences, with further studies implicating variations in breast-milk IL-7 as a possible mediator of these effects. Studies in adults have shown that size at birth is positively correlated with antibody responses to vaccination with polysaccharide antigens, thus providing evidence for long-term functional deficits. The present paper will review progress made to date within this field of research.

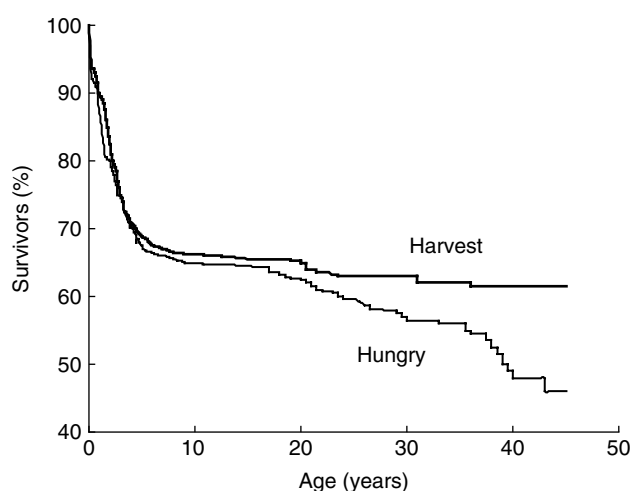
#### Gambia: Immune function: Programming: Thymus: Vaccine response

In rural Gambia many aspects of health and behaviour are defined by the distinct seasonal pattern, with a long dry season from November to June followed by a period of intense rainfall lasting from July to October. In brief, the dry ‘harvest’ season is characterised by low levels of active infections (Brewster & Greenwood, 1993) and periods of good growth in infants and children (Ulijaszek & Strickland, 1993), and a period of positive energy balance is observed in all adults. This positive energy balance comes as a result of adequate food supplies from the previous year’s harvest coupled with reduced farming activities in the absence of rainfall (Singh *et al.* 1989). Conversely, the annual rains (the ‘hungry’ season) bring about an increase in levels of infections (specifically

malaria and diarrhoeal disease) and poor growth during infancy, and a negative energy balance is observed in all adults as a direct consequence of depleted food supplies coupled with intensive agricultural activity. Pregnant women are not exempt from these effects, which lead to seasonal patterns in pregnancy weight gains (Prentice *et al.* 1981), length of gestation (Rayco-Solon *et al.* 2005b) and birth weight (Rayco-Solon *et al.* 2005a). It is known that much of the observed seasonal deficit in birth weight is nutritional in origin, since it is reversed by maternal dietary supplementation (Ceesay *et al.* 1997). Season of birth in this part of rural Africa can therefore be used as a proxy indicator of early-life exposures to both nutritional and infectious stresses.

**Abbreviation:** sjTREC, signal-joint T-cell receptor-rearrangement excision circles.

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**Fig. 1.** Kaplan-Meier survival plots by season of birth (the 'harvest' season is from January to June and the 'hungry' season is from July to December) for 3162 subjects (2059 alive and 1103 dead) resident in three villages in the rural West Kiang region of The Gambia. Hazard ratios for mortality: for adults aged  $\geq 20$  years, 10.3 ( $P = 0.00002$ ); for adults aged  $\geq 15$  years, 3.7 ( $P = 0.000013$ ). (From Moore *et al.* 1999, reproduced with permission.)

### Season of birth and survival in rural Gambia

The past two decades has seen much research and debate focused on the long-term health consequences of early-life exposures, with poor early nutrition now considered as an important risk factor for a number of chronic degenerative diseases such as heart disease and type 2 diabetes (Barker, 2004). Initial interest in this field was fuelled by epidemiological observations of associations between geographical patterns in social conditions in early life and mortality from chronic disease in adulthood (Barker & Osmond, 1986), and the active research that followed these observations has led to the 'developmental origins of health and disease' hypothesis. Much of the research within this field has relied on the long-term preservation of detailed records of birth, growth and death, with three cohorts from Hertfordshire, Preston and Sheffield in the UK paving the way for a number of key early publications (Barker, 1994). The main observations from these initial studies then precipitated a number of research programmes around the world that have utilised long-held demographic and birth records to explore the long-term consequences of early-life exposures.

The authors' contribution to this field started with a serendipitous observation arising from a combination of long-term demographic record keeping and the 'experiment of nature' created by the strong seasonality described earlier. Since the late 1940s the UK Medical Research Council has maintained a demographic record of all residents from three villages in the rural West Kiang region of The Gambia. In light of the growing interest in the long-term effects of early-life exposures, these records have been used to look at survival according to events in early life, using season of birth as a proxy measure for early-life exposures (Moore *et al.* 1997, 1999). The Kaplan-Meier survival plots in Fig. 1 illustrate the profound bias that was

observed in survival, with individuals born in the annual hungry season having rates of mortality in young adulthood that are up to 10-fold higher than those of their counterparts born in the harvest season. Using clinic and hospital records, where they were available, or verbal autopsy data for missing cases, cause-of-death information reveals an absence of deaths from chronic degenerative diseases and an excess of infectious or infection-related deaths. This finding led to the hypothesis that early-life exposures, correlated with season of birth, have compromised immune development, resulting in later deficits in function and ultimately survival. There are a number of seasonally-related factors that could potentially disrupt early immunological development, including a variety of nutritional, infectious or toxic exposures. However, it has been argued elsewhere (Moore *et al.* 1999; Prentice *et al.* 1999) that there is strong evidence that within this setting the most likely aetiology of the observed effect on survival is nutritional in origin, and a programme of work is currently exploring this aspect in detail.

### Programming the immune system

Evidence to support the hypothesis that the human immune system may be programmed by nutritional deficiencies during early life starts with the knowledge that many of the principle components of the immune system develop in fetal life; for example, the release of T-cells from the fetal thymus occurs as early as weeks 14–15 of gestation. Indeed, by 15–16 weeks the architecture of the human fetal thymus resembles that of the neonate (Klein & Horejsi, 1997). Specific nutritional deficiencies occurring during these critical periods of development could, therefore, have long-term immunological consequences. Indeed, maternal malnutrition has been observed to have greater effects on thymic and lymphoid tissue development than on other organs (Winick & Noble, 1966; Owens & Owens, 1989), presumably reflecting a physiological mechanism to protect the growth and development of other specific organs, such as the brain. In addition, there is evidence to demonstrate that such deficits in organ growth and development occurring *in utero* are more serious and longer lasting than those caused by later malnutrition (Beach *et al.* 1982).

The evidence to support the long-term consequences of these early insults comes from data that suggest that low-birth-weight infants may have sustained impairment of immune competence as infants and children when assessed by various *in vitro* methods. However, such findings are not universal (Pittard *et al.* 1984) and much debate now surrounds the validity of many of these early studies. It is well known, however, that intrauterine growth retardation leads to an increased susceptibility to infections in childhood (Ashworth, 1998), with data from Brazil showing hazard ratios for infectious deaths rising as high as 5.0 (Victoria *et al.* 1988).

Direct evidence to support the long-term programming of the human immune system is, however, limited to a small number of published studies in adolescents and adults. Using data from a longitudinal cohort study in the

Philippines (the Cebu Longitudinal Health and Nutrition Survey), McDade and colleagues (McDade *et al.* 2001a,b, 2004) have demonstrated associations between early growth and thymopoeitin production (McDade *et al.* 2001b), antibody response to vaccination (McDade *et al.* 2001a) and serum IgE levels (McDade *et al.* 2004) during adolescence. Of interest, all these relationships only become significant when adjusted for contemporary factors such as adolescent BMI or household sanitary conditions, suggesting an interaction between the fetal environment and later-life exposures in programming the immune system. Studies in adults appear limited to two papers from the UK showing relationships between impaired fetal growth and elevated levels of thyroid auto-antibodies (Phillips *et al.* 1993) or serum IgE levels at ages 50–70 years (Godfrey *et al.* 1994). Despite these differing outcome measures, the reports of these studies include within their conclusions the suggestion that these associations may indicate the long-term functional consequences of an early insult to the thymus. This area is now being investigated in more detail.

#### Early programming of the thymus

The thymus is a likely programming target because its development commences during early fetal life and it reaches its maximum size in relation to total body weight soon after birth. Furthermore, the thymus is central to the development of controlled adaptive immunity, as it plays a crucial role in the development of T-cells by providing the microenvironment in which bone marrow-derived progenitors undergo proliferation, T-cell receptor rearrangement and thymocyte differentiation into mature T-cells (Madhok *et al.* 2005). By the time of delivery the thymus is almost fully developed. It increases in weight over the first 6 months of life, after which time it begins to shrink in size. This shrinkage is associated with a concomitant decrease in the production of new T-cells, and it has long been assumed that this decrease in production is correlated with a reduction in the functional capacity of the thymus. However, the advent of more advanced methods for the assessment of thymus output has led to recent evidence that the role of the thymus continues beyond this time point (Mackall *et al.* 1995; Douek *et al.* 1998; Eysteinsdottir *et al.* 2004; Madhok *et al.* 2005). Although early data from a prospective study in Danish infants fail to show any association between thymus size at birth and childhood allergic disease (Benn *et al.* 2001), evidence is now emerging from the authors' group and other groups that supports the long-term consequences of an early insult to the fetal thymus.

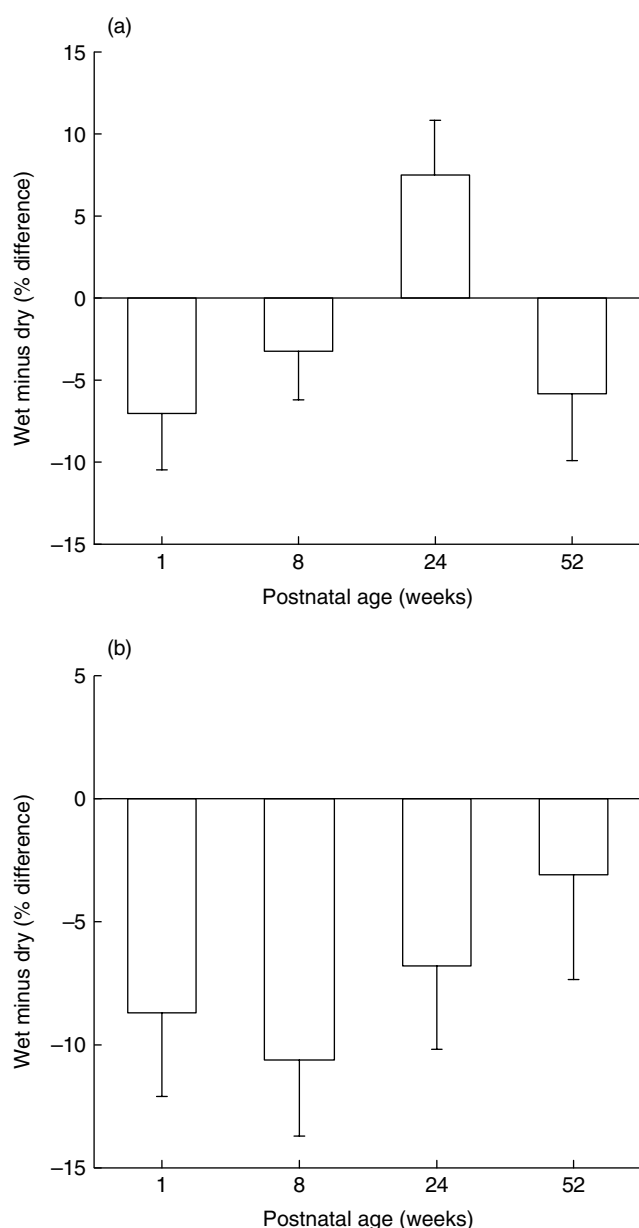
Postnatal thymus development can be assessed sonographically using a validated method in which the transverse diameter of the thymus and the sagittal area of its largest lobe are multiplied to give a volume-related thymic index. This index has been shown to correlate with thymus weight at autopsy (Hasselbalch *et al.* 1996b). Using this technique Hasselbalch *et al.* (1996a) have shown that formula-fed Danish infants have a reduced thymus size as compared with breast-fed infants. Furthermore, a study

from Guinea-Bissau in west Africa has shown that a small thymus at birth predicts an increase in risk of infection-related mortality in infancy (Aaby *et al.* 2002). As all deaths in this population group were found to be from infectious diseases, thymus size at birth may be an important predictor of postnatal immune competence. This sonographic technique has been used to assess thymus development in infants from The Gambia and from Bangladesh.

#### Thymus development in The Gambia

In The Gambia thymus development has been assessed in a prospective cohort of 138 infants recruited prenatally and followed until 52 weeks of age. Thymus size was assessed in infants at 1, 8, 24 and 52 weeks of age, together with the collection of growth and morbidity data. In relation to absolute size, mean thymic index increases dramatically up to 24 weeks of age, and then decreases to the measurement taken at 52 weeks (Collinson *et al.* 2003). At all ages thymic index is strongly associated with current weight ( $P \leq 0.001$ ), and in proportion to body size mean thymic index is greatest at 1 week, decreasing to the 52-week measurement. In addition, the results also show that infants have a characteristic thymic index, with tracking of thymus growth that is at least partially distinct from the postnatal effects of season and body weight. In relation to an early-life programming effect, thymic index at 1 and 8 weeks is associated with birth weight, but this relationship does not persist after adjusting for current weight. However, as is illustrated in Fig. 2, there is a seasonal effect on thymus size, with the smallest thymuses, from week 1 onwards, being observed in the hungry season after adjustment for infant weight (Collinson *et al.* 2003).

The functional consequence of the observed seasonal effect on thymus size is not yet fully understood. Recent data from Denmark show a correlation between thymic index at 10 months of age and CD8+ cell counts (Jeppesen *et al.* 2004), but further follow-up data on cohorts with early thymus size estimates are not yet available. In order to address this question in the Gambian cohort, T-lymphocyte populations and subpopulations have been assessed in parallel to the measurement of thymus size. Table 1 shows the key findings from this analysis. As illustrated, geometric mean cord and postnatal lymphocyte counts are higher in births occurring in the hungry season, with both season of birth and season of measurement effects. Absolute CD3+ (all T-cells), CD8+ and CD56+ (natural killer) cell counts are higher in hungry-season births, but absolute CD4+ counts and CD19+ (B-cells) counts show no association with birth season (Collinson, 2002). These results demonstrate a seasonal influence on cell counts that is present in cord blood (indicating *in utero* responses), is sustained at least over the first year of life and is not explained by current nutritional status (data not presented), possibly indicating long-term deficits in cellular function. The precise mechanisms for these observed seasonal effects on cell numbers, and the role that the apparent seasonal impact on thymus size may have, requires further investigation.



**Fig. 2.** Percentage difference in mean thymic index (transverse diameter of the thymus  $\times$  sagittal area of its largest lobe) between (a) hungry-season (wet season from July to October; wet) and harvest-season (dry season from November to June; dry) births and (b) hungry-season and harvest-season measurements adjusted for gender, gestation and current weight for a cohort of 138 Gambian infants. Values are the mean percentage difference with their standard errors represented by vertical bars. (From Collinson *et al.* 2003, reproduced with permission.)

In the absence of a direct marker for the accurate quantification of recent thymic emigrants, thymus output can be assessed by measuring the frequency of signal-joint T-cell receptor-rearrangement excision circles (sjTREC). These sjTREC represent the extrachromosomal excision products that are formed by the excision of DNA during recombination events in the genome to produce the  $\alpha$  chain of the T-cell receptor in the thymus, and hence quantification of the frequency of sjTREC levels is an

**Table 1.** Geometric mean absolute lymphocyte subpopulation counts at each age and overall, according to season of birth for a cohort of 138 Gambian infants\*

	Age (weeks)	Absolute counts ( $\times 10^9/l$ )		Statistical significance of the difference: $P =$
		Harvest-season birth†	Hungry-season birth†	
CD3+	0	2.30	2.53	0.41
	8	3.57	4.35	0.029
	16	4.10	5.26	0.014
	52	3.32	3.72	0.26
	All	3.40	3.85	0.01
CD4+	0	1.45	1.73	0.17
	8	2.49	2.73	0.26
	16	2.80	3.28	0.05
	52	2.07	2.19	0.55
	All	2.26	2.41	0.15
CD8+	0	1.29	1.44	0.44
	8	0.98	1.47	0.011
	16	1.34	1.65	0.093
	52	1.12	1.28	0.22
	All	1.16	1.42	0.0007
CD19+	0	0.78	0.73	0.68
	8	1.88	1.94	0.79
	16	1.97	2.50	0.034
	52	1.50	1.51	0.95
	All	1.54	1.66	0.23
CD56+	0	0.84	0.78	0.68
	8	0.27	0.35	0.095
	16	0.25	0.33	0.028
	52	0.21	0.29	0.0052
	All	0.33	0.42	0.0014

\*All values adjusted for gender, gestation and birth weight; overall values also adjusted for age at measurement.

†The hungry season is the wet season from July to October and the harvest season is the dry season from November to June.

indirect measure of thymus output. To assess seasonal influences on thymus output during infancy sjTREC levels were measured in blood samples collected from infants in the Gambian study at birth and 8 weeks of age. Despite considerable monthly variation, those infants born in the harvest season were found to have significantly higher sjTREC levels than those born in the hungry season (2.12 *v.* 0.92 sjTREC/100 T-cells,  $P = 0.006$ ) at week 8 but not at birth (N'Gom *et al.* 2004). Although the use of sjTREC levels as a marker for absolute thymus function has been questioned (Hazenberg *et al.* 2003), it can be argued that this finding implies that the higher sjTREC counts in infants born in the harvest season indicates a greater number of recent thymic emigrants, when compared with infants born in the hungry season, corresponding to the observed deficit in thymus size.

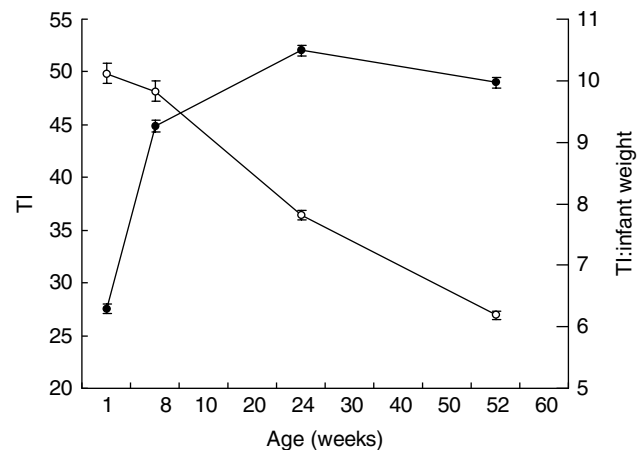
It is of interest that the greatest difference in thymus size is observed when the infants are 8 weeks of age. This finding is surprising, since at this age infants in this community are fully breast-fed, are growing well and have minimal incidence of active infections. Previous research from The Gambia has demonstrated seasonal variations in

a number of key antimicrobial factors in breast milk, with breast milk collected in the late rainy season containing 35% less IgA and IgG and 20% less secretory component and lysozyme (Prentice *et al.* 1984). In addition, these differences are accompanied by a slight fall in milk production during the rainy season. The observed deficit in thymus size in infants when measured in the hungry season could therefore reflect a deficiency of a specific component in breast milk. Indeed, this conjecture is supported by the observations from Denmark described earlier that suggest that breast-feeding promotes thymus development (Hasselbalch *et al.* 1996a). Whilst the role that immune factors in breast milk have on passive immunity is clearly established (Hanson *et al.* 2003), the influence that these factors may have on adaptive responses is less clearly understood. It is also well established that breast milk is a medium for a wide range of hormonal and cytokine signals, and the candidate factors that have already been identified include leptin, epidermal growth factor, transforming growth factors  $\alpha$  and  $\beta$ , IL-1, IL-6 and other cytokines (Houseknecht *et al.* 1997; Garofalo & Goldman, 1998; Hawkes *et al.* 1999). It is therefore plausible that seasonal variations in such factors may play a role in influencing the differential development of the human thymus during the early post-natal period.

Recent work in The Gambia in relation to these observations has focused on the potential role of the cytokine IL-7, which is known to be essential for normal thymocyte development and for the proliferation and survival of precursor T-cells (Suda & Zlotnik, 1991; Morrissey *et al.* 1994). However, until the study of N'Gom *et al.* (2004) no published evidence has existed in the literature to report the presence or variation of IL-7 in human breast milk; hence, the functional consequence of this factor, if any, is not understood. Using stored samples of breast milk from Collinson's (Collinson, 2002; Collinson *et al.* 2003) prospective birth cohort study described earlier, IL-7 levels have been quantified in samples collected at 1 and 8 weeks post partum. Despite considerable monthly variation IL-7 levels were found to be significantly lower in breast-milk samples collected at 1 week post partum in the hungry season compared with those collected in the harvest season (7 pg/ml *v.* 10 pg/ml,  $P = 0.02$ ; N'Gom *et al.* 2004). A similar, but non-significant, trend was found in the samples collected at 8 weeks post partum. The precise functional role of IL-7 in human breast milk requires further investigation, but this observation suggests that seasonally-variable exposures may exert direct effects on breast-milk composition, which may in turn be responsible for the observed seasonal differences in thymus size and function.

### Thymus development in rural Bangladesh

In an attempt to further understand these observations from The Gambia, thymus development has been assessed within a large-scale study of combined interventions to improve maternal and infant health in the rural Matlab region of Bangladesh (the MINIMat study). Within the MINIMat study all pregnant women were randomised to receive a combination of prenatal protein-energy and



**Fig. 3.** Changes in mean thymic index (transverse diameter of the thymus  $\times$  sagittal area of its largest lobe; TI) with age (●) and in mean TI expressed in relation to body weight (TI:infant weight; kg; ○) in Bangladeshi infants born from the rural Matlab region of Bangladesh (the MINIMat study). Data available on: week 1,  $n$  1173; week 8,  $n$  1724; week 24,  $n$  1750; week 52,  $n$  2103. Values are means with their standard errors represented by vertical bars.

micronutrient supplements. The prenatal arm of the study was completed in June 2004 with a total of 3282 singleton infants born. All key findings from the main study in relation to birth weight and the other primary outcomes will be reported elsewhere.

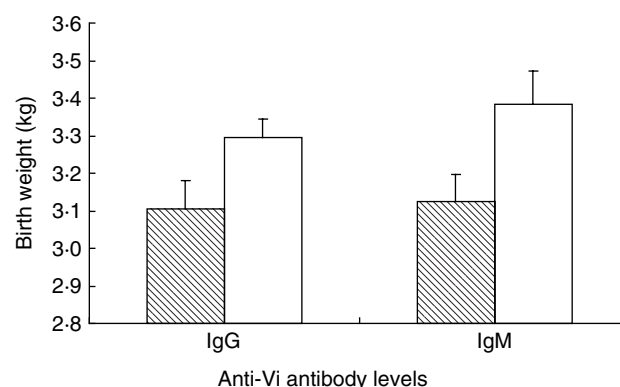
Within the MINIMat study, thymus development has been assessed during the first year of infancy in a large subgroup of infants to look more specifically at prenatal and postnatal influences on thymus development. In approximately 1750 infants thymus size was assessed by ultrasonography at weeks 8, 24 and 52, with sonographers trained according to the same techniques as those used in The Gambia. In addition, infants who were born in health facilities ( $n$  1173) had an additional measurement made at day 1 of age. Fig. 3 illustrates absolute thymic index and thymic index:body weight by age at measurement. As in The Gambia, absolute thymus size increases rapidly from the measurement at birth to the measurement at 24 weeks of age, decreasing to the 52-week measurement. Thymus size relative to body weight shows a constant decrease from the measurement taken at birth to the final measurement taken at 52 weeks of age. Analysis of the data is ongoing, but preliminary observations support the previous observations from The Gambia, suggesting that thymus size in Bangladeshi infants is also influenced by both seasonally-related factors and by exposures early in life, as measured by birth weight. Further work in this large cohort of well-characterised Bangladeshi infants will also explore the functional implications of these differences in thymus size.

### Long-term functional consequences of early undernutrition

As highlighted earlier, evidence linking fetal growth to a direct measure of functional deficits in later immune

function is limited to one study from the Philippines that has demonstrated an association between small size at birth and an impaired antibody response to vaccination with a single dose of purified Vi surface polysaccharide extracted from *Salmonella typhi* (McDade *et al.* 2001a). In this study the predicted probability of mounting an adequate antibody response to the Vi vaccine was found to be lower ( $P = 0.023$ ) in adolescents who were both prenatally and currently undernourished (0.32) compared with adequately-nourished adolescents (0.49–0.70; McDade *et al.* 2001a). This observation has subsequently been replicated in a cohort of adults in Lahore, Pakistan (Moore *et al.* 2004). In the early 1960s an urban slum settlement in Lahore was selected for a community-based follow up of infant health. Between 1964 and 1978 a total of 2468 infants were recruited into this study and followed partly longitudinally into childhood (Jalil *et al.* 1989). These subjects are now adults and have recently been traced for follow up in a study looking at the effect of poverty, early-life malnutrition and infections on adult health and mortality. In parallel to the main follow up a selected cohort of 257 of the adults from this cohort (mean age 29.4 years; 146 males) has participated in a study of early-life predictors of adult immune function that used a variety of measures including response to vaccination. Each subject received a single dose of the same Vi vaccine as that used in the Philippines study of McDade *et al.* (2001a) and two doses of rabies vaccine. Antibody titres were measured on pre-vaccination (Vi only) and post-vaccination serum samples (Vi and rabies). Vaccine responses were not found to be consistently associated with any of the contemporary adult variables measured, including month of study, gender, current age and indicators of socio-economic status. However, both anti-Vi IgG and IgM antibody responses to the Vi vaccine were found to be significantly related to birth weight, with a lower antibody response observed in subjects who had a lower birth weight (for the anti-Vi IgG antibody response,  $P = 0.031$ ; for the anti-Vi IgM antibody response,  $P = 0.034$ ; Fig. 4). The antibody response to the first dose and the second dose of the rabies vaccine, however, was not found to be associated with birth weight. The contrasting effects on typhoid and rabies responses observed in this study seem to suggest that the antibody generation to polysaccharide antigens, which are T-cell-independent antigens, is compromised by fetal growth retardation.

A follow up of these adults has recently been carried out to see if the impaired ability of lower-birth-weight subjects to mount an adequate antibody response to vaccination is maintained following a subsequent follow-up dose of the same vaccine. Pre-vaccination antibody titres were found to be strongly correlated with post-vaccination titres from the previous study ( $r = 0.832$ ), with geometric mean antibody titres increasing from 2.86 (95% CI 2.38, 3.46) ELISA units before vaccination to 5.10 (95% CI 4.28, 6.09) ELISA units 21 d post vaccination ( $P \leq 0.0001$ ). Of key interest, this second dose of the same vaccine was not observed to overcome the deficit observed in the previous study, and post-vaccination antibody titres remained positively correlated with birth weight ( $P = 0.0284$ ). In the same study a single dose of a conjugated Haemophilus B



**Fig. 4.** Birth weight *v.* antibody response to a single dose of purified Vi surface polysaccharide extracted from *Salmonella typhoid* Vi for adult subjects who showed an increase in anti-Vi antibody titres that was <4-fold (▨) or ≥ 4-fold (□). The subjects were from a cohort of adults in Lahore, Pakistan who had participated in a community-based follow up of infant health between 1964 and 1978 and had been traced as adults. For details of subject and procedures, see p. 316 of proofs. Values are means with their standard errors represented by vertical bars. (From Moore *et al.* 2004, reproduced with permission.)

polysaccharide-conjugate vaccine was also administered. No significant associations were observed between size at birth and antibody responses to this vaccine. This lack of a response to the polysaccharide conjugate, together with the continued failure of lower-birth-weight subjects to respond to the Vi vaccine supports the suggestion of a possible defect in processes leading to the antibody generation to T-cell-independent antigens. An ongoing study in The Gambia is exploring whether this defect is unique to the Vi vaccine, or whether antibody responses to other polysaccharide vaccines are also correlated with exposures early in life.

Taken alongside the observations from The Gambia, these findings add another level of complexity, and suggest that more than one component of the immune system could be impaired by early-life nutritional insults. Further studies will aim to elucidate the functional mechanisms for this observed impairment in antibody response in individuals who have a lower birth weight.

## Conclusions

Evidence is accumulating to suggest that nutritional deficiencies during prenatal or early postnatal life may disrupt the development of the human immune system, resulting in functional deficits later in life. Such deficits may ultimately be linked to an increase in the risk of premature mortality from infectious disease. Whilst much of the data reported in the current paper comes from resource-poor countries, in which infectious diseases still contribute to a large proportion of deaths, the findings may have implications for the aetiology of other immune-related outcomes, such as allergy, autoimmune diseases and cancer immunosurveillance. Further research is therefore required to help understand the true relationship between early-life nutritional status and later immune function.

### Acknowledgements

The work presented in the present review is the product of a dedicated team working within the UK Medical Research Council's Nutrition Programme in The Gambia, and at collaborative field sites in Bangladesh and Pakistan. We specifically acknowledge the MINIMat study team, especially Dr Shams El Arifeen and Dr Yukiko Wagatsuma, for their contribution to the collection of the thymus ultrasound data in Bangladesh, and we thank Professor Fehmida Jalil and Professor Lars Å. Hanson for their leadership on the Lahore studies. This work was primarily funded by the UK Medical Research Council, with additional support from the Nestlé Foundation. Ethical permission for the Gambian studies was granted by the joint UK Medical Research Council/Gambian Government Ethics Committee. For work in Pakistan approval was given by the Medical Ethics Committee for Research, King Edward Medical College, Lahore, Pakistan and by the Ethics Committee of Göteborg University, Sweden. In Bangladesh ethical permission was granted by the Research Review Committee and the Ethical Review Committee, ICDDR,B-Centre for Health and Population Research, Dhaka, Bangladesh. All studies were conducted with informed consent from all subjects and/or their parents or guardians.

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