THE EFFECT OF SUPPLEMENTAL NUTRITION IN PREGNANCY AND EARLY CHILDHOOD ON FUTURE RISK OF CARDIOVASCULAR DISEASE: LONG TERM FOLLOW UP OF A COMMUNITY TRIAL

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Declaration

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree. Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol. The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED:..... DATE:13/06/07

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Abbreviations used in the text

AIx	Augmentation index
BMI	Body mass index
BP	Blood pressure
CHD	Coronary heart disease
CVD	Cardiovascular disease
CSB	Corn Soya Blend
CI	Confidence interval
DXA	Dual-energy X-ray absorptiometry
HDL	High density lipoprotein
HOMA	Homeostasis model assessment
FMI	Fat mass index
FFMI	Fat free mass index
ICC	Intraclass correlation coefficient
ICDS	Integrated Child Development Services
IQR	Inter-quartile range
IRS	Insulin resistance syndrome
LDL	Low density lipoprotein
meq	milliequivalent
mmol	millimol
mmHg	millimetres of mercury
mU	milliunit
NIN	National Institute of Nutrition
OR	Odds ratio
SD	Standard deviation
SDS	Standard deviation score
SEM	Standard error of the mean
SLI	Standard of living index
WHR	Waist to hip ratio

CHAPTER 1. INTRODUCTION

1.1. Overview of research area

Cardiovascular diseases (CVD) are a major and growing public health problem worldwide.[1] Already the leading cause of death in the high-income countries, their rapidly rising prevalence in the low- and middle-income countries highlights the need to better understand their risk factors and to control them.[2]

In the 20th century, most aetiological research focussed on the role of adult risk factors.[3] However, the difficulties in changing established behaviour and a better understanding of the natural history of CVD gradually shifted the attention to pre-adulthood.[4] The origins of atherosclerosis (a process of progressive narrowing of blood vessels, of which cardiovascular diseases are an end-stage syndrome), and its risk factors in childhood and adolescence are now well established. Atherosclerotic lesions have been shown in blood vessels well before adulthood, and their presence correlated with classic risk factors of CVD.[5] Longitudinal studies have been able to show convincingly how wide variations in established risk factors emerge in childhood, and then `track' along to adulthood.[6] CVD risk factors measured in childhood have been shown to predict later CVD morbidity and mortality, and to some extent, independently of their effect on adult risk factors.[7] While the beginnings of atherosclerosis in childhood are now recognised, its risk factors are far less understood.[4] Among these early risk factors, a central role for nutrition has emerged. Relative overnutrition with consequent obesity clearly seems to be important in later childhood and adolescence.[8-10] However, the attention has recently shifted to undernutrition at a much earlier stage in life (fetal life and infancy), and is also the theme of this thesis.[11;12]

Although the importance of deprived living conditions in early life had been highlighted by Forsdhal in 1970s,[13;14] it was Professor David Barker and colleagues at the Medical Research Council's Environmental Epidemiology Unit at Southampton who progressed this idea in a major way during the 1990s through the formulation of the `fetal origins of adult disease hypothesis'.[12;15] From their observations in epidemiological studies linking small size at birth to later risk of CVD, they concluded that cardiovascular and other related diseases are `programmed' in-utero through the persistence of endocrine, physiological, and metabolic adaptations that the fetus makes when it is undernourished.[11;12] Although this hypothesis (or elements of it) has its detractors, it has generated an immense amount of interest in the contribution of undernutrition, specifically inadequate diet, in early life towards lifelong risk of CVD.[16] The idea that inadequate diet in early life may result in some form of heightened sensitivity to the lifestyle-related risk factors is of some importance to the enfolding CVD epidemic in the low- and middle-income countries, where undernutrition and urbanisation now frequently co-exist.[17]

Much of the evidence in support of a role for inadequate diet in early life comes indirectly through animal experiments[18;19] and observational studies in humans that have demonstrated associations between various anthropometric measures relevant to early life (as proxies for inadequate diet) and CVD risk.[11;12]

Animal experiments of dietary restriction in intrauterine life and infancy provide strong evidence of programming of several CVD risk factors.[18;19] However, the extreme nature of some of these interventions and important developmental differences between animals and humans puts limits to the inferences that can be drawn from these experiments. Observational studies in humans have used various anthropometric measures as indices of early diet and nutrition.[11;12] Of these, the most consistent evidence comes from studies that have used birthweight as a proxy for fetal nutrition. Despite initial concerns about selection

bias and inappropriate adjustments for confounders,[20;21] the associations between lower birthweight and later risk of CVD are now clearly established. What remains unclear though is whether low weight at birth represents poor maternal diet in pregnancy or poor nutrition of the fetus due to placental and other `supply line' factors.[22;23]

Direct evidence of a role for poor diet in early life towards later risk of CVD is extremely limited. The importance of balanced protein-calorie reduction has been studied in two natural experiments of starvation and one small randomised controlled trial of nutrition supplementation, but the results have been conflicting.[24-27] The small sample sizes of these studies and the differences in the timing and duration of exposure may explain this inconsistency. More specific deficiencies in pregnancy nutritional intake that have been investigated to any extent include those of protein and calcium, and these have been equally inconclusive.[28-32] Difficulties in characterisation of the exposure (i.e. dietary intake of specific nutrients) in large-scale epidemiological studies and the well-fed nature of study populations (with dietary intakes predominantly in excess of requirements) may be responsible for opposing results.

Research on the role of diet in infancy has mainly focussed on the type of feeding (breast versus formula) and sodium intake. Breast feeding appears to reduce CVD risk, but only modestly.[33-36] There is evidence to suggest that higher sodium intake in infancy may be positively associated with blood pressure in the short term, but clear evidence on its longer-term effects is lacking.[37;38] There are hardly any studies on the role of dietary inadequacy in early childhood beyond infancy.

These studies suggest that despite indirect evidence from diet experiments in animals and observational studies in humans using anthropometric markers as proxies, there is little direct evidence to support a role for inadequate diet in

early life and later risk of CVD. Very few studies have been conducted and those that have been conducted were limited by small sample sizes, insensitive exposure or outcome measures, and observational study designs (with their inherent confounding structures). However, probably the most important limitation of these studies has been their setting. Almost all studies were conducted in generally well-nourished populations, in which the effects of relatively inadequate diets (if they exist) are less likely to manifest. Even in those studies where dietary inadequacy was established, birthweights never fell below 3.0 kg.[24;25;27]

Clearly, there is need for further studies conducted in settings where undernutrition is prevalent. Such studies need to be adequately powered through the use of sufficient sample sizes and sensitive measurement techniques. Use of sensitive measurement techniques that can detect early changes also offer the best insurance against the confounding effects of adult risk factors. Finally, wherever possible, intervention designs need to be employed to break up the problematic confounding structures and to additionally improve study power (by introducing sufficient variation in the exposure). The opportunity to conduct a study that met most of these criteria arose through the existence of an earlier trial of nutritional supplementation conducted in 29 villages near Hyderabad (south India), approximately 15 years ago.

1.2. The community trial

Integrated Child Development Services (ICDS) is a community-based outreach programme aimed at improving the health, nutrition and development of children in India.[39-42] The centrepiece of this programme is the provision of free food: a cereal based meal prepared from locally available ingredients, but providing on average 500 kilocalories and 20 grams of protein to pregnant/lactating women, and 300 kilocalories and 10 grams of protein to

children up to 6 years. The supplement has to be collected daily by the woman (and/or her children) from the ICDS centre (run by a local woman trained for this programme), but they are not obliged to eat it there. To ensure that the impact of food supplementation on child's nutritional status is not undermined by ill-health, diarrhoea and frequent infections, the programme is complemented by a) health check-up and referral services, b) health and nutrition education for the mothers, and c) delivery of other vertical programmes (immunisation, and control of anaemia and Vitamin A deficiency) through the ICDS centre. These other programmes are available universally, but it is anticipated that a common point of delivery increases their uptake in ICDS programme areas.

The ICDS programme was introduced as a pilot project in 1975, but since then has been incrementally expanded so that it now covers approximately three-fourths of the country's rural population (5 million expectant and nursing mothers, and 23 million children under the age of six), making it the largest national food supplementation programme in the world.[42;43] Despite the scale and financial implications of this programme, and the national commitment for universal coverage, no long-term evaluations of its benefits to the population have been carried out. Short term evaluations, on the whole, support the effectiveness of ICDS scheme, both in terms of process indicators (uptake of individual programme components) and in outcome measures (prevalence of child undernutrition).[43-46] However, the quality of these studies has generally been quite poor and several found only modest improvements.

Using the opportunity afforded by the stepwise expansion of this programme during the 1980s and 1990s, the National Institute of Nutrition (NIN) in India conducted a trial to assess the impact of food supplementation in pregnancy on birthweight of the offspring. A cluster of villages with a total population of 30,000 was chosen from each of the two adjacent administrative areas (called `blocks') near Hyderabad city, one of which already had the ICDS programme in

place (intervention area), while the other was awaiting implementation (control area). Since the 100+ villages in each of the two blocks were spread over an unfeasibly large area, villages were restricted geographically: contiguous villages falling within a 10 km radius of a prominent central village in each block were selected. This process resulted in 15 villages from the intervention area and 14 villages from the control area being included in the study.

A team of investigators resided full-time in the field for the duration of the study. Following a lead-in period of six months (involving training, pilot data collection and household enumeration to identify women in the reproductive age group i.e. 15-44 years), the trial was conducted to include all births in the area between 1st January 1987 and 31st December 1990. The `at risk' (of pregnancy) women were monitored monthly to detect missing of menstruation, and those identified as pregnant were followed closely during pregnancy until delivery (undergoing clinical examinations in each of the three trimesters). The field team attempted to visit the home as soon as possible after delivery to collect data on the outcome of the pregnancy, and to weigh the newborn.

The supplement given to those in the intervention arm (women and children) was `upma', a savoury preparation made from Corn-Soya blend (CSB) and soybean oil. No other nutrients were added to the supplement. The other universal programmes: immunisation; anaemia control, Vitamin A prophylaxis and basic health services existed to a similar extent in both the intervention and control areas, although their uptake may be presumed to have been higher in the intervention area.

The data collected were computerised and preliminary findings were reported in an abstract published in an in-house publication of the National Institute of Nutrition, while the study was still ongoing. That abstract reported an 88 g

($p<0.01$) improvement in birthweight among children born in the supplemented villages.[47] However, departmental reorganisation towards the end of the study, and lack of funding for statistical support in the project resulted in the final data never getting published (Dr KV Rameshwar Sarma, personal communication, June 2002).

A follow-up of the children born in this trial offered an ideal opportunity to study the role of early nutrition on later cardiovascular disease risk for several reasons:

1) The area has high levels of chronic undernutrition; so one could realistically expect to find long-term programming effects of inadequate early diet, if they exist;

2) Although not satisfying the rigorous standards of a randomised design, the controlled trial design provided a reasonable degree of control over confounding, while at the same time improving study power by increasing exposure variation;

3) Some of the most crucial confounders that normally plague such research were either completely absent (i.e. maternal smoking) or severely restricted (i.e. socio-economic heterogeneity and urbanisation with the consequent heterogeneity in diet and physical activity) in this population; and

4) The age group (adolescence) was ideal for studying early life exposures, as the participants were old enough to have adequate variability in the outcome measures (study power) and to demonstrate persistence of any effects, but young enough so that observed associations were not distorted by effects of adult lifestyle factors in the intervening years.

1.3. Aims and objectives

The primary aim of this thesis was to investigate the role of dietary inadequacy in early life on later risk of CVD.

The specific objective was to test the hypothesis that:

1. Supplemental nutrition in early life is associated with a lower prevalence of CVD risk factors in adolescence (increased blood pressure and arterial stiffness, dyslipidaemia, and abnormal glucose-insulin homeostasis)

Before addressing this primary hypothesis, it was important to answer two other related questions. First, whether the determinants of CVD risk factors in this low-risk population are similar in their relative importance to those within high-risk populations.[48] While the risk factors for CVD are well characterised in high-risk populations, they are much less so in relatively low-risk populations. Second, the long-term effects of early supplemental nutrition on later body size and composition are not known. This is relevant as supplemental nutrition could potentially both decrease (by increasing height)[49] and increase (by increasing adiposity) risk of CVD.[48] The possibility of a positive supplementation-adiposity association was of particular concern in view of the positive birthweight-adiposity association seen in more affluent populations.[50;51] Concerns about improved nutrition in early life fuelling obesity epidemics have already been raised.[52;53] These issues resulted in two secondary objectives. The specific hypotheses relating to these objectives were that:

2. The determinants of CVD risk factors in low-risk populations reflect the direction and strength of the associations seen in high-risk populations (i.e. increasing levels of CVD risk factors are associated with decreasing

socio-economic position and height, and with increasing urbanisation and adiposity)

3. Supplemental nutrition is associated with taller stature and greater adiposity

1.4. Organisation of the thesis

Chapter 2 gives a brief overview of the state of current knowledge about the early origins of cardiovascular diseases, before going on to outline the indirect evidence relating to the role of early diet; specifically data from animal experiments and observational studies in humans using anthropometric data as proxy for diet. Chapter 3 reviews the research that has directly investigated the role of early diet on later CVD risk. Chapter 4 describes the methods used in the design and conduct of the study. Chapter 5 describes the characteristics of the study participants, the distributions of the clinic variables, and the reliability of the collected data. Chapters 6 and 7 present the results of the analyses relating to the determinants of CVD risk in this population and the effects of supplemental nutrition on CVD risk, respectively. Finally, Chapter 8 discusses the findings, their strengths and limitations, and their implications.

CHAPTER 2. EARLY ORIGINS OF CARDIOVASCULAR DISEASE

2.1. Introduction

Coronary heart disease, stroke and peripheral vascular disease are end stage disease syndromes of atherosclerosis, a process of progressive narrowing of blood vessels resulting in reduced blood supply to the organs. The word atherosclerosis itself comes from the Greek words `athero' (meaning gruel or paste) and `sclerosis' (hardness). Atherosclerosis is believed to start as deposits of cholesterol and its esters (called fatty streaks) in the inner wall (intima) of the large muscular arteries (see Figure 2.1).[54;55] In some persons and at certain arterial sites, more lipid accumulates and is covered by a fibromuscular cap to form a fibrous plaque.[54] Further changes in the fibrous plaque render it vulnerable to rupture, an event that precipitates occlusive thrombosis and clinically manifest disease. In adults, elevated levels of non-HDL-cholesterol and reduced levels of HDL-cholesterol concentration, hypertension, smoking, obesity, and diabetes are associated with advanced atherosclerotic lesions and increased risk of clinically manifest atherosclerotic disease.[3]

The link between early diet and CVD has been recognised for some time. Holman had declared atherosclerosis to be a paediatric nutrition problem as early as 1961.[56] His conclusions at the time, however, were based on early observations of fatty streaks and plaques in the aorta and coronary arteries in childhood, coupled with the emerging evidence that dietary fat and cholesterol were important determinants of plasma cholesterol concentrations which contributed to the progression of atherosclerosis. Over the ensuing years, the beginning of atherosclerosis in childhood has been confirmed, as has the emergence and importance of the majority of risk factors (not just cholesterol), hitherto considered as adulthood risk factors, towards later cardiovascular

disease morbidity and mortality.[6] While the central role of relative overnutrition and consequent obesity in the emergence of these risk factors is now established, the role of undernutrition in early life (or even before) is contentious, and is the theme of this thesis.[16] In this chapter, I will first outline briefly the evidence that supports the emergence and importance of atherosclerosis and its established risk factors in childhood and adolescence, before presenting, in some detail, a critique of the current state of knowledge on the role of early undernutrition.

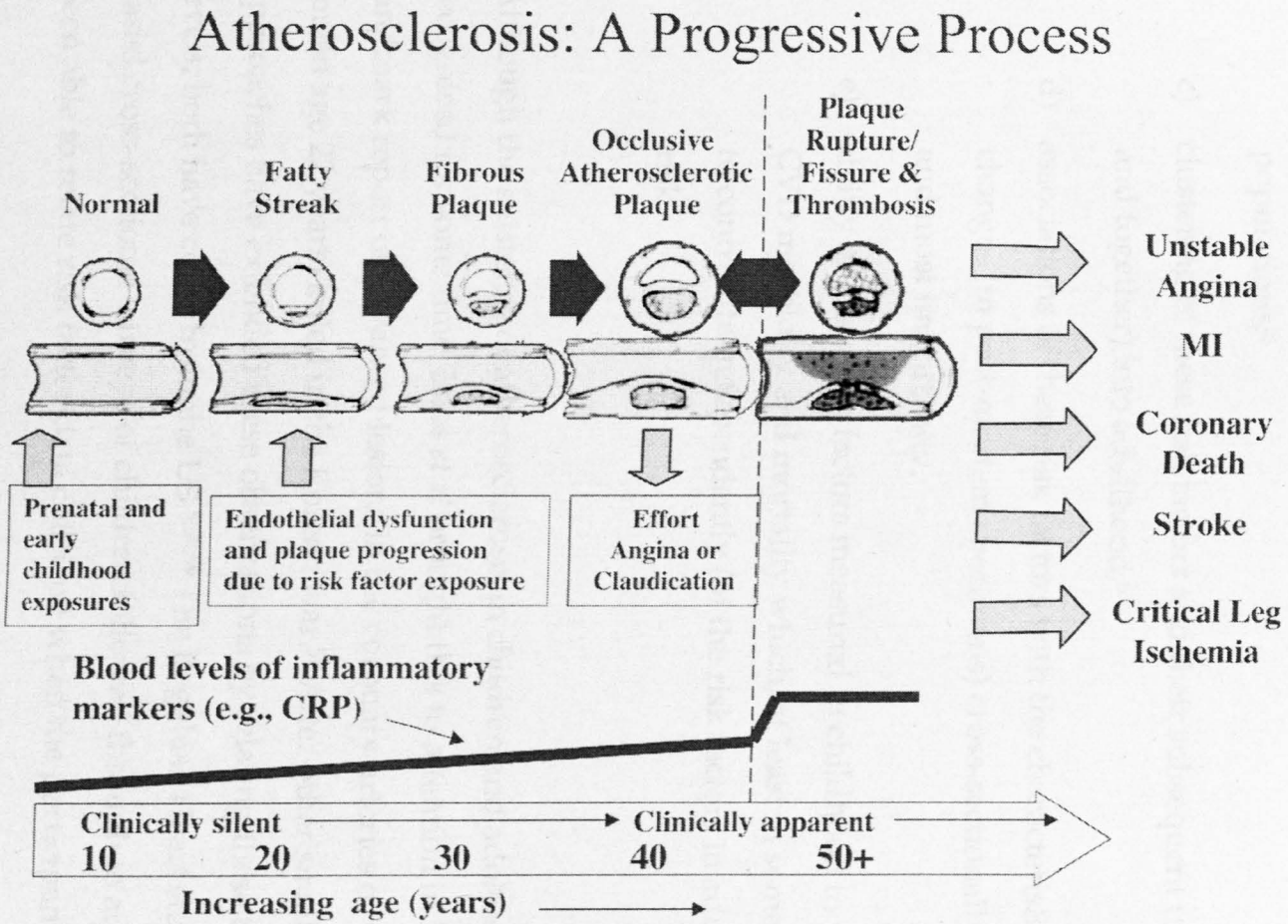


Figure 2.1: Evolution of atherosclerosis (reproduced by kind permission of the Oxford University Press and the author)[55]

2.2. Origins of cardiovascular disease in childhood and adolescence

There are several lines of evidence that confirm the beginnings of atherosclerosis and its risk factors in early life and their relevance to later disease. These include the documentation, in childhood and adolescence, of:

a) characteristic changes in pathology specimens of blood vessels;[5]

b) wide variations in the levels of established risk factors within populations;[6]

c) clustering of these risk factors and their subsequent tracking (singly and together) into adulthood;[6]

d) associations of these risk factors with the characteristic pathological changes (in post-mortem specimens) cross-sectionally in childhood;[5] and most importantly,

e) ability of these risk factors measured in childhood to predict later CVD morbidity and mortality which, at least in some cases, appears to contribute independently (of the risk factors in adulthood) to the risk.[7]

Although the existence of atherosclerosis in children and adolescents had been recognised for some time, Enos *et al* brought this to attention in 1953 with their landmark report of advanced lesions in the coronary arteries of young soldiers (mean age 22 years) killed in the Korean war.[57] After other smaller studies, two approaches have extended these observations by relating these to risk factor levels; both have come from the US.[5;58;59] The Bogalusa Heart study (population based cross-sectional surveys of children followed through to adulthood) has been able to relate risk factor data collected when the participants were alive,

aged 3-18 years, to atherosclerotic lesions on post-mortem autopsy (93 deaths between the ages of 2 and 39 years).[58] The Prevalence and Extent of Atherosclerosis in Adolescents and Young Adults (PADY), a much larger study, conducted autopsies on 2,876 persons dying at ages 15-34 years (who were free of known CVD) and related these to their CVD risk factor levels assessed from records and examination at the time of autopsy.[5;59] Both these studies have confirmed the presence of atherosclerotic lesions well before adulthood, the links between fatty streaks and more advanced lesions, and the relationship of these lesions to classic risk factors like hypertension, lipid concentrations, obesity and smoking.

Several longitudinal studies with CVD risk factor data collected at regular intervals in childhood and adolescence have been able to follow these children through to adulthood. These include the Bogalusa Heart Study,[60] the Muscatine study,[61] Project HeartBeat,[62] and the Cardiovascular Risk in Young Finns Study,[63], among others. Reports from these studies show the co-existence of multiple risk factors, especially in relation to obesity and insulin resistance.[64;65] They have also shown a wide variation in the levels of these risk factors, and even more importantly, `tracking' of these risk factors through to adulthood.[6] `Tracking' refers to persistently high values of continuously distributed traits (such as blood pressure or cholesterol) on repeated measurement (i.e. a consistency of values or ranks over time).[6] These studies have shown that some degree of tracking is present for most of the risk factors (although their strength is debated), and tends to be greater for traits measured with less measurement error and at shorter interval between measurements.[60;61;63;66] As an example, the range of spearman correlation coefficients for total cholesterol concentrations, measured 12 years apart, was: 0.48 - 0.58 in The Cardiovascular Risk in Young Finns study (aged 3-18 years at baseline);[63] and 0.42 - 0.53 in the white and 0.38 - 0.66 in the black children (both aged 2-14 years at baseline) of the Bogalusa Heart Study.[60]

Evidence is also accumulating about the long-term effects of early life risk factors.[7] Strong associations have been shown between the risk factors measured in childhood and adolescence and carotid artery intima-media thickness: measured at ages 25 - 37 years in the Bogalusa Heart Study,[67] and 24 - 39 years in the Cardiovascular risk in Young Finns study.[68] Obesity (boys and girls, aged 2 - 15 years),[10] blood pressure (males, aged 15 - 29 years),[69] cholesterol (males, mean age 22 years),[70] and multiple risk factors (males and females, aged 18 - 39 years) have all been associated with CVD mortality.[71;72] In the Chicago Heart Association Detection Project in Industry study, 7,302 women aged 18 to 39 years (without prior CHD) were screened between 1967 and 1973.[71] Women were divided into low-risk (<2 risk factors) and high-risk (≥ 2 risk factors) on the basis of the number of unfavourable risk factors (systolic and diastolic blood pressure, serum cholesterol level, BMI, presence of diabetes, and smoking status), using national guidelines for cut-offs as appropriate. Multivariable-adjusted CVD mortality hazard ratio for low-risk women was 0.19 (95% CI: 0.08 to 0.45), compared with high-risk women.

The evolution of CVD risk factors is of particular importance during adolescence. The body size and composition change dramatically as the person goes through stages of puberty.[6;62] Levels of hormones change and sex-specific patterns emerge. Many of the important behavioural patterns associated with later risk of CVD (such as smoking, diet and physical activity) get established at this age.[6] All these reasons make adolescence an extremely important and informative age to study the risk of CVD. However, because of the speed of change, one would require at least annual, if not more frequent, data collection to allow a clear understanding of the evolution of the risk factors, and most studies do not have such data. Consequently, the current level of knowledge is limited to data from one or two studies (such as Project HeartBeat), and these show ethnic differences

in patterns, thus highlighting the limitations of extrapolating these to other populations.[73]

Data from Project HeartBeat show sex and ethnic differences in the trajectories of concentrations of lipids over the age period 8 to 18 years.[6] Overall patterns suggest that the concentrations of cholesterol (total and LDL fraction) decline between the ages of 10 and 14 years, after which they remain largely stable. HDL-cholesterol fraction appears to change less with age, but rises somewhat between the ages of 14 and 18 years. The concentrations of triglycerides rise till the age 13, after which they appear to plateau. As opposed to lipids, blood pressure appears to rise year on year over the entire age range of 6 to 18 years, although the increase is modest in girls after age 14 years.[74] Such detailed longitudinal data are not available for glucose and insulin. Cross-sectional data from Bogalusa Heart and other studies show that glucose values rise slightly with age to a peak at 13 years, and fall slightly at older ages.[75] Cross-sectional studies also suggest an increase in insulin levels with age.[76]

Detailed longitudinal data are available for some measures of body size. After a transient decrease in the first year of life, BMI increases continuously throughout childhood in both males and females.[6] This increase reflects general growth of body mass relative to growth in height. The proportionate contributions of lean and fat mass differ by sex.[6;62] While females have a greater and nearly constant proportion of fat mass, the rise in BMI in males is largely due to a greater proportionate increase in lean mass, relative to fat mass, from the start of adolescence. The pattern for abdominal circumference resembles BMI.[62] In contrast, percentage of body fat (measured by skinfolds or bioelectrical impedance) and sum of multiple skinfolds are greater in females than males at all ages, and show a sharp decrease in males after a peak at age 10 (percentage body fat) or 12 (skinfolds) years.[62]

2.2.1. Behavioural and social antecedents

Differences in behavioural patterns (such as those of diet, physical activity and smoking), to a large extent, explain the differences in biological markers of CVD risk e.g. lipid concentrations, obesity and hypertension.[3] Such behavioural differences often originate and get established in childhood and adolescence, and may be the mechanism underlying tracking of biological markers of CVD risk and their later expression as disease.[16;77] The strongest evidence is for smoking, which often begins in childhood and is considered unlikely to become a habit if not started before adulthood.[6;77] Dietary patterns and physical activity levels that contribute to the development of obesity often track into adulthood, particularly among children who are obese as adolescents.[77;78]

Cross-sectional studies show clear associations between behavioural and biological risk factors in childhood.[79] However, the contribution of childhood differences in behavioural risk factors towards later risk, independent of the differences in adulthood (i.e. outside of `tracking'), is less well established. A systematic review investigated the published literature on childhood determinants of adult obesity.[80] It found no consistent evidence that childhood diet or physical activity patterns determined obesity in adulthood. The review highlighted the limited nature of existing evidence, with studies being few in number and generally underpowered (most studies had fewer than 400 participants). Physical activity patterns and dietary intake are both extremely hard to measure accurately. This may explain both the lack of large studies and the absence of associations (since random error introduced as a result of imprecise measurements would serve to bias the association towards the null). A systematic review of intervention studies in adults found that improvements in blood pressure seen with increased levels of physical activity were limited to the duration of physical activity.[81] This could potentially explain the lack of long-

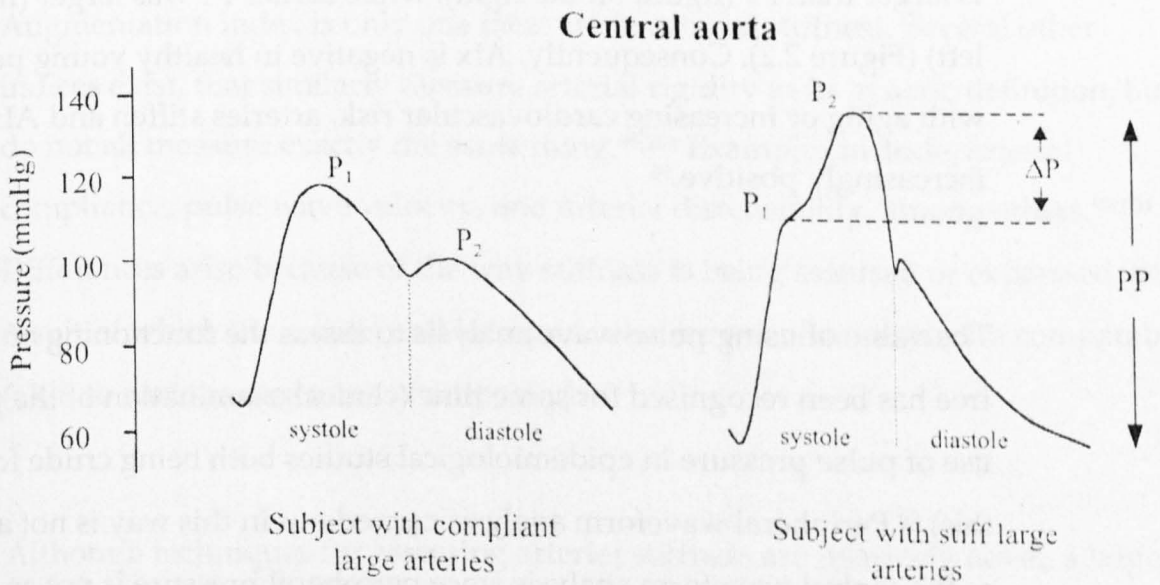
term benefits of increased activity in childhood; however, the evidence is too limited to arrive at any conclusions. Recently, there has been considerable interest in the effects of television viewing on obesity, which is believed to operate through a combination of reduced physical activity and dietary imbalance.[82] In the Framingham children's study, 106 children were enrolled during preschool years (mean age 4 years) and followed into early adolescence (mean age 11 years).[83] Children who watched more television during childhood had the greatest increase in body fat over time. Results from other studies, however, are mixed with not all studies finding such an association.[82;84] Although relevant, these findings from predominantly US based studies may not be entirely generalisable to other settings.

There is recognition that socio-economic differences could underlie much of the observed differences in CVD risk in childhood.[85;86] In adults, CVD and its risk factors show clear socio-economic gradients, although these gradients can be context-specific, as evidenced by the higher prevalence of CVD among the more affluent in the low-income countries,[2;86;87] and the less affluent in the high-income countries (certainly in the last few decades).[85;86;88] Evidence from high-income countries suggests that low socio-economic position in childhood is predictive of increased CVD morbidity and mortality.[85;86;89] This effect has been found to be independent of adult socio-economic position, which has prompted considerable debate about the potential mechanisms by which childhood socio-economic position might influence CVD risk in later life.[85;88-91]

A recent systematic review examining the socio-economic gradients of CVD risk within UK children found consistent evidence for cigarette smoking and height (lower prevalence of smoking and greater height among those from more affluent households), but no clear evidence for an association with blood pressure, cholesterol and adiposity.[92] On the other hand, there is good evidence, from UK and elsewhere in Europe for a consistent association between socio-

economic position in childhood and CVD risk factors when measured in adulthood (lower prevalence among those more affluent), although the evidence is more consistent for some risk factors (obesity and blood pressure) than others (lipid concentrations).[90;93] Several lifecourse models have been proposed to explain this late manifestation of early life socio-economic position on CVD risk, which variously imply combination, accumulation and/or interactions of different environments.[90;94] One review evaluated the existing evidence in view of such models and concluded that there was more support for a cumulative model i.e. accumulation of negative socio-economic conditions or experiences (also called `accumulation of risk model' by Ben-Shlomo *et al*).[90;94] Irrespective of the model typologies, it seems plausible that social patterning of obesity (which itself manifests late) has a central role in the social patterning of CVD risk.

2.2.2. Arterial stiffness: risk factor or marker of disease



P1 = systolic pressure peak; P2 = diastolic pressure peak; ΔP = P1 – P2; PP = Pulse pressure

Figure 2.2: Aortic waveform pattern in participants with compliant and stiff arteries

Arteries are compliant structures and they serve to buffer the pressure changes resulting from the intermittent ventricular ejection of the blood into the aorta.[95] By absorbing part of the energy, and releasing it in diastole, the arteries help to smoothen the peripheral blood flow. This results in the characteristic pressure waveform, as in the case of the ascending aorta shown above in Figure 2.2.[96;97] The first peak P1 is produced during systole by the pressure of the forward-going wave resulting from contraction of the ventricle. The second peak P2 is produced in diastole by summation of the outstanding forward-going wave and pressure from the reflected waves. The augmentation index (AIx) is defined as the difference between the first and second peaks (ΔP), expressed as a percentage of pulse pressure (PP).[96] Normally, the reflected wave arrives at the ascending aorta during diastole, after closure of the aortic valve, so that it does not influence the central systolic pressure. However, with arterial stiffening, the wave returns sooner (before the aortic valve has shut and the ventricle is still in systole) and adds to, or augments, the central systolic pressure. Note that now P2 is larger than P1 (figure on the right), while earlier P1 was larger (figure on the left) (Figure 2.2). Consequently, AIx is negative in healthy young people, but with aging or increasing cardiovascular risk, arteries stiffen and AIx becomes increasingly positive.[96]

The value of using pulse wave analysis to assess the functioning of the vascular tree has been recognised for some time (clinical examination of the pulse or the use of pulse pressure in epidemiological studies both being crude forms of this).[98] Peripheral waveform analysis carried out in this way is not as informative as the central waveform analysis since peripheral pressure is not as influenced by changes in systemic arterial stiffness as central pressure.[99] However, with the recent development of techniques that allow central pressure waveforms to be analysed non-invasively, interest in the use of pulse wave analysis has gathered pace. In one such technique (O'Rourke system), a tonometer (a probe with a

sensor that can record pressure changes with high fidelity) is used to flatten but not occlude a peripheral artery (say the radial artery on the wrist) (Figure 2.3).[98;100] Circumferential pressures are equated and an accurate recording of the pressure waveform is obtained, which is then transformed into the corresponding central arterial waveform, using a generalised transfer function validated against invasive pressure recordings.[100-103]

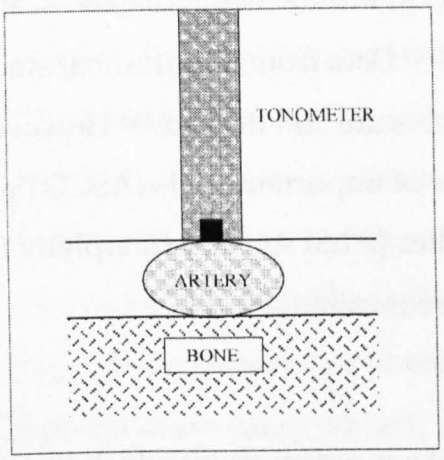


Figure 2.3: Tonometer applied to an artery

Augmentation index is only one measure of arterial stiffness. Several other indices exist, that similarly measure arterial rigidity in its generic definition, but do not all measure exactly the same thing.[98;104] Examples include, arterial compliance, pulse wave velocity, and arterial distensibility, among others.[98;104] Differences arise because of the way stiffness is being assessed or expressed, as a result of which the results and interpretation are not always directly comparable (akin to various measures of adiposity).

Although techniques for assessing arterial stiffness are relatively novel, a large body of evidence already exists that supports its role as an important independent risk factor of cardiovascular disease, and also as an important predictor of outcome.[105;106] Measures of arterial stiffness have been shown to correlate well with other cardiovascular risk factors such as hypercholesterolaemia, diabetes, and sedentary lifestyle,[107-109] while risk factors

identified in childhood and adolescence have been shown to predict arterial stiffness in adulthood.[110] Arterial stiffness has been shown to independently predict coronary, cardiovascular, and all cause mortality in high-risk populations,[111-116] and cardiovascular risk in less selected populations.[117-119] In patients with end stage renal disease, improvement in arterial stiffness predicts reduction in mortality, independent of improvement in other risk factors and blood pressure.[120;121] Direct comparisons have found that arterial stiffness correlates strongly with common carotid intima media thickness, an important clinical measure of atherosclerosis.[122;123] Data from longitudinal studies using more direct measures of arterial stiffness are still limited.[118] However, it is incorporated in protocols of a number of important trials (ASCOT and FIELD) and prospective epidemiological studies (ALSPAC and Caerphilly Prospective Study), and data from these studies are awaited.[109]

Arterial stiffness has been proposed as a mechanism by which hypertension develops.[118;124] More importantly for the purposes of this study, it has been proposed as the pathway by which poor nutrition in early life leads to hypertension and cardiovascular disease.[125] Its correlations with low birthweight (although inconsistently)[126-129] and insulin resistance,[109;130-132] both highly prevalent in Indians,[133-136] argued for its inclusion in this study. Arterial stiffness also offers strong practical advantages in rural fieldwork, as the equipment is lightweight and portable, and can be operated by non-specialist staff with limited training.[137]

2.3. Possible origins of cardiovascular disease before childhood

The idea that cardiovascular disease may have its origins not just in childhood and adolescence but even before (i.e. intrauterine period and infancy) was

advanced by Professor David Barker and colleagues at the Medical Research Council Environmental Epidemiology Unit at Southampton.[15] Originally searching for an explanation of the association between high neonatal mortality and cardiovascular disease mortality (50 years later) in geographical regions of England and Wales, they hypothesised that it was mediated by low birthweight (since low birthweight was associated with neonatal mortality). They then conducted a series of historical cohort studies following up men and women in middle and late life whose body measurements at birth had been recorded, and showed that small size at birth was associated with coronary heart disease (CHD), stroke, diabetes and the associated risk factors.[15] They interpreted this as evidence of long-term effects of fetal undernutrition and consequently formulated the `fetal origins of adult disease' hypothesis, which proposes that cardiovascular and other related diseases may be `programmed' in-utero through the persistence of endocrine, physiological, and metabolic adaptations that the fetus makes when it is undernourished.[11;12] Although this hypothesis, especially in its entirety, is not accepted by many, it has generated an immense interest in the role of early life undernutrition in later disease.[16;20;23;138] The idea that undernutrition in early life may result in some form of heightened sensitivity to lifestyle related risk factors has great relevance for much of the developing world, where undernutrition and urbanisation now frequently coexist.[17;139;140]

At this point it is important to clarify the terminology used, since the terms `diet' and `nutrition' are often used interchangeably in the literature. Diet is defined as the regular food and drink of a person, while nutrition refers to the process by which the consumed food and drink is used for liberation of energy, replacement of tissues and growth.[141] Growth refers to an increase in size or number. Anthropometric measurements of body size and composition (commonly referred to as `nutritional status') or change in the measurements over time (`growth') provide a crude measure of dietary intake, since they can be

influenced by factors unrelated to diet, some of which may even operate intergenerationally. Where the interest is in diet (which it often is because it offers a more discrete option for intervention, unlike nutritional status which may require improvements in general living conditions for a few generations), assessment of nutritional status through anthropometry provides less direct evidence than assessment of diet itself.[141] The focus of this thesis is on the role of diet in early life, and the direct evidence relating to this is discussed in detail in the next chapter (i.e. where diet was the primary exposure). Evidence from animal experiments and studies using anthropometry as a proxy for diet are regarded as indirect lines of evidence and consequently summarised in relatively less detail in the remaining half of this chapter.

2.3.1. Animal experiments

Indirect evidence for the role of diet in early origins of cardiovascular disease comes from a series of animal experiments. Although poor fetal nutrition in animals can be induced by a variety of interventions that alter the fetal supply line (such as uterine artery ligation), they often lead to mixed effects that include elements of hypoxia and reduced nutrient supply.[23] More informative are experiments that involve dietary manipulation, and these have been the focus of recent research; specifically, global caloric restriction, reduction of dietary protein content, and dietary fat supplementation.[18;19] Rats have been studied most frequently due to reasons of feasibility, despite important differences in their development as compared to humans.[18] Much of the organ development in rats takes place after birth, primarily in the weaning period. Primates, guinea pigs and sheep offer models that are more similar to humans, but have been studied less extensively due to the costs involved.

In rat experiments, global food restriction in pregnancy has been frequently shown to result in hypertension among the offspring.[18;142] Female offspring of

wistar rats fed severely reduced diet (30% of ad libitum intake) in pregnancy had increased blood pressure.[143] Similar effects have been reported even with more modest dietary restriction (70-80% of ad libitum intake), and limited to the latter part of pregnancy.[144] Experiments on protein restriction, however, have given mixed results. Offspring of pregnant rats fed on the Hope Farm diet do not appear to get hypertension, while those on the Southampton diet do.[18] Although the protein content of these two diets is very similar, they differ importantly in other nutrients (Southampton diet has starch as the source of carbohydrate and corn oil for lipids, while Hope Farm diet contains glucose and soy oil), and these differences have been suggested as possible explanations for the differences in findings.[18] Raised blood pressure has been reported in offspring of animals fed on diet rich in animal lard and saturated fats,[145] but not on polyunsaturated fatty acids. In one study, a low-protein diet that contained more fat and starch led to increased BP in the offspring, while a low-protein diet that contained more sugar failed to program for hypertension.[146] High or low sodium intake has also been reported to program offspring hypertension.[142] Once developed, prenatally programmed hypertension seems to persist indefinitely and to get progressively worse with age.[142]

As opposed to hypertension, fetuses seem to be resistant to metabolic imprinting of weight and adiposity from intrauterine undernutrition.[19] Offspring of initially well-nourished dams restricted by 50% caloric intake in pregnancy and followed up to adulthood had body weight and body composition similar to the control group, despite a 20% decrease in birthweight.[147] Offspring of dams given restricted diets in the first two trimesters but allowed free access in the third trimester were heavier and fatter than the control group, suggesting compensatory maternal hyperphagia as the cause of offspring's later obesity.[148] Among artificially fed baboons given formulas of different energy densities, those overnourished in the preweaning period show greater adiposity than those undernourished, even at 5 years of age.[149] Cumulatively, animal experiments

suggest that nutritional status during early postnatal period has more profound and persistent effects on obesity than prenatal period.[18;19]

Dyslipidaemia has been studied less frequently, possibly as the rat is less ideal as a model. Compared to humans, rats have low serum LDL concentrations, carry cholesterol mostly as HDL, and are generally resistant to diet-induced atherosclerosis.[18] Plasma lipid profiles (cholesterol and triglycerides) were found to be normal in two global diet restriction models.[18] In one experiment with protein-restricted diet in rats, concentrations of triglycerides were found to be lower, not higher, as anticipated from human birthweight data.[150] In fact, protein-restriction models often fail to achieve low birthweight, with some even reporting higher birthweight.[18] Infant baboons that were breast-fed had more atherogenic lipid profile and more atherosclerotic lesions on autopsy than those fed formula milk.[151] The cholesterol levels among the formula fed baboons were similar despite differing concentrations of various formulae used, suggesting that other contents of breast milk may be more relevant.

Moderate maternal food restriction (30% reduction in ad libitum intake) in guinea pigs resulted in glucose intolerance, and raised plasma insulin was seen even after 15% reduction of intake.[152] Impaired glucose tolerance was also seen in offspring of pregnant rats fed protein-restricted diets or a diet rich in animal lard.[18] Experiments have shown alterations to the structure and function of fetal pancreas following maternal protein restriction.[18] Long-term effects of prenatal exposure to hyperglycaemia have been modelled by streptozocin-induced gestational diabetes, with persistent hyperglycaemia seen in the offspring.[153] These findings support the epidemiological finding of U-shaped relationship between birthweight and adult diabetes.[154] Both global restriction of diet and low-protein diet in various postnatal periods (suckling and early postweaning) seem to result in persistent alterations in fasting insulin, but not in glucose

concentrations, suggesting that critical window for imprinting of insulin sensitivity may extend beyond suckling period.[19]

Vascular endothelial dysfunction and concomitant increased blood pressure has been found among offspring of Wistar rats fed 50% of normal intake during pregnancy.[155] Protein restriction during development produces endothelial dysfunction in adult Wistar rat offspring, as determined by blunted endothelium-dependent vasodilator dysfunction in resistance arteries.[156] Maternal diets rich in animal fat also produce endothelial dysfunction in offspring.[18] Endothelium-dependent relaxation appears to be an accompaniment to most of the nutritional developmental programming models in the rodents.[18]

Summary of animal experiment evidence

There is clear evidence from animal experiments to support the role of diet (during pregnancy and early postnatal life) in programming of glucose-insulin homeostasis. There is less data to support the programming of lipid metabolism, although the rat is not an ideal model to assess this. There is no evidence to support programming of obesity in-utero, although diet in early postnatal period may be important. There is evidence, albeit inconsistent, on the programming of hypertension. Impaired vascular endothelial dysfunction appears to be one of the most robust phenotypes observed with poor diet in early life. The prevalence of this defect in different models support its central role in the evolution of various phenotypes which include, among others, hypertension, arterial stiffness, and atherosclerosis.

Although these experimental models provide important insights and support for epidemiological findings, caution needs to be applied in extending these to humans. The dietary interventions in animals are often more extreme than generally observed in humans. Even within experiments, variations in feeding

regimens, dietary challenges, and techniques used to measure outcome can contribute to marked differences in findings.[18] In most animal experiments, the animals are fed normal diet following dietary manipulation, and this may not reflect the situation in humans, where development of CVD risk is often contingent on later diet.[18] Finally, there are important differences in structure and function of developing humans and animals that allow only limited inferences to be drawn.[23] As Gilman has suggested, 'One should use the animal experimental data only as models, as they are intended'.[138]

2.3.2. Anthropometric evidence

Indirect evidence for the role of poor diet in early life comes from epidemiological studies that have used anthropometric markers of undernutrition and related those to later CVD and its risk factors. Of the anthropometric markers, the most frequently studied has been birthweight, possibly because it is most frequently available. Others have investigated the associations of CVD risk with anthropometric measures and growth in childhood, height and components of height in adulthood, and maternal anthropometry during or before pregnancy. The evidence from these is summarised according to the type of measure used below.

Birthweight

Several studies have examined the association of birth size with coronary heart disease (CHD) and stroke, and they have consistently found strong inverse associations.[4] These results are seen both for self-reported birthweight and recorded birthweight, although most studies have come from Europe. While the inverse association between birthweight and CVD is generally consistent, the strength of this association varies somewhat between sexes, anthropometric measure at birth (weight or length or ponderal index), and type of outcome (CHD and haemorrhagic or ischaemic stroke).[4]

The results of investigations into associations between birth size and atherosclerosis (intima media thickness (IMT) of carotid arteries assessed by carotid ultrasonography) have been more mixed, despite carotid atherosclerosis itself being associated with CHD and stroke incidence.[157-159] Studies have generally found only modest associations,[157] although in one study this may have resulted from overadjustment for potential intermediaries (such as blood pressure and lipids).[158] In a large study of 9,817 participants (Atherosclerosis Risk in Communities study), there was no association between birthweight and carotid IMT: a 1 kg greater birthweight was associated with 0.004 mm higher IMT (95% CI: -0.002 to 0.012).[159] The use of self-reported birthweight in this study may have attenuated any modest association, if present.

Researchers have also examined the associations between birthweight and various risk factors for CVD such as blood pressure, adiposity, dyslipidaemia, and abnormal glucose homeostasis. Blood pressure is the most investigated of these risk factors, as evidenced by in excess of a hundred studies to date, and several systematic reviews.[21;160;161] The majority of these studies show an inverse association between birthweight and blood pressure, with an effect size of about 1.5 to 2 mmHg per kg change in birthweight.[21;160;161] One review suggested that this estimate may be inflated somewhat by publication bias (small studies with large effects getting preferentially published), as evidenced by smaller effect sizes reported in large studies.[21] However, very large studies have often used routinely collected data, which can result in underestimation of the true association.[162] There is also a suggestion from the reviews that the association may be somewhat attenuated in adolescence, and this is of some importance to this thesis. Body composition changes dramatically in adolescence and this could have distorted the association. Most studies were unable to adjust for pubertal stage of the participants.

Similar to blood pressure, the inverse association between birthweight and glucose-insulin homeostasis is well documented, and has also been subject of a systematic review.[163] The review found that most studies show an inverse association between birthweight and some measure of adverse glucose homeostasis (such as fasting glucose, insulin, 2 hr post-load glucose, measures of insulin resistance and Type 2 diabetes). In some populations, a U-shaped or reverse-J shaped association is reported, which could potentially arise from a higher prevalence of maternal diabetes (and its association with higher birthweight) in these populations.[154] Studies in children generally showed an inverse association with fasting insulin, but the relationship with fasting glucose was inconsistent. This could arise if changes in insulin metabolism preceded those in glucose homeostasis in the evolution of clinical diabetes. This observation again is of relevance to the present thesis.

Compared to the consistent inverse associations seen for blood pressure and glucose-insulin homeostasis, the associations of birthweight with adiposity and dyslipidaemia are either weak and inconsistent (lipids) or go in the opposite direction (adiposity). The association between birthweight and adiposity has been examined in at least three systematic reviews, and has been found to be generally positive, although the evidence was less consistent in middle-aged subjects.[50;51;80] The majority of the studies used BMI an index, rather than more specific measures of adiposity. One review evaluated the studies according to the measure of body composition used, and found that birthweight tended to be associated positively with lean body mass and negatively with relative adiposity.[50] This finding though of considerable interest needs to be treated with caution, as most studies did not have accurate measures of body composition. This review also found that, in most studies, birthweight was positively associated with waist circumference (though not waist-hip ratio or truncal-peripheral skinfold ratios). However, on controlling for current BMI, there was consistent negative association with truncal-peripheral skinfold ratios (rather less

for waist-hip ratio). The interpretation of these data is complicated, as these apparently related measures do not measure exactly the same thing.

The relationship between birthweight and lipid concentrations, especially total cholesterol, in later life has also been the subject of several systematic reviews.[164-167] The association of birthweight with concentration of total cholesterol is generally inverse but weak.[164;167] A quantitative review that collated published and unpublished data from a large number of studies (n=58), arrived at a weighted estimate for total cholesterol of 0.04 mmol/L (95% CI: 0.03 to 0.05) per kg change in birthweight.[167] This review also found evidence of between-study heterogeneity, with stronger associations reported from smaller studies. Another review suggested that there may be sex differences in this association (stronger effect in males); however, this result was driven by one very large study.[165] A qualitative review examined the association of birthweight with total cholesterol, HDL and LDL fractions of cholesterol, and triglyceride concentrations and found no consistent relationship.[166] They also found no patterns by age, sex, or generation of the study participants. The majority of the studies were noted to be quite small.

Childhood and adult anthropometry

Small size (height, weight or BMI) in infancy has been associated with later disease outcomes, and this has been taken to reflect the effect of poor nutrition in intrauterine life. These associations have been documented in the Hertfordshire, Helsinki, and New Delhi birth cohorts.

In a follow up of 290 men born (1920-30) and still living in Hertfordshire, UK, the prevalence of CHD fell from 27% in those who weighed ≤8.2 kg at one year of age to 9% in those who weighed >11.8 kg (p for trend=0.03).[168] This trend occurred in both smokers and non-smokers and within each social class, and was

independent of birthweight. These results were based on only 42 cases of CHD. In the Helsinki 1 cohort (4,630 men born in Helsinki during 1934-44), however, 357 men developed CHD between 1971-1991.[169;170] The hazard ratio for a 1 SD increase in weight at one year, adjusted for birthweight, was 0.84 (95% CI: 0.75 to 0.94). This finding has been replicated in the Helsinki 2 cohort (8,760 men and women born in Helsinki during 1934-44).[171] During follow up between 1971 and 1998, 357 men and 87 women had been either hospitalised or died because of CHD. The hazard ratios associated with a 1 SD increase in BMI at age two years, independent of BMI at age eleven years, was 0.76 (95% CI: 0.66 to 0.87) for males and 0.62 (95% CI: 0.46 to 0.82) for females. In the New Delhi birth cohort (1,492 men and women born in New Delhi during 1969-72), the prevalence of impaired glucose tolerance at the time of examination, aged 26-32 years, was 10.8% and diabetes 4.4%.[172] The BMI at age two years was inversely associated with impaired glucose tolerance or diabetes, independent of BMI at age 12 years.

Unlike the associations with independent anthropometric measurements in childhood, the issue of growth (i.e. change between anthropometric measurements over time), specifically growth in infancy, is more contentious. Three systematic reviews have examined the association between growth in infancy (and childhood) and later obesity, and they all found consistently positive associations.[51;80;173] One review examined the relationship between growth and blood pressure and also found a positive association.[160] Positive associations have also been reported between growth in childhood and other CVD risk factors.[170;172] However, growth studies have often been based on just two measurements taken several years apart, and the long time intervals between measurements do not allow important time periods to be identified. Among the studies that had serial measures of body size in childhood, there is consistency in the associations of rapid growth in childhood with adverse CVD profile, but the reports for growth in infancy are conflicting, showing beneficial, adverse and no effect of rapid weight gain in infancy on later CVD risk.

Law *et al* examined the BP of 346 British men and women aged 22 years, whose size had been measured in the first ten years of life.[174] Using conditional (on previous size) methods of analyses, they estimated the effects of growth at different time periods on blood pressure, free from concerns about bias arising from regression to the mean. Systolic BP increased by 1.6 mmHg (95% CI: 0.6 to 2.7) for every SD increase in childhood weight gain after infancy. However, weight gain in infancy did not affect blood pressure. Other studies have also reported no associations of later CVD risk with weight at one year.[175]

Some studies have, however, suggested that poor growth in infancy may be associated with adverse CVD outcome. In a small study of Chinese adults from Hong Kong, those who had gained less weight between 6 and 18 months had higher systolic blood pressures at age 30 years.[176] Similarly, the data from the Helsinki and New Delhi cohorts suggested a trend for more adverse outcome among those with poorer weight gain in infancy.[171;172] In the Helsinki 2 cohort men and women, the hazard ratio for CHD associated with an increase in BMI of 1 SD (from age 2 to 11 years) was 1.28 (95% CI: 1.15 to 1.42).[171] Among those who later developed CHD, the mean BMI z-scores fell between the birth and one year of age. Similarly, in the New Delhi cohort, the odds ratio for disease (glucose intolerance or diabetes) associated with an increase in BMI of 1 SD from 2 to 12 years of age was 1.36 (95% CI: 1.18 to 1.57).[172] The SD scores for BMI fell between birth and two years of age among children in whom impaired glucose tolerance of diabetes developed later, although the decrease was reported to be not statistically significant.

Singhal *et al*, on the other hand, argue that relative undernutrition in infancy may be beneficial for those born small.[177] They have proposed that CVD may be programmed by adverse effects of faster growth, especially in early infancy (`the growth acceleration hypothesis').[177] Their conclusions are based on the results of

two nutrition trials conducted in preterm infants, who were randomised to receive either nutrient-enriched preterm formula or banked breast milk (in trial 1) and nutrient-enriched preterm formula or term formula (in trial 2).[178;179] The last follow up was conducted at age 13-16 years, and outcomes assessed included measures of insulin resistance, lipids, and blood pressure. A total of 216 (23%) adolescents participated. In the two trials combined, fasting 32-33 split proinsulin concentration was greater in children given nutrient-enriched diet, but not fasting insulin or glucose.[179] In trial 1 alone (n=130), mean arterial pressure and diastolic blood pressure were higher in those on nutrient-enriched diet (as compared to breast milk), but not systolic blood pressure.[178] There were no differences in blood pressures between the two arms of trial 2. Blood pressures were also not different between two arms of either trial in an earlier follow up at age 8 years, when follow up was 82% complete. In non-randomised analyses, blood pressure and fasting 32-33 split proinsulin were associated with greater weight gain in first 2 weeks of life, independent of birthweight.

These conclusions need to be treated with caution. The follow up was limited, and multiple outcomes were tested. Preterm infants are an extreme group and not generalisable to all infants. In addition, there is a methodological concern. The assigned diet was reportedly given till the baby was 2,000 g or was discharged. Since the babies who were given nutrient-enriched diet put on weight faster, they presumably came off the diet sooner and were also likely to be discharged earlier. The duration of stay in neonatal unit is not stated, nor adjusted for in the analyses. It is important not only in relation to differences in duration of exposure (diet), but also in the non-specific effects of stay in a neonatal unit.

The reasons for this inconsistency in findings relating to growth in infancy are unclear. One potential explanation could lie in the setting of these studies. All three studies that reported adverse effects of poor growth in infancy were from

areas with relatively poor nutritional conditions at the time study participants were born (Hong Kong and New Delhi in 1970s, and Helsinki in 1930s and 1940s). It is possible to speculate that unlike well-fed populations, poor growth in infancy may lead to adverse CVD risk in the undernourished populations. Clearly more data are needed to clarify this issue.

The association between greater height and lower CVD risk is well established.[7] Height itself may not be a very specific marker of early nutritional experience, but recent evidence suggests that one component of stature (i.e. leg length) may particularly reflect nutrition in early life, since up until puberty, most increase in total height is due to increase in leg length.[180] Leg length has been shown to be associated with CHD mortality, insulin resistance and type 2 diabetes, and prevalent CHD.[180-182]

Maternal anthropometry

Maternal anthropometry during or at the start of pregnancy has also been used as a marker of pre-pregnancy energy stores, and by extension an index of nutritional status of the fetus. In the studies conducted so far, results have been inconsistent, with studies reporting a mixture of inverse (n=4), positive (n=3), mixed (n=3) or no association (n=1) between maternal anthropometry and CVD risk among the offspring.

Among the studies showing an inverse association is a study of 77 11-year olds from Jamaica.[183] Maternal triceps skinfold thickness at 15 weeks gestation and weight gain between 15 and 35 weeks gestation were independently and inversely related to systolic BP, after adjustment for current weight. Systolic BP rose by 10.7 mmHg (95% CI: 5.7 to 15.6) for each log mm decrease in mother's triceps skinfold thickness, and by 0.6 mmHg (95% CI: 0.1 to 1.0) for each kg decrease in mother's weight gain during pregnancy. Maternal anthropometry

data, however, was available for only 55 participants. In another study involving follow up of 296 11-year olds from UK with available obstetric records on the mothers, there was weak inverse association between maternal triceps skinfold thickness (or pregnancy weight gain between 18 and 28 weeks) and systolic BP of the offspring.[184] Systolic BP increased by 0.9 mmHg (95% CI: -2.5 to 4.3) for each log mm decrease in triceps skinfold thickness.

In a follow up of 627 men and women (mean age 45 years) from China, whose mothers' obstetric records were preserved from the time of pregnancy, maternal BMI at 15 and 38 weeks of pregnancy was inversely associated with fasting glucose and insulin levels, but not with blood pressure and lipid concentrations.[185] Loos *et al* also reported an inverse association between maternal BMI before pregnancy and fasting insulin in 423 twin-pairs aged 18-34 years from Belgium.[186] Fasting insulin increased by 1.3% (95% CI: 0.1 to 2.6%) for each unit (kg/m^2) fall in maternal BMI. However, they did not find an association with pregnancy weight gain as the exposure or with fasting glucose as the outcome. Maternal BMI and weight gain were self reported and obtained through completion of a questionnaire by the women at the time of the follow up.

The studies showing positive associations include the Helsinki cohort (3,302 men born in Helsinki during 1924-33 and followed for mortality), which showed that maternal BMI (pre-delivery) was positively associated with risk of death due to CHD.[187] The hazard ratio for CHD was 1.24 (95% CI: 1.10 to 1.39) for every standard deviation increase in mother's BMI. Lawlor *et al* examined the association of parental characteristics with systolic BP at five years of age (n=3,864) in the Mater-University study; a prospective cohort of women (and their offspring) from Brisbane recruited in early pregnancy.[188] Maternal pre-pregnancy BMI was positively and independently associated with offspring systolic BP, before and after adjustment for a number of variables. The increase

in systolic BP, after adjustment for potential confounders, was 0.70 (95% CI: 0.39 to 1.04) per standard deviation increase in maternal BMI. Laor *et al*, similarly found a positive association between mother's BMI before pregnancy and systolic and diastolic BP in 10,833 17-year olds from Israel.[189] Data came from linkage of Jerusalem perinatal study (maternal characteristics) with data from military draft records (BP and BMI at age 17 years). Blood pressure, however, was not associated with weight gain in pregnancy.

Other studies have arrived at more mixed conclusions. In a follow up of 2,026 adolescents aged 14-16 years from Philippines (Cebu Longitudinal study), maternal triceps skinfold thickness measured at 30 weeks gestation was inversely associated with systolic BP in boys (after adjustment for current BMI of the adolescent), but not in girls.[190] Blood samples were available for a random sub-sample (296 boys and 307 girls) of these adolescents, in which the relationship of maternal fat mass area (MAFA; calculated from triceps skinfold thickness and midarm circumference measured at 30 weeks) with lipid concentrations was examined.[191] In males, MAFA was inversely related to LDL and total cholesterol, while in females, MAFA was positively related to LDL and total cholesterol. The associations were generally weak with wide confidence intervals.

In a study of 675 children from Gambia aged 1-9 years, the relationship between mother's weight at 6 months of pregnancy (or weight gain in last trimester) and offspring BP was analysed.[192] Among children aged 1-7 years, mother's pregnancy weight and weight gain were both positively associated with systolic BP of the offspring, while in children aged 8-9 years, pregnancy weight was not related, and weight gain showed an inverse relationship with systolic BP. Finally, the association between maternal weight gain in pregnancy and weight at 20 weeks gestation was also investigated in 518 children from Argentina, whose mothers had taken part in a calcium supplementation trial in pregnancy.[193] There

was no association between maternal weight (or weight gain in pregnancy) and BP in the offspring.

Summary of anthropometric evidence

For birthweight, there is consistent evidence of an inverse association with CHD and stroke, although the evidence for an association with atherosclerosis as assessed by ultrasonography (intima media thickness) is inconsistent. Among the risk factors for CVD, birthweight is consistently associated inversely with higher blood pressure and abnormal glucose-insulin homeostasis. The relationship of birthweight with lipid concentrations is generally inverse, but weak and inconsistent. The association of birthweight and BMI is consistently positive.

Rapid growth in childhood and adolescence is associated with an adverse CVD profile. However, the evidence regarding rapid growth in infancy is conflicting, with studies reporting both positive and inverse associations with later CVD risk. Increased height is consistently associated with lower risk of CVD; leg length (a putative marker of childhood undernutrition) is the component of height that seems to be most strongly associated.

The association of CVD risk with maternal anthropometry measured at or before pregnancy is inconsistent, with studies reporting both positive and negative associations.

There are some common limitations to evidence from the anthropometric data. Firstly, the CVD risk factors are highly correlated with BMI and in many cases are dependent on adjustment for this to become manifest. Since earlier anthropometric measures such as birthweight lie on the same pathway as BMI (and are generally correlated), it becomes difficult to appreciate the extent to

which these effects are independent. This problem becomes compounded in the analyses of growth data where a series of correlated measures are all on the same pathway. The second concern is about the interpretation of these associations. Since both anthropometric measures in early life and CVD risk factors in later life are socially patterned, it is possible that the associations reflect the shared effect of material circumstances rather than diet. This is relevant because as highlighted earlier, a person's anthropometry is a sum total of multiple environmental exposures, experienced by the generations present and past, rather than a simple reflection of recent or past diet.

The problem with using anthropometry as marker of diet becomes greater under two circumstances: (a) when birthweight is used as a proxy for maternal nutrition, as maternal nutrition does not equate fetal nutrition and many other factors apart from maternal diet influence birthweight, and (b) when anthropometric data is used as marker of undernutrition and subtle imbalances in nutrients within populations that are generally well fed, as relative differences in anthropometry in such settings in all likelihood do not reflect recent diet. Unfortunately, much of the existing research has involved the use of birthweight data, and has been conducted in high-income countries. Studies using dietary intake data, especially when conducted in low-income countries, are likely to prove more informative. The evidence from studies that have used some form of dietary exposure is detailed in the next chapter.

CHAPTER 3. EARLY DIET AND CARDIOVASCULAR DISEASE

The evidence relating to the role of early diet can be examined according to the timing of exposure: maternal diet in pregnancy, diet in infancy, and diet in early childhood. Studies that have examined the role of maternal diet in pregnancy generally fall under one of the three categories (i.e. balanced protein-calorie reduction, relative protein reduction, or reduction in the intake of selective nutrients, chiefly calcium) and they will be presented as such. Research on the role of diet in infancy has mainly focussed on the type of feeding (breast versus formula) and sodium intake, with a couple of studies examining the role of long chain polyunsaturated fatty acids. There are very few studies on dietary undernutrition in early childhood beyond infancy. Since the focus of this research is on long-term effects of early life undernutrition, the postulated mechanisms for which are quite distinct from those related to overnutrition in early life, the body of research revolving around long-term effects of obesity in early life were not considered.

3.1. Maternal diet in pregnancy

The evidence relating to this is presented below according to the nature of nutritional deficiency: balanced protein-calorie, relative protein or selected nutrients (mainly calcium).

3.1.1. Balanced protein-calorie deficiency in pregnancy

Since evidence for this comes mainly from three studies (two natural experiments of starvation and one randomised controlled trial), the methods are

described in detail with the first set of results, and subsequently referred back to avoid repetition. The limitations of these studies are discussed together at the end.

Blood pressure

The Dutch famine birth cohort study is based on a follow up of children born in a single hospital in Amsterdam during 1943-47.[194] All children born during 1945 were considered to be potentially exposed to famine (n=1,380). The famine (that occurred in the western part of Netherlands at the end of the Second World War) was defined on the basis of a drop in daily rations for adults to fewer than 1,000 kilocalories per day. The caloric intake from protein, carbohydrate and fat was proportionally reduced. The exposed category was further sub-divided into those exposed during early, middle, and late gestation on the basis of 13-week periods from the time of conception. A random sample of children born before (650 out of 1,305) and after (650 out of 2,391) the famine period (i.e. before: 1943-44; after: 1946-47) was regarded as unexposed.

During 1995-96, 741 men and women (28% of birth cohort; mean age of 50 years) born in this cohort and living in or around Amsterdam were examined.[24;194] Of these, 443 were unexposed (210 born before famine and 233 conceived after famine), and 298 (120 late gestation, 110 mid-gestation, and 68 exposed in early gestation) were exposed to the famine. Blood pressure (measured four times in a clinic) could be recorded in 739 participants.[195] No effect of prenatal exposure on blood pressure (systolic or diastolic) was observed. The mean difference in systolic blood pressure, adjusted for age and sex (mmHg; exposed minus unexposed) was: 1.3 (95% CI: -1.9 to 4.4) for late gestation, -0.6 (95% CI: -3.8 to 2.7) for mid-gestation, and -1.7 (95% CI: -5.6 to 2.2) for those exposed in early gestation. Multivariable adjustments for maternal (weight at the end of pregnancy and weight gain), birth (gestational age, birth weight and socio-economic status), and adult characteristics (BMI, socio-economic status and use

of anti-hypertensive medication) made little difference to the results. Birthweight, however, was inversely associated with systolic blood pressure: a 1 kg increase in birthweight was associated with a decrease of 2.7 mmHg (95% CI: 0.3 to 5.1) in systolic blood pressure.

The other starvation experience based study was conducted in the residents of Leningrad (now St Petersburg), who were born during the German blockade of the city in 1941-44.[196] Those born during 1941-42 (n=1,229), when the starvation conditions were at their worst, were considered to be exposed. The average daily ration for most citizens of Leningrad at this time provided 300 kilocalories, and virtually no protein. The exposed category was further sub-divided into those exposed to the siege in-utero and in infancy and those exposed in infancy alone. A group of adults (number not stated in the paper) born in the province of Leningrad but outside the city (and thus the siege limits) during the same period (1941-42) were invited to form the unexposed category. These participants came from two sources: the radial keratotomy clinic of the local hospital where patients had been referred for surgery for refractive eye problems and six local workplaces.

Twenty nine per cent (n=361, mean age of 53 years) of the men and women considered exposed to the famine were followed up: 169 were exposed during the intrauterine period and infancy, and 192 were exposed in infancy alone. Among the non-exposed, 188 (response rate not given; mean age 53 years) persons took part in the study. Blood pressure was measured in triplicate using a random zero sphygmomanometer. In analyses adjusted for sex, systolic blood pressure was similar across the three categories of participants, while diastolic blood pressure was similar in the two exposed categories, but marginally lower in the unexposed category. Mean systolic blood pressure (mmHg), adjusted for sex, was: 134.7 (95% CI: 131 to 138.4) in intrauterine plus infancy exposure group, 134.4 (95% CI: 131.3 to 137.5) in infancy exposure alone group, and 130.9 (95% CI:

127.7 to 134.1) in the unexposed group; p-value for test for trend across categories was reported as non-significant (exact value not presented). Further multivariable adjustments were not carried out, although results after exclusion of those on anti-hypertensive medication were similar.

The Instituto de Nutricion de Centro America y Panama (INCAP) conducted a longitudinal study on growth and development between 1969 and 1977 in four villages near Guatemala city.[197] The villages were provided with improved medical care and randomised within pairs to receive either of two supplement types: Atole (containing 900 kcal/L, 6.4 gm protein/100 ml, and micronutrients) or Fresco (containing 330 kcal/L, proteins nil, and micronutrients). Supplement was consumed twice daily and recorded for all pregnant and lactating women, and their offspring up to the age of seven years.

During 1997-98, 450 men and women (34% of the birth cohort; mean age of 24 years) who were residing in one of the four original villages or in Guatemala City were followed up.[26;27] Blood pressure was measured three times in all the participants using an oscillometric syphgmomanometer.[26] There was no association between the supplement group or intake of energy from supplement (prenatal or postnatal) and blood pressure. The regression coefficients for the association between supplement group (Atole *vs* Fresco) and blood pressure were: 0.17 mmHg (95% CI: -1.68 to 2.02) for systolic, and 0.69 mmHg (-0.82 to 2.19) for diastolic blood pressure. The effect estimates are for fully adjusted models, including age, sex, adult anthropometry (BMI and waist-hip ratio), socio-economic status at birth, current residence (urban *vs* rural), attained education, current physical activity level, smoking and alcohol consumption. Unadjusted estimates were not presented.

Adiposity

In a study published in 1976 (prior to the formulation of the `developmental origins of adult disease hypothesis'), Ravelli *et al* investigated the prevalence of obesity in Dutch military conscripts who were born during 1944-47.[198] 408,015 men aged 19 years at the time of induction in 1964-67 were eligible for inclusion. During the last six months of the Second World War (October 1944 to May 1945), the western part of Netherlands experienced an acute famine as a result of a food embargo by the occupying Nazi forces, while other areas that had already been liberated by the Allies remained relatively unaffected. Based on the time (before, during or after the famine) and place of birth (famine affected or unaffected area), the cohort was sub-divided into 10 distinct birth cohorts to compare the prevalence of obesity among those exposed to the famine in various stages of gestation to those who were unexposed.

Data was analysed on 307,700 (75% of the eligible) participants: 94,800 were in the exposed cohorts and 212,900 in the unexposed cohorts. Obesity was defined as weight for height equal to or greater than 120 per cent of the World Health Organisation (WHO) standard. Obesity rates were lower in those exposed in the last trimester of pregnancy and the first months of life ($p<0.005$), and higher in those exposed in the first half of pregnancy ($p<0.0005$), as compared to those living in the unexposed areas. Similar differences were seen on comparing these two cohorts to the cohorts immediately preceding or following them, and on stratification by social class (manual versus non-manual).

The association between maternal starvation in pregnancy and subsequent obesity in the offspring was also investigated in the Dutch famine birth cohort study, described earlier (n=741), and gave broadly similar results, but in women only.[199] The BMI of 50-year old women exposed to famine in early gestation was higher by 7.4% (95% CI: 0.7 to 14.5) than that of the unexposed women. BMI did

not differ in women exposed in mid-gestation (-2.1%; 95% CI: -7.0 to 3.1) or in late gestation (-1.3%; 95% CI: -6.3 to 3.9). In men, BMI was not affected by exposure to famine in any gestation. Adjustment for maternal and adult offspring characteristics hardly changed the results. The authors did not adjust for birth characteristics, as they considered these to be intermediary variables. BMI was not associated with birthweight in either sex.

Unlike the findings from the studies based on the Dutch famine of 1944-45, the results from the Leningrad siege study and the Guatemala trial did not suggest an association between early undernutrition and later obesity. For example, in the Leningrad siege study, the BMI (kg/m^2) of women was: 26.9 (95% CI: 26.1 to 27.7) in the intrauterine plus infancy exposure group, 27.0 (95% CI: 26.2 to 27.8) in the infancy alone exposure group, and 26.7 (95% CI: 25.9 to 27.5) in the unexposed group.[196] Similarly, in the Guatemala trial, the BMI (kg/m^2) of women born in the two supplementation groups was: Atole 23.4 (SD 3.7) and Fresco 23.9 (SD 4.7) ($p>0.05$ for comparison).[26] Results for men in both these studies were similar.

Dyslipidaemia

Only two studies have examined this association (the Dutch famine birth cohort study and the Leningrad siege study) and arrived at opposite conclusions.[196;200] The Dutch study reported an association between exposure to famine in early gestation and a more atherogenic lipid profile.[200] Of the various components of lipid profile examined, only the association with LDL-HDL cholesterol ratios was found to be statistically robust. LDL-HDL cholesterol ratio was higher by 13.9% (95% CI: 2.6 to 26.4) in those exposed in early gestation, as compared to those unexposed. BMI of those exposed in early gestation was higher, and the authors suggest that as a possible explanation for the findings (presumably due to current dietary differences). However, the effect of adjustment for BMI is presented separately for the two sexes (while the unadjusted estimates are

presented for the two sexes combined), and in a statement that is hard to follow, thus making it difficult to appreciate the exact effect of BMI adjustment. The effect of adjustment for other maternal and offspring characteristics is reported as unimportant (data not provided). Birthweight was not associated with LDL-HDL cholesterol ratio.

The Leningrad siege study did not investigate the association of starvation with LDL-HDL cholesterol ratio, but found no evidence for an association with total, HDL and LDL cholesterol, and triglycerides.[196]

Abnormal glucose homeostasis

Three studies have studied this association.[27;196;201] In the follow up of the Dutch famine cohort, glucose tolerance tests with standard glucose load were performed in 702 participants.[201] Assays for glucose, insulin, proinsulin, and 32-33 proinsulin were performed at 0, 30 and 120 minutes. After adjustment for sex and adult BMI, the 120-minute plasma glucose concentration was higher by 0.5 mmol/L (95% CI: 0.1 to 0.9) among participants exposed during late gestation, by 0.4 mmol/L (95% CI: 0 to 0.8) among those exposed during mid-gestation, and by 0.1 mmol/L (-0.4 to 0.6) among those exposed during early gestation, as compared to those not exposed. Associations unadjusted for BMI were not presented in the paper. Fasting proinsulin was higher in those exposed in any stage of gestation, but not insulin or 32-33 proinsulin. Other glucose and insulin measures were not different between the exposed and unexposed participants. Adjustment for other confounding variables (maternal age, parity, current socio-economic status and current smoking) is reported to have made little difference to the results. Birthweight was inversely associated with 120-minute glucose concentrations, falling by 3.8% (95% CI: 1.6 to 5.9) for each standard deviation (SD) increase in birthweight.

The Leningrad siege study, however, found no evidence for an association between exposure to starvation during intrauterine life or infancy and concentrations of glucose and insulin (fasting, 30-minute and 120-minute), and fasting proinsulin, 31-32 proinsulin, and C-peptide.[196] In the Guatemala trial, there was no association between the type of supplement received and plasma glucose levels in men.[27] However, intake of Atole supplement was associated with a 0.29 mmol/L (SE 0.13, P=0.03) lower fasting plasma glucose level in women (compared to those who received Fresco). In women only, energy intake from prenatal supplementation was also inversely associated with fasting glucose. An important limitation of this study was the method of blood collection: capillary blood taken by pricking the finger is not considered to be a very reliable method for assessing glucose homeostasis.

Coronary heart disease

Prevalence of CHD at the age of 50 years was examined in members of the Dutch famine birth cohort.[202] CHD was defined by the presence of angina pectoris according to Rose questionnaire, Q waves on the ECG, or a history of coronary revascularisation. The prevalence of CHD was higher in those exposed in early gestation than in those not exposed (8.8% versus 3.2%; odds ratio adjusted for sex 3.0, 95% CI: 1.1 to 8.1). The association was robust to adjustments for maternal and offspring birth characteristics, but was attenuated after adjustment for BMI (adjusted OR 2.5, 95% CI: 0.9 to 7.1). The prevalence was not increased in those not exposed in mid- or late gestation. Birthweight was not associated with CHD (p=0.13). The total number of cases in this study was 24, of which six were among those exposed in early gestation. No other study has examined the association between prenatal diet and CHD.

Table 3.1: *Balanced protein-calorie deficiency in pregnancy and offspring CVD risk*

Study	Population	Exposure	Results	Comments
Natural experiments of starvation from the Second World War				
Dutch famine study[24]	Born in single hospital in Amsterdam, 1943-47 Mean age: 50 years N= 741 (M+F); 28% of birth cohort	Exposed to famine (N=298; 120 late gestation, 110 mid-gestation, 68 early gestation) Unexposed to famine (N=443; 210 born before famine, 223 conceived after famine)	No association of famine exposure with BP Adiposity (women only), dyslipidaemia (LDL-HDL cholesterol ratio only) and prevalence of CHD associated with famine exposure in early gestation Abnormal glucose homeostasis (120-minute glucose only) associated with famine exposure in late gestation	Unlike other groups, the participants from the early gestation group included those living outside Amsterdam (due to low recruitment in this group)
Leningrad siege study[25]	Born in Leningrad province, 1941-42 Mean age: 53 years N=549 (M+F); 29% of exposed birth cohort (data not provided for unexposed)	Exposed to famine (N=361; 169 intrauterine period and infancy, 192 infancy alone) Unexposed to famine (N=188; recruited from local hospital and workplaces)	No association of famine exposure with BP, adiposity, dyslipidaemia or abnormal glucose homeostasis	The unexposed group was recruited from a mixture of local hospital and workplaces, for which the methodology and response rates are not described
Randomised controlled trial of nutritional supplementation				
Guatemala trial[26;27]	Born in four villages near Guatemala city, 1969-77 Mean age: 24 years N=450 (M+F); 34% of birth cohort	Atole supplement (900 kcal/L, 6.4 gm% protein, micronutrients) Fresco supplement (330 kcal/l, proteins nil, micronutrients)	No association of supplement type with BP or adiposity Exposure to Atole supplement associated with lower fasting glucose (in women only)	Villages were randomised within pairs to receive either of the two supplement types Glucose was assessed in capillary blood collected by finger prick method

Summary

There is no evidence to support an effect of balanced protein-calorie reduction in pregnancy on offspring blood pressure (summarised in Table 3.1). Offspring of women exposed to undernutrition in early gestation during the Dutch hunger winter of 1944-45 appear to be predisposed to greater adiposity in later life, but this effect was not noted in other settings. There was weak and mixed evidence for dyslipidaemia and abnormal glucose homeostasis in those exposed to prenatal undernutrition. There is little evidence to support the role of prenatal undernutrition in CHD in adult life.

Almost all the evidence on balanced protein-calorie reduction comes from three studies (the Dutch Famine birth cohort, the Leningrad siege study, and the Guatemala trial) and their limitations can be considered together. The important issues relate to sample size, selection bias, exposure assessment and generalisability.

All three studies are small and inadequately powered. The likelihood of chance findings is increased by attempts to look at sub-groups (e.g. stages of gestation or by sex of the participant) and further tests for numerous interactions, thus reducing the effective sample sizes quite dramatically. For example, in the Dutch Famine birth cohort, a number of important conclusions relate to those exposed in early gestation, for which the total number of participants is 68. Furthermore, the association of obesity reported for women is based on 38 female participants only. It is easy to see how a chance introduction of one or two overweight women could reverse the weak association and change conclusions entirely. Similarly, the association of supplementation with glucose levels in the Guatemala trial is based on 167 women. In addition, 8% of the independent data were imputed.

All three studies raise concerns about selection bias. The proportion of eligible participants followed up were 34% in the Guatemala trial, 29% in the Leningrad siege study (exposed only, response rate for unexposed not provided), and 28% in the Dutch Famine cohort. The low follow up rate is less important in the Guatemala trial, where the included and excluded group of participants were broadly similar in baseline characteristics. In the Dutch Famine cohort, the differences between included-excluded participants are not presented, although birthweight is reported to be not different. One issue is that unlike other exposure groups in which participants living in or around Amsterdam were invited, in the early gestation group the invitation was extended to everyone since the recruitment in this group was low. Differences in current lifestyle of those living in Amsterdam to those living outside (perhaps rural areas) may have distorted the results, especially as many of the important findings of this study stem from this group of participants. In the Leningrad siege study, differences between included and excluded participants were not presented. Importantly, the unexposed comparison group was reportedly selected from a local clinic and six workplaces (and combined), but the exact procedure for this and the response rate are not provided.

Exposure was assessed at the population level in the Leningrad Siege and Dutch Famine cohort studies, and that too was crudely estimated on the basis of average rations for the population. Supportive evidence was available in the Dutch cohort in the form of reduction in birthweight of those exposed. The reduction in birthweight, however, was not seen in those exposed in early gestation; in fact, they had the highest birthweights of all the groups, including those not exposed to the famine. A likely explanation for this is that those exposed to the famine in early gestation were also exposed to plentiful food in late gestation. Efforts to compensate for reduced diet in early gestation may have led these women to overfeed themselves and possibly their infants. The established link between higher birthweight and later obesity may then be the

explanation for the observed association with starvation in early gestation, and potentially also with other obesity related outcomes (dyslipidaemia and CHD) reported in this study. This hypothesis is also suggested as a potential explanation of the findings by the authors of the Dutch conscripts study, who found higher BMI in those exposed to famine in early gestation, and lower BMI in those exposed in late gestation. Data from animal experiments show the same phenomenon.

In the Guatemala trial, daily intake of supplement was recorded and the data relating to energy intake were presented. Although the energy intake from postnatal supplement intake in children showed an important difference (450 *vs* 51 kJ/day), the difference in energy intake from prenatal supplement intake was much smaller (483 *vs* 372 kJ/day). This is surprising as one supplement type contained nearly three times more energy than the other, suggesting important differences in the intake volume. This finding or its implications are not discussed by the authors, but are relevant to the conclusions reached from the primary analyses comparing women in the two supplement categories. The small difference in intake of the supplement may explain the predominantly null findings of this study.

Apart from these limitations, there are also issues of generalisability. The Dutch hunger winter and Leningrad siege were unusual and clearly delineated natural experiments that cannot be applied easily to other settings. Moreover, despite the sharp decline, birthweights never fell below 3.0 kg (the lowest mean birthweight was 3.2 kg in the late gestation category of the Dutch study). The Guatemala trial may be more relevant to settings where poor maternal diet is a public health issue, although here as well, the lowest mean birthweight was 3.0 kg (girls in the control arm). Admittedly, many factors other than maternal diet in pregnancy can influence birthweight; still, the relatively high birth weights in these studies may potentially explain the predominantly null findings. Mean birthweights in

settings with chronic undernutrition (i.e. low-income countries) are generally much below 3.0 kg.[136]

3.1.2. Relative protein deficiency in pregnancy

Blood pressure is the only outcome that has been studied, and six studies have reported on it.[28-30;190;203;204]

The earliest study was based on a follow up of men and women (born in a maternity hospital in Aberdeen during 1948-54) whose mothers' had taken part in a survey of diet in late pregnancy.[28] Maternal diet was assessed in the third trimester by self-completed seven-day diet diaries and weighing of all food portions, and converted to nutrient intake (amounts of macronutrients and proportion of calories derived from them). Blood pressure was examined in 253 (46% of the eligible) offspring at a mean age of 41 years. In the main analyses, there was no association between mother's intake of any individual nutrient and offspring blood pressure (data not presented). The authors, however, discovered a complex interaction between maternal intake of animal protein and carbohydrate: at daily animal protein intakes less than 50g, a higher carbohydrate intake was associated with higher offspring blood pressure, and at daily protein intakes above 50g, lower carbohydrate intake was associated with higher blood pressure. This interaction was not pre-specified (but driven by the observation that blood pressure showed a weak inverse association with increasing maternal energy intake from animal protein) and needs replication.

Shiell *et al* tried to replicate this association by conducting a follow up study of men and women (born in a maternity hospital in Motherwell during 1967-68) whose mothers' food intake had been recorded during pregnancy.[29] The consultant obstetrician at that time in Motherwell encouraged women to eat 1 lb

(0.45 kg) of red meat per day and discouraged carbohydrate-rich foods. Furthermore, they were encouraged to eat corned beef between meals to make up the required intake. Frequency of consumption was recorded for 10 food items in early and late pregnancy. Blood pressures were measured in 626 offspring (44% of the eligible) at a mean age of 29 years (56% female). Offspring of women who reported greater consumption of meat and fish in the second half of pregnancy had higher systolic blood pressure (β-coefficient: 0.19 mmHg per portion per week; 95% CI: 0.04 to 0.35; p=0.02). Adjusting for offspring's BMI and birthweight did not attenuate this association. The authors regarded these results as a replication of the results by Campbell *et al.* This is surprising as the predominant finding in the study by Campbell *et al* was *an inverse association* between maternal animal protein intake and offspring blood pressure (the majority (83%) of women ate less than 50 g of animal protein daily, in whom there was an inverse association with offspring BP). Frequency of selected food items is a crude measure of diet. Furthermore, the authors do not report adjustment for socio-economic position, which is relevant in this study as the uptake of dietary advice and the ability to purchase meat would depend on material circumstances. The authors do admit an alternative explanation of these findings i.e. high saturated fat and salt content of the tinned meats that the mothers were advised to eat.

The link between maternal diet during pregnancy and offspring blood pressure has also been examined in the Dutch Famine birth cohort by using the reports of official weekly rations to estimate the percentage of energy derived from macronutrients.[204] Adult blood pressure was not associated with total caloric, protein, carbohydrate, or fat intake during any week of gestation. It was, however, inversely related to the average protein/carbohydrate ratio in the third trimester, but not in the first or second trimester. The systolic blood pressure decreased by 0.6 mmHg (95% CI: 0.1 to 1.1) for every 1% increase in protein/carbohydrate ratio. This association was seen not only in those exposed

to the famine, but also in those unexposed (i.e. those born before or conceived after the famine).

Since birthweight was directly associated with maternal protein/carbohydrate ratio in late gestation (and inversely with adult blood pressure), it and adult BMI could be potential confounders or intermediaries. However, adjustment for birthweight and BMI was not presented for directly comparable models, but for models employing an unusual analytical strategy. The models were adjusted for a 0.8 mmHg increase in blood pressure per year (instead of directly adjusting for age) on the grounds that maternal nutrition intake and age of the participant were highly correlated (r= -0.62), both having been derived directly from the participant's date of birth. The figure of 0.8 mmHg was taken from published literature, since the authors considered the actual increase in blood pressure found in the study (1.8 mmHg per year) to be too high. Such adjustment appears unnecessary, as linear regression techniques are generally robust at this level of correlation between the covariates. Furthermore, the fact that crude results highlighted in the study were not transformed in this way, while those adjusted for important confounders were, simply serves to confuse the interpretation. The authors admit that official daily rations may not be an accurate measure of food intake (since food came from many other sources) and the real food intake by individuals may have been twice as high. This information was novel and had not been highlighted in the earlier reports from the Dutch Famine cohort that have all used total calorific intake from rations to define exposure. Although this does not alter the conclusions from the other reports, it does have implications for the quantification of the association between levels of starvation in pregnancy and offspring birthweight.

The Cebu Longitudinal Health and Nutrition Survey (CLHNS) is a community-based survey (a metropolitan area) in Philippines involving follow up of a cohort of 3,080 infants born in 1983-84.[190] A single 24-hour dietary recall at 30 weeks of

gestation provided data on total energy intake and percentage of energy from macronutrients. Blood pressure was measured in 2,026 children (66% of the cohort) at a mean age of 15.5 years (52% girls). Systolic BP was inversely related to the mother's percentage of dietary energy from protein in boys (β-coefficient: -0.18; $p \leq 0.05$), but the effect was attenuated on adjustment for current BP of the mother (β-coefficient: -0.15; $p \leq 0.1$). Among girls, however, SBP was inversely associated with mother's percentage of dietary energy from fat. All models were adjusted for birthweight and current BMI and energy intake of the child, among other covariates. Girls in the study were a year younger than boys (since they were surveyed before boys at follow up). Results reportedly did not change after adjustment for maturational variables. A single dietary recall is not regarded as a robust measure of dietary intake.

Project Viva is an ongoing cohort study of women and children recruited from eight offices of a large group practice in Massachusetts, US, during 1999-2002.[203] The women completed semi-quantitative food-frequency questionnaires in pregnancy to measure gestational protein intakes (in the first and second trimesters). Systolic BP was measured up to five times with an automated device in the offspring (n=947; 47% of the cohort) at the age of six months. The mothers not included in the analyses were more likely to be non-white and to have lower education. There was no association between maternal protein intake and infant's systolic BP. After adjustment for covariates (maternal, birth and infant characteristics, and energy intake), the BP increase was 0.14 mmHg (95% CI: -0.12 to 0.40) for a 1% increase in energy from protein during the second trimester. The authors suggest the high level of protein intake in this well-nourished cohort as a potential explanation for the null effect. Infancy may be too young an age for differences in BP to manifest. The inverse association between birthweight and blood pressure, however, was present in this study.

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a population based cohort study of 13,678 children from Bristol, England, born in 1991-92.[30] Mothers completed a food-frequency questionnaire at 32 weeks of gestation, from which intake of 12 nutrients (plus protein/carbohydrate ratio) were derived. Blood pressure was measured in children at the mean age of 7.5 years. Analysis was based on 6,944 mother-offspring pairs (51% of the cohort) with relevant data. Those who attended the clinics were similar to those who did not attend. There was no association between maternal intake of any of the macronutrients (or protein/carbohydrate ratio) and offspring BP. Dietary intake was assessed by a single unquantified food-frequency questionnaire, which may not provide a valid estimate of the intake. The authors suggest that the association between diet in pregnancy and offspring BP may not be present in well-fed populations.

Summary

The evidence for an association between relative protein deficiency in maternal pregnancy diet and CVD risk among the offspring is inconsistent (summarised in Table 3.2). Six studies have examined this association, and BP is the only outcome that has been investigated. Two studies have reported an inverse association; one study reported an inverse association only in boys; one study reported a positive association; and two of the larger studies found no association at all. One explanation for the inconsistency in findings could lie with the method of exposure assessment. Most studies relied on one-off measures of diet in pregnancy (either food frequency or 24-hour diet recall), while some others used even cruder measures (such as reports of official weekly rations or frequency of few selected food items). Any true underlying association could be potentially missed due to measurement error and regression dilution bias. Moreover, the studies were generally based in well-fed populations, with protein intakes in excess of the dietary requirements. Gross dietary imbalances are

unlikely to be present in these populations, while finer imbalances are unlikely to be picked by the imprecise instruments used in these studies to characterise diet.

Table 3.2: *Relative protein deficiency in pregnancy and offspring blood pressure*

Study	Population	Exposure	Results	Comments
Aberdeen study[28]	Born in maternity hospital in Aberdeen, 1948-54 Mean age: 41 years N=253 (M+F); 46% of eligible birth cohort	Self-completed 7-day diet diaries and weighing of food portions in third trimester	No association of mother's intake of any individual nutrient and offspring BP At animal protein intake <50g/day, higher maternal carbohydrate intake associated with higher offspring BP At animal protein intake >50g/day, lower maternal carbohydrate intake associated with higher offspring BP	Protein-carbohydrate interaction was not pre-specified The majority (83%) of women consumed animal protein <50g/day, in whom there was an inverse association between protein intake and offspring BP
Motherwell study[29]	Born in maternity hospital in Motherwell, 1967-68 Mean age: 29 years N=626 (M+F); 44% of eligible birth cohort	Frequency of consumption for 10 food items recorded in early and late pregnancy	Higher maternal consumption of meat and fish in second half of pregnancy associated with higher systolic BP (β-coefficient: 0.19 mmHg per portion per week; 95% CI: 0.04 to 0.35; p=0.02)	No adjustment for socio-economic position in multivariable models Mothers were advised to eat tinned meats (high fat and salt content) to augment their protein consumption
Dutch famine study[204]	Born in single hospital in Amsterdam, 1943-47 Mean age: 50 years N= 741 (M+F); 28% of birth cohort	Reports of official weekly rations at the time used to estimate nutrient intake of participants	No association of mother's intake of any individual nutrient and offspring BP Inverse association of maternal protein-carbohydrate intake ratio with offspring BP (in third trimester only)	Observed inverse association with protein-carbohydrate ratio noted in those exposed and unexposed to famine Food available from several sources other than official weekly rations

Table 3.2: *Relative protein deficiency in pregnancy and offspring blood pressure (continued)*

Study	Population	Exposure	Results	Comments
Philippines CLHNS study[190]	Born in a metropolitan area in Philippines, 1983-84 Mean age: 16 years N=2,026 (M+F); 66% of birth cohort	Single 24-hour dietary recall at 30-weeks of pregnancy	Mother's % of dietary energy from proteins inversely associated with offspring SBP (in boys only) Mother's % of dietary energy from fats inversely associated with offspring SBP (in girls only)	Observed associations were not pre-specified Single dietary recall not a robust measure of intake
US Project Viva[203]	Born in a large medical practice, Massachusetts, 1999-2002 Mean age: 6 months N=947 (M+F); 47% of birth cohort	Semi-quantitative food frequency questionnaires in the first and second trimesters	No association of maternal protein intake with offspring systolic BP	High levels of protein intake in this well-nourished cohort
Bristol ALSPAC study[30]	Born in Bristol area, 1991-92 Mean age: 8 years N=6,994 (M+F); 51% of birth cohort	Single food frequency questionnaire at 32-weeks gestation	No association of maternal intake of any macronutrient (or protein-carbohydrate ratio) with offspring BP	Single unquantified food frequency questionnaire used to estimate intake Well-fed population

3.1.3. Micronutrient deficiency in pregnancy

Although there are isolated reports on the role of other micronutrients and outcomes other than blood pressure, the majority of the research has focussed on the role of prenatal calcium intake and offspring blood pressure. So far, seven studies have reported on this, two of which have involved follow up of children born in randomised controlled trials of calcium supplementation for prevention of pre-eclampsia.[28;30-32;205-207] BP has been the outcome investigated by all studies except one that also looked at concentrations of lipids and glucose-insulin profile.

McGarvey *et al* (1991) were the first to report on this.[206] Delivering mothers and their normal term infants born at an obstetric hospital in Rhode Island in 1985-86 were recruited (n=212). The total number of women invited to participate in the study were not reported in the paper. Maternal prenatal diet was assessed postpartum by a semi-quantitative food-frequency questionnaire, and used to estimate calcium, magnesium, potassium, and calorific intake during pregnancy. Infant blood pressure was measured at 2-3 days and again at 1, 6, and 12 months of age. Loss to follow up was high and only 70 children (33%) could be examined at 12 months age. Maternal prenatal calcium intake was inversely related to systolic blood pressure at 1 month of age ($r= -0.28$; $p<0.01$), and diastolic blood pressure at 12 months of age ($r= -0.20$; $p<0.05$). Potassium and magnesium were also associated to either systolic or diastolic BP, at differing ages. The intake of the three nutrients was strongly correlated with each other and associated positively with socio-economic position and breast feeding in infancy. However, the authors did not adjust for these (except for one model that had BP measured at one month as the outcome) on the grounds that socio-economic position and breast feeding did not independently predict the outcome (i.e. blood pressure).

Belizan *et al* conducted a follow up of children born to women who had participated in a randomised controlled trial for prevention of pre-eclampsia in pregnancy.[32] In this trial conducted in three public hospitals and one private hospital, in 1987-90, daily supplements of 2g elemental calcium were given from 20 weeks gestation until delivery. Of the 614 women randomised at the private hospital only, 518 (84%) were followed up and blood pressure measured in their offspring at a mean age of 7 years. The mothers and the examiners assessing blood pressure remained blinded to the exposure, and the participants in the two arms were similar in their baseline characteristics. There was no clear primary effect of calcium supplementation on blood pressure: the mean systolic blood pressure was 1.4 mmHg (95% CI: -3.2 to 0.5) lower in the calcium supplementation group, but the confidence intervals were broad and also consistent with a higher blood pressure in the calcium supplementation group. In sub-group analyses, the effect was found to be predominantly among children whose BMI was above the median for this population.

Another study of a similar nature involved follow up of women who took part in the Calcium for Prevention of Preeclampsia Trial.[205] The trial was conducted in five US medical centres in 1992-95, and the participating women were randomised to receive 2g calcium or placebo daily from 13-21 weeks gestation until delivery. Follow up was limited to one centre (Portland) only: of the 497 trial participants invited, BP could be measured in 260 infants at 3 months (52% of cohort) and 57 toddlers at the age of 2 years (11% of cohort). Systolic BP in calcium-supplemented group was 2.2 mmHg lower ($p>0.05$) at 3 months and 4.8 mmHg lower ($p<0.05$) at 2 years, as compared to the placebo group. The association noted at 2 years was based on 37 children in the calcium supplementation arm and 18 children in the placebo arm. Despite a nearly 90% loss to follow up, no comparisons were provided for participants included and excluded in the study, nor any attempts made to adjust for potential

confounders. The confounders clearly could not be assumed to be balanced at this stage.

In the US prospective cohort study Project Viva (described earlier), maternal food-frequency questionnaires completed in pregnancy were used to estimate calcium intake.[31] Blood pressure measurements were available on 936 six-month old infants (46% of birth cohort). In unadjusted analyses, maternal consumption of calcium was reported to be modestly, inversely associated with systolic blood pressure (trend across quartiles, p=0.06). The actual estimates or the results of adjusted analyses were not presented. Instead, the authors focussed on sub-group analyses: calcium from supplements only (showing a strong inverse association with systolic BP) and calcium from foods only (showing no association with systolic BP). The association of systolic BP with calcium supplement intake was seen in second trimester only; no association of systolic BP with calcium intake (as supplement or food derived) in the first trimester was seen. Approximately two thirds of supplemental calcium in this study came from multivitamins, making it difficult to attribute the effect specifically to calcium. Furthermore, the mean intake of calcium in pregnant women in this population, as in many developed countries, exceeded the recommended requirements.

During 1988-95, twins born in Tasmania were recruited into the Tasmanian Infant Health Study (THIS) soon after birth to investigate the Sudden Infant Death syndrome (SIDS).[207] Mothers were asked, retrospectively, if they had consumed any supplements during pregnancy. Of the 463 children recruited to the study during 1991-93, 294 (63% of eligible; representing 147 twin pairs) agreed to participate in the follow up at a mean age of 9 years. Blood pressure was measured in all the 294 children, while blood samples (used to assay lipids, glucose, and insulin) could be collected on 230 of these. There was no association between maternal calcium supplementation and offspring's blood pressure, fasting glucose or insulin. However, children whose mothers' took calcium

supplements had lower plasma triglycerides, and total and LDL cholesterol levels. These associations were modest but robust to adjustments for maternal education. Other measures of socio-economic status were not used to examine confounding; this is relevant since calcium intake is likely to be socially patterned. Data on supplement intake was retrospectively collected and the effective sample size was very small (bearing in mind the non-independence of twins).

Finally, the role of prenatal calcium supplementation has been reported secondarily in two other studies, where the primary focus was on the role of macronutrients in diet.[28;30] The methodology of both these studies (the ALSPAC study and the Aberdeen study) has already been described. Associations of offspring blood pressure with maternal calcium intake during pregnancy were also examined, among a variety of other nutrients. Both studies reported no association of calcium intake (as assessed by dietary intake) with blood pressure (measured at 7.5 years or 41 years).[28;30]

Summary

There is no clear evidence for an effect of calcium supplementation in pregnancy on the offspring's risk of CVD (summarised in Table 3.3). Of the seven studies that have reported on the role of calcium supplementation in pregnancy, five were observational studies of dietary intake and two involved follow up of children born within trials of calcium supplementation in pregnancy to prevent pre-eclampsia. BP was the outcome assessed in all the studies. No association was found in three of the five observational studies. Of the two studies that did report inverse associations, one failed to adjust for socio-economic position despite reporting a strong social gradient for calcium intake. The other study that reported an inverse association found no main effect, but reported a sub-group finding of an inverse association with calcium supplement intake (but no association with dietary intake). The study population was from high socio-

economic background with dietary calcium intake exceeding requirements. Both trials reported inverse associations with calcium intake but were unconvincing. The larger of the two trials had an effect estimate (1.4 mmHg; 95% CI: -3.2 to 0.5) that was not statistically robust, while the effect estimate from the other trial (4.8 mmHg; $p<0.02$) was based on a total of 55 children (37 children in the calcium arm and 18 children in the placebo arm) from a total of 497 trial participants (loss to follow up of 90%). Only one study examined outcomes other than BP, and this study found no association with glucose-insulin levels, although it did find a weak inverse association with lipid concentrations (the mechanism for which the authors were unable to explain). The majority of these studies were conducted in generally well-nourished populations with adequate calcium intake. Calcium intake is prone to confounding by simultaneous intake of other micro-nutrients (since it is often taken as multivitamin tablets) and behavioural differences that are socially patterned (since vitamin intake itself shows social gradients).

Table 3.3: *Calcium intake in pregnancy and offspring blood pressure*

Study	Population	Exposure	Results	Comments
Observational studies				
Rhode Island study[206]	Born in maternity hospital in Rhode Island, 1985-86 Mean age: 1 month, 6 months and 1 year N=212 (M+F); 33% of birth cohort at 1 year	Semi-quantitative food frequency questionnaire completed postpartum	Maternal prenatal calcium intake inversely associated with SBP at 1 month of age (r= -0.28;p<0.01) and DBP at 12 months of age (r= -0.20; p<0.05)	Calcium intake strongly correlated with socio-economic position in the study, but not adjusted for in the analyses
US Project Viva[31]	Born in a large medical practice, Massachusetts, 1999-2002 Mean age: 6 months N=936 (M+F); 46% of birth cohort	Semi-quantitative food frequency questionnaires in the first and second trimesters	Calcium intake from supplements inversely associated with offspring BP No association of calcium intake from diet with offspring BP	These sub-group analyses were not pre-specified
Tasmania THIS study[207]	Twins born in Tasmania, 1988-95 Mean age: 9 years N=294 (M+F; 147 twin pairs); 63% of eligible cohort	Mothers were asked retrospectively if they had taken any calcium supplements during pregnancy	No association of maternal calcium supplement intake with offspring BP	No association of maternal calcium supplement intake with offspring glucose or insulin but inverse associations with triglycerides, total and LDL-cholesterol levels
Bristol ALSPAC study[30]	Born in Bristol area, 1991-92 Mean age: 8 years N=6,994 (M+F); 51% of birth cohort	Single food frequency questionnaire at 32-weeks gestation	No association of maternal dietary calcium intake with offspring BP	Single unquantified food frequency questionnaire used to estimate intake

Table 3.3: *Calcium intake in pregnancy and offspring blood pressure (continued)*

Study	Population	Exposure	Results	Comments
Aberdeen study[28]	Born in maternity hospital in Aberdeen, 1948-54 Mean age: 41 years N=253 (M+F); 46% of eligible birth cohort	Self-completed 7-day diet diaries and weighing of food portions in third trimester	No association of maternal dietary calcium intake with offspring BP	
Follow up of randomised controlled trials of calcium supplementation in pregnancy for prevention of pre-eclampsia				
Argentina trial[32]	Born in a private hospital in San Luis, 1987-90 Mean age: 7 years N=514 (M+F); 84% of birth cohort	Randomly assigned with intervention group receiving daily supplements of 2g of elemental calcium from 20 weeks of pregnancy onwards	Inverse association of maternal calcium supplementation with offspring SBP in those whose BMI was above the median	No clear overall association of maternal intake of supplemental calcium with offspring BP BMI sub-group analyses were not pre-specified
Calcium for Prevention of Preeclampsia trial[205]	Born in one centre of a US multicentre trial, 1992-95 Mean age: 3 months and 2 years N=260 (M+F; 52% of birth cohort) at 3 months N=57 (M+F; 11% of birth cohort) at 2 years of age	Randomly assigned with intervention group receiving daily supplements of 2g elemental calcium from 13-21 weeks onwards	Inverse association of maternal calcium supplementation with offspring SBP	Despite a 90% loss to follow up, no comparisons for included-excluded participants, nor adjustments for potential confounders in analyses

3.2. Diet in infancy & early childhood

Research on the role of diet in infancy has focussed mainly on the type of feeding (breast versus formula) and sodium intake. Two studies have examined the importance of long chain polyunsaturated fatty acids. There are hardly any studies on dietary undernutrition in early childhood beyond infancy.

3.2.1. Breast feeding in infancy

The role of breast feeding in infancy and later CVD risk (blood pressure, adiposity, dyslipidaemia, abnormal glucose homeostasis, and CVD mortality) has been examined thoroughly in several recent systematic reviews, which are summarised below.

Blood pressure

Two recent systematic reviews have examined the role of breast feeding in infancy on blood pressure in later life and arrived at similar conclusions.[35;208] Both reviews aimed to determine whether breast-fed infants had lower mean blood pressures, as compared to infants fed artificial milk by bottle. In the review by Owen *et al*, the pooled SBP was 1.10 mmHg (95% CI: -1.79 to -0.42) lower, and the pooled DBP was 0.36 mmHg (95% CI: -0.79 to 0.08) lower in breast-fed, as compared to bottle-fed infants.[208] In the review by Martin *et al*, the blood pressures were similarly lower in breast-fed infants, as compared to the bottle-fed infants: SBP (pooled difference: -1.4 mmHg; 95% CI: -2.2 to -0.6) and DBP (pooled difference: -0.5 mmHg; 95% CI: -0.9 to -0.04).[35] There was marked heterogeneity in effect estimates of the studies. Both reviews found evidence of publication bias, with smaller effect sizes reported from larger studies (>1000 participants). There was little evidence that heterogeneity was explained by quality of exposure assessment (prospective or recall), exclusivity of breast

feeding, decade of birth (before or after 1980, relevant due to secular changes in composition of formula milk) or the age at which blood pressure was measured. In studies where relevant data were available, there was some attenuation of the effect estimates following adjustment for socio-economic factors (investigated by Martin *et al* only) but none for current body size (investigated by Owen *et al* only).

The results of the two reviews are very similar, despite differences in the studies included. The pooled estimates in the review by Owen *et al* include outcome assessment at all ages, while those from review by Martin *et al* are for post-infancy measurements of blood pressure only. Martin *et al* presented data for infancy BP measurements separately, the pooled estimate for which was similar to the estimate for older age groups. After combining the sex-specific estimates from the same study (two studies in Martin *et al*) and excluding repeat measurements in same participants at different ages (one study in Martin *et al*), there were 18 unique estimates of systolic BP (for all ages, including infancy) in both reviews. Of these, only 11 studies were common to both reviews. Differences in remaining studies appear to have arisen because of attempts to get additional data directly from the investigators (done by Owen *et al*, but not Martin *et al*) and later date of conclusion of the publication search (April 2003 in Owen *et al*, and May 2004 in Martin *et al*) allowing inclusion of more recently published or in-press studies. Apart from this, the methodology of the two reviews was very similar, with clear criteria, systematic approach and presentation. Neither review used quality scores or double data extraction.

Adiposity

Several systematic reviews have examined the association between breast feeding and subsequent adiposity and, on the whole, concluded that the effect is modest, if any, and likely to be confounded by other factors.[34;80;209] In a review

limited to published research (n=28 studies), Owen *et al* examined the influence of breast feeding on odds of obesity in later life.[209] Obesity was defined in the studies by some measure of weight for height (mostly BMI), with cut-offs typically ranging from 90th to 97th centile. The authors found that breast feeding was associated with a reduced risk of obesity, compared with formula feeding (odds ratio: 0.87; 95% CI: 0.85 to 0.89). However, in six studies that adjusted for all three major potential confounding factors (parental obesity, maternal smoking and social class), the association was markedly attenuated.

To investigate the role of these confounding factors in greater detail, the authors conducted a further review in which they tried to quantify the association between infant feeding and BMI in later life, with and without adjustment for potential confounders.[34] They reviewed the published literature and also obtained data from previously unpublished sources, requesting researchers to provide a series of pre-specified estimates for BMIs. Analysis of 36 mean differences in BMI (355,301 participants) showed that breast feeding was associated with a slightly lower BMI (kg/m^2) than formula feeding (mean difference: -0.04; 95% CI: -0.05 to -0.02). Adjustment for socio-economic status, maternal smoking in pregnancy, and maternal BMI in 11 studies abolished the effect (mean difference: -0.01; 95% CI: -0.05 to 0.03). There was evidence to support publication bias, with larger mean differences seen in smaller studies. There was some evidence to suggest that the relationship of breast feeding to mean BMI differed with age and was stronger in adolescents. These findings were robust to differences in the method of ascertainment of infant feeding status and time period of study, although there was weak evidence to suggest that prolonged breast feeding was related to lower BMI. From this study, the authors concluded that the perceived benefit of breast feeding in reducing BMI is small and likely to be influenced by selective publication of studies and confounding factors.

To reconcile the apparently differing conclusions from the two reviews, the authors propose two explanations: inclusion of unpublished data in the later review (and therefore less publication bias) and possibility that breast feeding is associated with lower prevalence of obesity but bears no relationship to mean BMI. The authors speculate that such a situation could arise if breast feeding was associated not only with a lower prevalence of obesity, but also with a lower prevalence of underweight. A related interpretation, not proposed by the authors, concerns the accuracy and interpretation of BMI at the extreme ends of the range. While high BMI performs satisfactorily as a measure of excess underlying adiposity, BMI may not measure adiposity as accurately in lean populations, thus distorting the association with mean BMI.[210;211] Such distortion is reflected to some extent in the stronger association of infant feeding in adolescence, when BMI is a poor marker of underlying adiposity. Furthermore, a relatively higher BMI at the lower end of the range may confer, conversely, a more favourable health profile. Because of these reasons, the interchangeable use of mean BMI and extremely high BMI (cut-offs), when the real interest is in excess adiposity as a risk factor for CVD, may not be appropriate. This issue may have limited relevance for the above review as few studies were from low-income countries, but may become important if more such studies were included in further reviews.

Dyslipidaemia

Only one systematic review has examined the association between type of infant feeding and blood cholesterol.[212] In that review, the authors examined the association of breast feeding with total cholesterol in 37 studies (26 observations in infancy, 17 in childhood or adolescence, and 9 in adulthood). Mean total cholesterol (mmol/L) in childhood or adolescence was not related to type of infant feeding (mean difference: 0.00; 95% CI: -0.07 to 0.07). However, mean total cholesterol in infants was higher among those breast-fed (mean difference: 0.64; 95% CI: 0.50 to 0.79), whereas mean total cholesterol in adulthood was lower

among those breast-fed (mean difference: -0.18; 95% CI: -0.30 to -0.06). Patterns for LDL-cholesterol were similar to those for total cholesterol, at all ages (although, they were based on fewer observations). The authors concluded from this that while the association between infant feeding and higher cholesterol in infancy is likely to be a direct consequence of nutritional differences between breast and bottle milk, the inverse association seen later in life reflects long-term programming of cholesterol metabolism. The authors postulated that the long-term changes in cholesterol metabolism were induced through the high cholesterol content of breast milk.

The social class patterns of breast feeding have changed over the years, and socio-economic position and current body size are likely confounders of an association between type of infant feeding and cholesterol levels.[213] The importance of such confounding has been demonstrated in the association of infant feeding with BMI. In the above review, the authors acknowledged these confounders but failed to conduct any formal analyses (such as meta-regression) to examine their influence on the observed association. In view of this limitation, the conclusions from this review require further examination. A further review like the one conducted for BMI by Owen *et al*, with adjustment for potential confounders, may clarify the situation.[34]

Two further studies have been reported since the publication of the above review, and neither found an association between breast feeding and cholesterol concentrations; however, they were both conducted in children or adolescents.[214;215] In a small clinical trial, infants either received human milk until weaned (n=15), or were randomised to receive for 12 months, either standard cow's milk formula (n=17) or cow's milk formula modified with added cholesterol (n=15).[215] Cholesterol contents of human milk (HM), modified cow's milk formula with added cholesterol (MCF), and cow's milk formula (CF) were 120, 80, and 40 mg/L, respectively. When examined at 18 months, there were no

differences in blood lipid profiles of the three groups. For example, total cholesterol levels (mmol/L) among the three groups of infants were: 3.58 (standard error of the mean (SEM) 0.16), 3.80 (SEM 0.15), and 4.12 (SEM 0.16) (p for trend=0.09). The authors interpreted this as evidence against imprinting of cholesterol biosynthesis in early life. However, the study was too underpowered for any strong conclusions.

The association between infant feeding and lipid profile has also been examined in 2,192 randomly selected school children aged 9 and 15 years from Estonia (n=1,174; 76% response rate) and Denmark (n=1,018; 75% response rate).[214] These children were examined as part of the European Youth Heart Study, set up to investigate CVD risk factors across 4 countries with standardised methodology. There was no association between breast feeding and total or LDL-cholesterol. The sex, age, country, and pubertal stage-adjusted difference between those who were ever, compared to never, breast-fed was -0.02 mmol/L (95% CI: -0.11 to 0.07).

Abnormal glucose homeostasis

A recent systematic review examined the influence of initial infant feeding on type 2 diabetes, hyperglycaemia, and insulin resistance.[36] To differentiate acute from long-term influences of breast feeding, studies in infants were examined separately from those in children and adults. Subjects who were breastfed had a lower risk of type 2 diabetes in later life than those who were formula fed (N=7 studies; odds ratio: 0.61, 95% CI: 0.44 to 0.85; p=0.003). Excluding those with diabetes, there were seven studies in children and adolescents with fasting glucose levels and six studies with serum insulin. Pooled estimates from these studies showed no difference in fasting glucose, but a marginally lower mean fasting insulin concentration in breastfed, as compared to those who had been formula fed as infants (percentage difference: -3%, 95% CI: -8% to 1%; p=0.13).

Breastfed infants had consistently lower levels of blood glucose and insulin, as compared to the formula fed infants. The authors concluded that published evidence is consistent with a protective effect of breast feeding on diabetes risk; however, further studies were needed with adjustment for potential confounders. Both these caveats are important. Not only was the number of studies in review small, several came from atypical groups such as Pima Indians (very high prevalence of diabetes), preterm infants, or survivors of Dutch famine from the Second World War. Although, formal tests for publication bias were negative, they are not very useful with so few studies. There is potential for confounding with birthweight, current body size or parental diabetes. The effect of adjustment for these confounders was modest, but such data were available only for few studies.

CVD mortality

A systematic review was conducted to investigate the association of breast feeding with cardiovascular and ischaemic heart disease mortality.[33] Only four studies were available for meta-analysis, and the participants among these were born between 1904 and 1939. Cardiovascular disease mortality was similar in breast-fed versus bottle-fed participants (pooled rate ratio: 1.06; 95% CI: 0.94 to 1.20). There was no statistical evidence of between-study heterogeneity. There was a moderate degree of between-study heterogeneity for ischaemic heart disease estimates (I^2 value, indicating the degree of between-study variation attributable to heterogeneity, was 66%), with pooled rate ratio for breast-fed versus bottle-fed of 1.19 (95% CI: 0.89 to 1.58). The number of studies in this review is quite small. Furthermore, as the authors also point out, the modification of formulae over the last few decades makes any findings from these older cohorts less relevant to modern birth cohorts.

Summary

There is consistent evidence for a lower BP in those breast-fed as infants, with the effect estimate in the range of 1.1 to 1.4 mmHg. There is only modest evidence for lower mean BMI and obesity in those breast-fed as infants; in addition, the observed association may be confounded by socio-economic position. The association of breast feeding with cholesterol concentrations is age-dependent: positive in children, absent in adolescents, and inverse in adults. The association in adults is modest, and evidence for confounding by socio-economic position has not been rigorously examined. Limited evidence suggests an inverse association between breast feeding and abnormal glucose-insulin homeostasis. There is no evidence for an association between breast feeding and CVD mortality, although data available is limited.

3.2.2. Sodium intake in infancy

Unlike the evidence for type of feeding in infancy (mainly from observational studies), the evidence for the role of early sodium intake is from randomised trials only.[37;38;216;217]

The earliest of the four trials was reported by Whitten *et al.*[217] Twenty seven male black infants were fed on provided foods, and randomised to receive added salt in the food (or not) for 5 months, starting at the age of 3 months. The diets provided 1.9 and 9.3 meq of sodium per 100 kcal to the two groups of infants. No difference in blood pressure was noted at 8 months and 8 years of age. Furthermore, there was no indication that salted foods imprinted a preference for salt at 8 years. Although the exposure appears to be adequate in terms of the magnitude (the higher salt intake corresponded to the 99th percentile of sodium intake by US infants in 1969), it is possible that the duration of exposure was not long enough for persistent effects.

Hofman *et al* conducted a double-blind randomised trial, in which newborn infants were randomly assigned (immediately after birth) to receive a normal-sodium diet (N=245) or a low-sodium diet (N=231) during the first six months of life.[37] Women resident in a defined area of the Netherlands were recruited in the seventh month of pregnancy, and all infants born during 1988 were eligible for inclusion into the study. All infants in the trial received formula milk and solid foods (advised to start after 13 weeks of birth) free of charge for six months. The sodium concentration of the low-sodium formula was similar to that of human milk, and it was three times lower than that of the normal-sodium diet (6.3 versus 19.2 mmol/L). The sodium content of the solid food in each group corresponded to that of the milk received in the same group. Women were allowed to breast feed (sodium from that intake was incorporated in the analyses), but not any other food. The compliance to the intervention was confirmed by urinary excretion in three casual urine samples. Both the parents and the investigators were blind to the assignment.

Systolic BP was measured every month until the 25th week. There was a clear trend for increasing difference in systolic BP between the two groups over the follow up period. At 25 weeks, systolic BP (adjusted for birthweight and systolic BP in the first week) was 2.1 mmHg lower (95% CI: 0.5 to 3.7) in the low-sodium group, as compared to the normal-sodium group.

Subsequently, the authors tried to follow up these children in adolescence.[38] Of the 466 infants who completed the trial, only 167 could be examined at a mean age of 15 years: 71 (31%) from the low-sodium group and 96 (39%) from the normal-sodium group. The groups were not balanced at this stage, with lower levels of education and higher proportion of parents with hypertension reported in the low-sodium group. The mean differences in systolic BP (low-sodium minus normal sodium group) were: -1.5 mmHg (95% CI: -4.7 to 1.8) before

adjustment, and -3.6 (95% CI: -6.6 to -0.5) after multivariable adjustment (for sex, birthweight, education of child and mother, parental hypertension and maternal systolic BP). The sodium excretion in overnight urinary samples at the time of follow up was found to be similar in the two groups, suggesting no major differences in current diet (or poor validity of single, as compared to 24-hour urinary samples, for this purpose). These results are important, but the evidence at this point must be regarded as observational, with loss of randomisation and only limited follow up.

Lucas *et al* have also reported on the association between sodium intake in infancy and blood pressure at later ages, using data from their feeding trials in preterm infants.[218] The feeds given to infants differed not only in sodium, but also in other nutrients. Since it is not possible to independently assess the effects of sodium in these diets (only dietary groups have been compared), they do not merit inclusion here. They have already been included in the evidence on type of feeding and growth.[178;179]

Finally, a small but elegant trial has been reported by Pomeranz *et al* from a hospital in Israel.[216] Fifty-eight Jewish term infants, whose mothers refused to breast feed, were randomly allocated to having the formula feed diluted with tap water (sodium concentration 8.5 mmol/l; n=33 infants) or Eden Spring Mineral Water (sodium concentration 1.4 mmol/l; n=25 infants). This was done because although the modern milk formulae contain low sodium (identical to breast milk), the final sodium concentration in formula feed can vary considerably depending on the sodium content of the water used to prepare it. After 8 weeks, the spring water formula feed group reverted to tap water formula feeds as well. Fifteen breast fed babies served as a control group. Weekly body size and BP measurements were recorded for the first 8 weeks, with a final measurement at 24 weeks. Urinary sodium/creatinine ratio was determined in the first two months to confirm differences in sodium intake.

Increases in weight and height were similar across the groups. At 6-8 weeks of age, blood pressures were significantly greater in high-sodium tap water group. At week 24, the difference had reduced somewhat. Systolic blood pressures in the three groups at week 24 were: 93.2 mmHg (SD 6.3) in low-sodium spring water formula group, 95.1 mmHg (SD 6.0) in high-sodium tap water formula group, and 88.3 mmHg (SD 4.4) in the breast-fed group; the p-value was less than 0.05 for the comparison of tap water formula group with breast-fed group, but not spring water formula group. This study used a novel approach and the findings have implications for much of the developing world, where sodium levels in tap water are generally high. However, they need to be replicated as the final sample was very small: there were 11, 20, and 7 infants in the spring water formula, tap water formula, and breast-fed groups, respectively, at the 24 week follow up.

Summary

There is randomised evidence to suggest that sodium intake in infancy may be positively associated with BP in the short term (summarised in Table 3.4). Clear evidence on the longer-term effects of sodium intake in infancy is lacking.

Table 3.4: *Sodium intake in infancy and BP in later life*

Study	Population	Exposure	Results	Comments
US trial[217]	Black male infants born in a US hospital, 1969 Mean age: Follow up at 8 months and at 8 years of age N=27 (M); 13 control, 14 intervention	Randomised to receive normal diet with or without added salt (started at 3 months of age and given for 5 months)	No difference in BP at 8 months or 8 years of age	Sodium intake corresponded to 1.9 meq and 9.3 meq (per 100 kcal) in control and intervention groups
Dutch trial[37;38]	Born in a defined area of the Netherlands, 1988 Mean age: Follow up monthly until 25 weeks and then at 15 years N=466 (M+F) in infancy; N=167 (36%) at follow up aged 15 years	Randomised to receive formula milk (later solid foods) with low sodium (N=231) or normal sodium (N=245) content (for first six months of life)	Low sodium intake group had lower SBP at 25 weeks (2.1 mmHg; 95%CI: 0.5 to 3.7) and at 15 years (3.6 mmHg; 95%CI: 0.5 to 6.6) of age, in multivariably adjusted models	Sodium intake corresponded to 6.3 mmol and 19.2 mmol (per litre) in the low and normal sodium groups The difference in SBP at 15 years of age was not statistically robust in univariate models (3.6 mmHg; 95%CI: -1.8 to 4.7)
Israeli trial[216]	Jewish infants born in a hospital in Israel, 2002 Mean age: Follow up weekly until 24 weeks N=73 (M+F); 15 breast-fed and 58 whose mothers were unwilling to breast feed; N=38 (66%) at 24-week follow up	Those whose mothers were unwilling to breast feed were randomised to receive formula feed diluted with tap water (N=33) or low sodium spring water (N=25) (for the first 8 weeks)	SBP at 24-weeks: 93.2 mmHg (SD 6.3) in spring water group, 95.1 mmHg (SD 6.0) in tap water group and 88.3 mmHg (SD 4.4) in breast-fed group; p-value reported as less than 0.05 for comparison of tap water with breast-fed group but not spring water group	Sodium intake corresponded to 8.5 mmol and 1.4 mmol (per litre) in the tap water and spring water groups Results at 24-weeks based on comparison of 11 (spring water), 20 (tap water) and 7 (breast-fed) infants only

3.2.3. Long chain polyunsaturated fatty acids intake in infancy

The role of long chain polyunsaturated fatty acid (LCPUFAs) intake in infancy has been examined in two trials.[219;220] A multi-centric randomised controlled trial was originally conducted to investigate the role of LCPUFA supplementation in cognitive development.[220] Newborns were randomised to receive formula feeds with LCPUFAs (n=111) or without LCPUFAs (n=126), for the first four months of life. A third group of infants who were breast-fed were included (though not randomised) to be the reference group (n=139). Of the trial participants, blood pressure was measured in 219 children at a mean age of 6 years: 69 (59%) in the LCPUFA supplementation group, 71 (56%) in the formula without supplementation and 83 (60%) in the breast-fed group. Diastolic blood pressure was lower in the supplemental formula group (mean difference: -3.6 mmHg; 95% CI: -6.5 to -0.6, p=0.018). Systolic blood pressure was also lower in the supplemental formula group, although the difference was not statistically robust (mean difference: -2.3 mmHg; 95% CI: -5.3 to 0.7, p=0.1). The confidence intervals for these estimates are quite wide, and the results need to be treated with caution. The researchers did not adjust for potential confounders, presumably on the assumption that this was a trial. However, bearing in mind the extent of loss to follow up and differences among those included and excluded from the study (and between comparison groups), this assumption is questionable. Interestingly, the blood pressures in the breast-fed group were similar to the LCPUFA supplemented group, although the authors did not present the mean differences.

A similar design was used by another group of researchers who followed up children of women who had participated in an earlier trial of LCPUFA supplementation.[219] The trial was designed to investigate the effects of LCPUFA (administered as fish oil supplements) on growth and development in infancy. Women were recruited through the Danish National Birth Cohort study, and those with low (study group) and high (reference group) fish intakes were

invited to participate. Mothers with low fish intake (n=122) were randomised to receive daily supplementation with 4.5 g of either fish oil or olive oil, in the first four months of lactation. The high fish intake reference group (n=53) did not receive anything. Of the 175 mother-child pairs who took part in the trial, 73 (42%) children could be followed up at the age of 2.5 years. Blood pressures were recorded, and data analysed as an observational study. There were no important differences between the trial groups. For example, systolic BP (mmHg) was: 112 (SEM 2) in fish oil group, 108 (SEM 2) in olive oil group, and 108 (SEM 2) in the high fish intake reference group. The authors suggest the generally high intake of LCPUFA in the Danish population as an explanation for the null finding. However, it may also be due to the small sample size: there were 30, 22, and 21 children in the fish oil, olive oil and high fish intake groups, respectively.

Summary

There is limited and inconsistent evidence to support a role for early intake of long chain fatty acids in lowering BP in later life.

3.2.4. Diet in early childhood

Although there is a growing body of evidence on the longer-term risks of CVD associated with overnutrition in childhood beyond infancy, very few studies have examined the role of undernutrition in this age group.

Few trials have examined the longer term effects of nutrition supplementation on height.[221-223] In a non-randomised controlled trial conducted in Britain in 1937-39 (n=1,010; some centres of the Carnegie Survey of Diet & Health), food supplements (varying foods) were given to some of the children for 12 months, either at school or as food parcels sent home.[222] Children were between the ages of 2 to 14 years at the time; the supplemented children were 3.7 mm (95% CI: 1.9

to 5.5) taller than controls at the end of one year. In a randomised controlled trial, free milk supplements (190 ml daily) given to economically deprived Welsh school children (aged 7-8 years; n=581 for the study) resulted in a 2.9 mm increase in height ($P<0.05$) after 21 months.[221]

A randomised controlled trial was carried out to examine the effects of free milk tokens (equivalent to 284 ml per day) given to pregnant women and their offspring on birth weight and childhood growth up to 5 years.[223] Consecutively born infants from the towns of Barry and Caerphilly in South Wales were recruited (1972-74); 951 (82%) completed the first 5 yrs of follow up. The difference in birthweight of the supplemented and unsupplemented children, and in height and weight at the age of five years was consistent with random variation. Therefore, in subsequent follow up studies, the children from the two groups were treated as a single cohort and analysed as such (rather than a trial).

Two studies have reported on dietary constituents and CVD mortality.[224;225] In the 1946 UK birth cohort, diet was assessed by a single 24-hour recall at the age of four years.[225] Fat and vegetable intakes were not associated with CHD morbidity at 53 years of age, although fruit intake appeared to be protective. In the Boyd Orr cohort study (4,028 participants from 1,234 families who took part in the Carnegie diet survey in Britain in 1937-39, and were followed for mortality until the year 2000), childhood intake of several dietary constituents studied was not associated with coronary mortality, although lower intake of vegetables and higher intake of fish were both associated with a higher risk of stroke.[224]

Summary

There are hardly any studies on the role of dietary insufficiency in early childhood beyond infancy in influencing later CVD risk. There is some evidence

to suggest that improvements in diet in childhood may result in short-term increase in height.

3.2.5. Summary of evidence on early diet and CVD risk and strategy for further investigation

Despite growing body of evidence from animal experiments and observational studies in humans using anthropometry (especially birthweight) as a marker of early undernutrition, direct evidence for long-term CVD risks associated with dietary insufficiency in early life remains unconvincing. Balanced protein-calorie, relative protein and micronutrient (mainly calcium) deficiency in pregnancy, and type of feeding (breast or formula) and sodium intake in infancy have been the exposures most studied, while BP has been the most frequently examined outcome. There is no clear evidence in support of dietary deficiencies in pregnancy, although evidence exists for a modest role of feeding type (especially for BP as an outcome) and sodium intake (short-term only) in infancy. So why have dietary epidemiological studies largely failed to support the evidence generated from animal experiments and anthropometric studies in humans?

Assuming a true effect exists (bearing in mind the previously outlined limitations of animal experiments and anthropometric data), the most likely explanation lies in the nature of these studies. Diet is an exceedingly difficult exposure to quantify accurately, and noise generated from the error in measurement is likely to mask any underlying signals. The underlying signals themselves would be expected to be weak in observational studies set in well-nourished populations (with limited variability in exposure). In addition to this, dietary intake in humans is socially-patterned, and therefore likely to be confounded by other socially-patterned behaviours such as smoking and physical activity. Nutritional intervention studies conducted in malnourished populations offer the best combination of conditions to unearth any real underlying associations. Setting up

prospective nutrition interventional trials to study long-term effects on CVD risk is not a realistic option. Therefore, despite some inherent limitations (such as imperfect exposure(s) assessment and loss to follow up), follow up of participants from previously conducted trials offer the most useful and expedient option for gathering this much needed evidence. Several such trials were conducted in the developing countries during the second half of the 20th century, and this thesis is based on a follow up of one such trial from south India.

CHAPTER 4. METHODS

4.1. Bristol to Hyderabad via Geneva: the journey from conception to execution

My interest in the `fetal origins of adult disease' hypothesis was kindled when I undertook a study module on this topic during a master's degree programme in epidemiology. Once I had decided to make this topic the basis for doctoral research, I realised that I needed three things to achieve my goal: an appropriate study, funding for conducting that study, and experts willing to guide and supervise my research. I began by looking for potential supervisors, as I believed that they would not only be able to refine my vague ideas but also guide me towards appropriate studies and sources of funding. I approached Professors Yoav Ben-Shlomo and George Davey Smith to be my supervisors, as I knew them to be experts with a critical approach to this hypothesis that I wanted. They very kindly agreed.

The next step was to identify an appropriate study setting and design. During discussions with my supervisors, it became quite clear to me that a follow up of an existing cohort in a developing country setting offered the most efficient and pragmatic way of testing this hypothesis. With my connection to India and the rising prevalence of CVD in that country, we quickly came to the conclusion that I should try to find a suitable birth cohort there. To that end, I made two trips to India, one funded by a Wellcome Trust travel grant and the other funded by myself. During these trips, I went to several cities and visited medical schools that I believed could have kept birth records. Despite several potential leads, no suitable cohort could be identified. In my efforts to find birth cohorts from India, I also attended conferences where I expected Indian researchers to attend in strength. It was during one such conference - the annual conference of the Global Forum for Health Research in Geneva, 2001 - that I met Dr K V Rameshwar

Sarma, Deputy Director of the National Institute of Nutrition, Hyderabad. He informed me about a cohort study that he had conducted nearly 15 years ago and indicated his willingness to collaborate on a follow up of this cohort, if funding could be arranged. Subsequently, one of the supervisors (Prof. George Davey Smith) met Dr Sarma in India to further establish the potential and feasibility of following this cohort.

The first attempt at obtaining support for this study was made to the Wellcome Trust, who unfortunately changed their areas of research interest around the same time and therefore could not entertain this proposal. However, I was able to secure funding for this study through a travel fellowship (Eden Fellowship in Paediatrics, £47,000) awarded to me by the Royal College of Physicians, UK. After funding, it took a further year to get approvals from various Indian agencies before the study could finally begin. The entire process from conception to execution took the best part of three years.

4.2. The baseline study

4.2.1. The ICDS programme

The Integrated Child Development Services (ICDS) scheme is a major human resource development programme of the Government of India, aimed at improving survival, growth and development in early childhood.[39-42] Its beneficiaries are children below 6 years, pregnant and lactating women, and more recently, women in the age group 15 - 44 years. The package of services provided by the ICDS scheme includes supplementary nutrition, immunisation, health checkups and referral services, nutrition and health education, and pre-school education. All services are delivered at a central point in each village (anganwadi, literally a courtyard) by a local village woman (anganwadi worker) especially trained for this programme. The programme preferentially targets low

income and deprived families identified through a household survey of the community.

The main component of this programme is supplementary nutrition.[39-42] A cereal based meal is prepared from locally available ingredients, and distributed at the anganwadi. The constituents of the supplement vary from region to region, but are expected to provide on average 500 kilocalories and 20-25 grams of protein to pregnant/lactating women, and 300 kilocalories and 8-10 grams of protein to children up to 6 years. The supplement has to be collected daily by the woman (and/or her children) from the anganwadi, but they are not obliged to eat it there. As a result, supplementation is received on average for ~300 days a year, but often gets shared by family members. The anganwadi serves as a child day-care centre, delivering pre-school education to children between the ages of three to six years, and monitoring their nutritional status, referring where necessary. The contact afforded by food collection is used by the anganwadi worker to provide nutrition and health education to the women, and to encourage them to attend antenatal checkups and seek timely health advice.

In addition to these core services, the ICDS programme also links to other national programmes in India: immunisation, and prevention of Vitamin A deficiency and anaemia. The immunisation programme includes vaccinations against diphtheria, poliomyelitis, tetanus, tuberculosis and measles. The Vitamin A prophylaxis programme offers 6 monthly megadoses (200,000 IU) of the vitamin as solution to children between the ages of 1 to 6 years. The anaemia control programme aims to provide iron-folic acid tablets to pregnant women and pre-school children, for ~100 days each. The adult tablets contain 100 mg elemental iron and 0.5 mg of folic acid, while the tablets for children contain 20 mg elemental iron and 0.1 mg folic acid. These national programmes are available universally in India (i.e. in the non-ICDS areas also), but it is anticipated that a common point of delivery increases their uptake in ICDS

programme areas by making it more convenient (hence the term `integrated' in ICDS).

Several evaluations of the ICDS programme have been carried out.[43-46] They confirm that ICDS services primarily cover the poor and disadvantaged families of the community, and improve the uptake of component national programmes to some extent. However, only about half of the potential beneficiaries utilise the services. Comparisons with non-ICDS programmatic areas show conflicting results; while some studies have shown marked differences in growth and nutritional status of children and their immunisation uptake, in favour of ICDS areas, others have reported only marginal differences.[43-46] Variations in study methodology (often of poor quality), as well as in the conduct of ICDS schemes across the country may explain some of these differences in results. No study has evaluated the impact of this programme on CVD risk.

The ICDS programme began in 1975 as an experiment in 33 blocks.[39] A block is an administrative area covering approximately 100,000 population (about 100 - 150 villages), and it is the level at which such programmes are introduced. Over the last three decades, the programme has expanded into a national programme that, in 1999, covered 4,200 blocks (approximately 75% of the rural population) with the ultimate goal of universal coverage.[41;42] Although blocks considered backward or underprivileged by the national socio-economic criteria are prioritised for introduction of the programme, its phased rolling out across the country has largely been due to financial and operational constraints. Already, the programme covers 4.83 million expectant and nursing mothers and 22.9 million children under the age of 6 years, making it the largest national programme for promotion of mother and child health in the world.

4.2.2. The ICMR-USAID collaborative study

The stepwise expansion of the ICDS programme during the 1980's and 1990's offered an ideal setting to conduct a study on the impact of supplemental nutrition on pregnancy outcome. Using this opportunity, researchers from the National Institute of Nutrition, Hyderabad, conducted a community trial, during 1987-90, with the following objectives:

a) To assess the impact of food supplementation on pregnancy outcome (specifically birthweight);
b) To study the role of maternal nutritional status and physical activity during pregnancy on outcome of pregnancy (birth weight) in rural communities; and
c) To study the growth of infants to assess the impact, if any, of food supplementation to mothers during pregnancy.

Methods

A cluster of villages making up to 30,000 population each was chosen from two adjacent blocks, one of which already had the ICDS programme in place (intervention arm), while the other was awaiting implementation (control arm). Each block had many more villages (100+; spread over a very large area) than those required by sample size calculations (~15 villages in each arm). A randomised selection of the villages would have required the fieldworkers to cover unfeasibly long distances. Therefore, it was decided to limit the number of villages geographically: all contiguous villages within a 10 km radius of a prominent central village (based on local knowledge) were selected in each of the two areas. An added advantage of this methodology was that the study villages in the experimental area were clearly separated from those in the control area by several uninvolved villages, thus preventing contamination of intervention (a common concern in community trials). Fifteen villages from the intervention area and 14 villages from the control area were selected as a result of this process. The

study was co-sponsored by the Indian Council of Medical Research (ICMR) and the United States Assistance for International Development (USAID).

A 12-member team of investigators including a medical officer, nutritionists and social workers resided full-time in the headquarters of both the experimental and control areas for the duration of the study. At the start, a complete household enumeration was undertaken to collect information on demographic and socio-economic profile of the population. All women in the age group of 13 - 45 years were identified, and their marital and reproductive status ascertained. From this, a list of all `eligible' women considered exposed to the risk of pregnancy was prepared. The last menstrual period (LMP) of all the eligible women was monitored monthly to identify pregnancies early in the antenatal period.

Once identified as pregnant, the women underwent clinical examinations in each of the three trimesters (at 16, 28 and 36 weeks). Data were collected with the help of interviewer-completed questionnaires on woman's household; past and present pregnancy history; lifestyle, physical activity, diet (24 hours dietary recall), and food supplementation during pregnancy. Physical examination included anthropometry (height, weight and mid-arm circumference); abdominal girth and fundal height; signs of swelling of the feet and vitamin deficiency; and BP measurement. A small blood sample was also taken for haemoglobin estimation.

Arrangements were made with the members of the household to inform the team about the delivery when it happened. The field team attempted to visit the home as soon as possible after delivery to collect data on the outcome of the pregnancy, and to weigh the newborn. Data were collected on the infants soon after birth and at 1, 3, 6 and 12 months of age. The data collected included questions relating to the infant's feeding practices and immunisation; anthropometry

(weight, length; head, chest, and mid-arm circumference); and assessment of the maturity and development of the infant. Data were also collected on the date and cause of death for any infants that died during the course of the study.

The following set of data collection forms were used for the study:

1. Village information (Form A)
2. Household information (Form B)
3. Pregnant woman's household information and habits (Form C)
4. Obstetric history (Form D)
5. Present pregnancy history (Form E)
6. Physical examination form (Form F)
7. Physical activity during pregnancy (Form G)
8. Diet survey (Form H)
9. Food supplementation during pregnancy (Form I)
10. Outcome of pregnancy (Form J)
11. Neonatal examination (Form K)
12. Infant feeding practices, immunisation, growth and development (Form L)

An infant beam balance with a 20g accuracy (John Chatillon & Sons Inc., New York) was used to weigh the newborn, and a portable bench beam balance (John Chatillon & Sons Inc., New York) with an accuracy of 0.5 lb (225g approximately) was used to weigh the mother. Mother's weight was taken in normal clothing (without footwear) while the infant was weighed naked. Mother's height was measured with an anthropometric rod (Galaxy Informatics, New Delhi) with an accuracy of 1 mm, while the infant's length was measured with a wooden anthropometer (Military Science House, Delhi), also accurate to 1 mm. A stretch-resistant fibreglass tape with an accuracy of 1 mm was used to measure head and chest circumference of the infant and abdominal girth and fundal height of the mother. Triceps skinfold thickness was measured with Holtain skinfolds calliper.

Haemoglobin was estimated using cyanmethaemoglobin method and duplicate samples with an agreement of 0.5 gms/dl only were considered for analysis. A detailed protocol was prepared for the study and the fieldworkers were standardised according to this protocol.

The supplement given to those in the intervention arm was *`upma'*, a savoury preparation made from Corn-Soya blend (CSB) and soybean oil (provided by CARE Foundation). The amount given to pregnant women (120g CSB and 16g oil) was expected to provide 600 kcal and 20g protein daily. A diet survey conducted in a sub-sample of pregnant women (n=338) estimated the calorie content from home diet to be 1265 (SD 555), with the supplement providing an additional 420 (SD 163) kilocalories (unpublished data from NIN; personal communication, Dr K V Rameshwar Sarma, September 2003). No other nutrients (micronutrients, iodised salt, etc) were added to the supplement. Children were given approximately half the amount of supplement given to the women. The other universal programmes (immunisation, Vitamin A prophylaxis and anaemia control) and the provision of basic healthcare existed to a similar extent in both the intervention and control areas, although their uptake may be presumed to have been higher in the intervention area.

Outcome of the study

The data from the study were computerised but only preliminary analyses could be carried out, as the study funding did not extend to statistical support. A preliminary abstract was published in an in-house publication, while the study was still ongoing. Of the 2,800 births (56% intervention arm) at the time, mean birthweight was 88 gms higher in the intervention area (2683 grams versus 2595 grams; $p<0.01$), as compared to the control.[47]

4.3. The follow up survey

I conducted this study during 2003-5 to establish the status of all women and their offspring who took part in the baseline study, and to conduct clinical examinations on those still resident in the area. I prepared a detailed protocol for this study, which was piloted before the study began.

4.3.1. Study population

The index participants for this study were the offspring of women who took part in the baseline study. Additional data were also collected on the mothers of the index participants (i.e. women from baseline study). All children born in the baseline study and still resident in the study area were eligible for inclusion in the present study. Those who had migrated between the study villages were also eligible for inclusion, but those moving outside the study area were not contacted because of feasibility reasons. Where a woman had more than one offspring born in the baseline study (due to a multiple birth or if she became pregnant more than once), all such offspring were considered eligible for inclusion. The impact of non-independence of data arising from relatedness of these offspring was subsequently addressed in the analyses.

4.3.2. Approvals, insurance and consent

Scientific approval

This proposal was peer reviewed and approved by the Royal College of Physicians, UK (June 2002) and the Scientific Advisory Committee of the National Institute of Nutrition, India (September 2002).

Ethical approval

Formal ethical approval was not sought from any of the UK agencies, as the study was to be conducted entirely within India. Informal advice was sought from one experienced multicentre ethics committee member in the UK (also a paediatrician), and he expressed satisfaction both with the protocol and the plan to seek advice only from the ethics committee in India. The National Institute of Nutrition has a well-established ethics committee that is used to dealing with such requests. Its members are experienced clinicians and researchers from within and outside the institute. The ethics committee initially sought some clarifications related to the scientific aspects of the study (mainly study power and choice of outcomes), which I was able to address satisfactorily. Ethical approval was granted in August 2003 (the letter of approval is reproduced in Appendix A).

Insurance

Clinical indemnity and insurance cover was arranged through the University of Bristol insurers.

Consent from participants

I regarded participant consent to be a particular issue in this study due to high prevalence of illiteracy. The baseline survey had already shown that the majority of women and many of their husbands were illiterate. Illiteracy not only impacts on the ability to give written consent, it also raises important issues about the participant's ability to understand fully the implications of their involvement, and to give a consent that is genuinely free. To address this issue, I took several steps:

1. The information sheet and consent form were prepared according to the guidelines laid down by the Nuffield Council on Bioethics for research in

developing countries.[226] With the help of the communications officer of the collaborating institute, these forms were appropriately modified. Local expressions were used so that the study population could easily understand them. The forms were translated into local language and back-translated into English to ensure that the meaning did not change. These forms were then tested with the help of study participants, village teachers and village head to verify that they would be understood correctly (see Appendix B for information sheet and consent form).

2. In each village, an open meeting was held 3-4 weeks before the planned day for clinics. The index participants and their parents, the village head and elders, the village schoolteacher, and the anganwadi worker were invited to this meeting. The team explained the study to those assembled, and gave them the opportunity to ask questions and to clarify their doubts. Written information sheet was also given to all to take away (to show and discuss with their literate relatives and friends). Those not able to come to this meeting were visited in person at their home. At this stage, the prospective participants were not asked to commit to participation, but to take time to consider this proposal with their families and friends.

3. Over ensuing weeks, eligible participants were visited personally at their homes to clarify any doubts in private, and to seek formal consent to participate in the study. Consent was sought from the mothers and assent from the children. The number of home visits was kept flexible - those who refused to participate were not visited again while those who were away at the time or asked for more time to consider were revisited.

4. On the first day of the clinic, the village head (or his/her representative) was requested to visit the clinic and observe the proceedings. If the village head was satisfied with the process, he/she was requested to sign a consent form allowing the study to be conducted in their village (see Appendix B).

5. At the time of the clinical examination, the children and their mothers (or any other accompanying guardian) were requested to sign a common consent form. Due to initial worries about the high rates of illiteracy, arrangements were made to digitally voice record the consent process. However, I was pleasantly surprised to find that even though most of the parents were illiterate, a large proportion of the children were literate. They were therefore able to explain the consent form to their mothers (or guardians) and also give their signatures. The mothers then counter-attested the forms, in most cases, with their thumb print impressions.

4.3.3. Framework of surveys

Two types of surveys were conducted in this study - the identification survey and the clinical survey. Both surveys were conducted on a village-to-village basis, and the survey area was interchanged between experimental and control villages every few months to even out the variations arising from seasons and team experience. The identification survey was carried out to establish the identity and whereabouts of the women and their offspring involved in the baseline study. In the clinical survey, clinical examinations were conducted on the participants found to be eligible in the identification survey. For this reason, the identification survey preceded the clinical survey by around 3 months. The identification survey was carried out by a team of two social workers (one male and one female), one of who was also involved in the baseline study. The clinical survey team consisted of a medical officer, a nutritionist, a biochemist and two social workers.

Identification of participants

The houses in the villages did not have addresses at the time of baseline study. Temporary number plates were put up in front of the houses at the time and removed at the end of the study. Consequently, no addresses were available for

follow up. In addition, the actual names of the infants born during the study were not recorded (since future follow ups were not anticipated). To assess the difficulties in identifying the study participants accurately as a result of the above issues (and to devise appropriate strategies), a pilot study was conducted, which highlighted several points:

1. The majority of the women could recall the baseline study (many women could even recognise one of the social workers in the follow up survey, who had previously been involved in the baseline study). Their particular memories were of fieldworkers residing in the village, and visiting their homes to administer questionnaires and to weigh the newborn.

2. Lack of home addresses was not an important issue due to the small size of the villages and their communal way of life. It was generally possible to arrive at a village with a list of names (of women and their husbands) and be pointed immediately to the family's home (or to be told the whereabouts of the family if they had moved). The anganwadi worker was particularly useful in this respect, as she routinely has to conduct a village census for her own work and therefore tends to know the names of all the women and young children resident in the village.

3. Many women were not aware of the precise date of birth for their children, but the local event calendars, which are routinely used by the collaborating institute in this setting, could be used to arrive at a reasonable estimate of the date. People in this setting tend to remember personal events in relation to the local festivals and events (e.g. child was born one week after Diwali). The local event calendars contain an exhaustive list and dates of such events, which can be used to estimate date of birth.

4. In the pilot, the fieldworkers carried the database print-outs of offspring-related information (e.g. date of birth, birth order and sex) to the field, and tried to establish with the help of the mother, her offspring that most

closely matched the existing record. I noticed a tendency in some of the fieldworkers to try and influence the woman's responses based on prior information. This was a particular problem with date of birth (which had to be negotiated to some extent with the help of local calendars), but also to some extent with birth order (where the rigour with which history of dead children was elicited and included in the birth order depended on the match).

Bearing in mind the issues highlighted by the pilot, a novel procedure for the correct identification of the index participants was devised. This procedure involved:

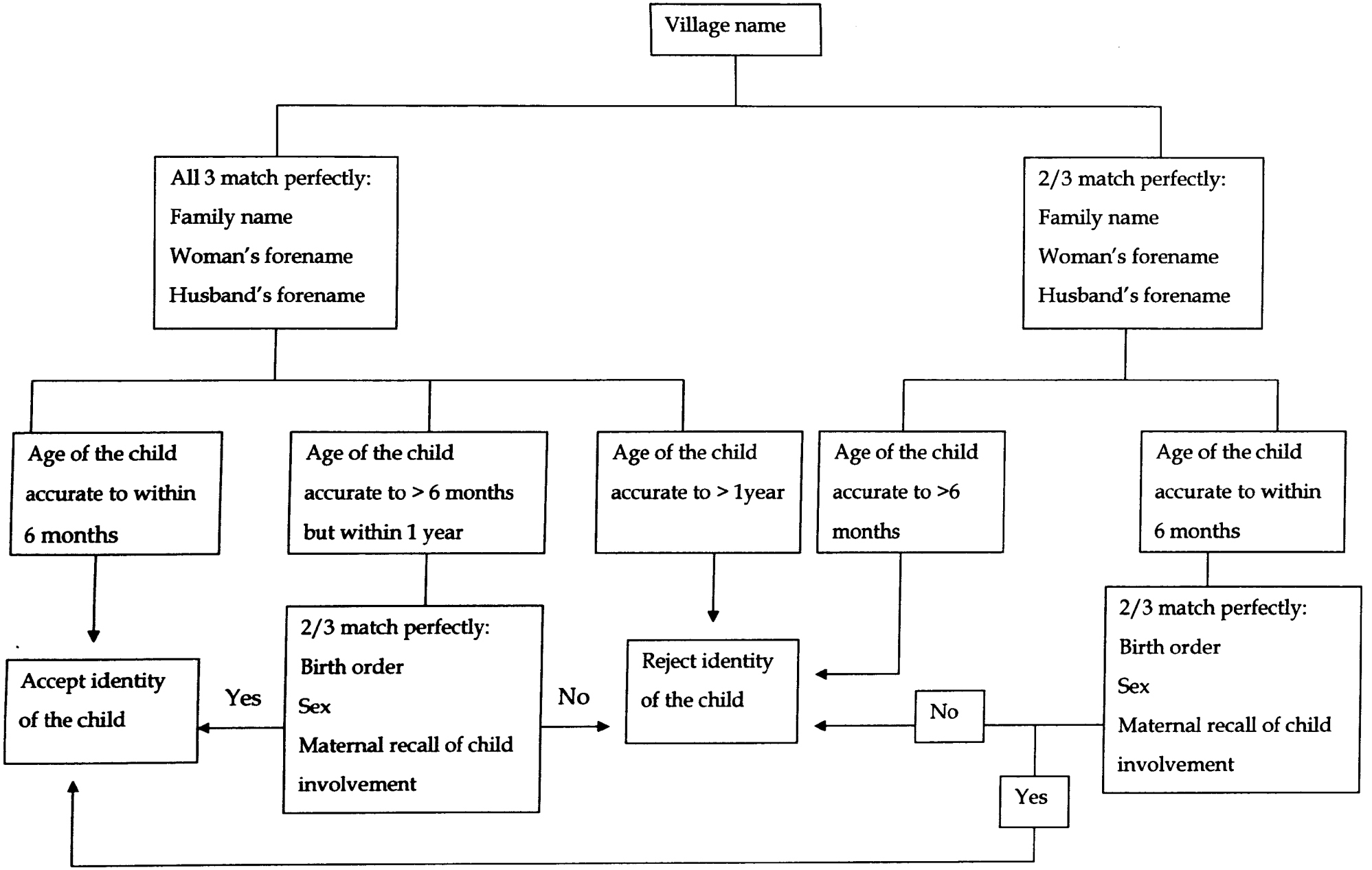
a) An algorithm for matching data on index participants; and

b) Separation and `blinding' of the identification survey team from the team involved in matching data on index participants

The fieldworkers were only provided with the names of the women (and of their husbands) involved in the baseline study from that village. They were not aware of how many of the woman's offspring (or their age, sex, and birth order) were involved in the baseline study. On arrival at a village, the survey team established the whereabouts of these women with the help of the anganwadi worker. The team then visited each of these women personally and completed the identification form. In this form, they were required to elicit, for every child born to the woman, the date of birth, sex, and her recall of the offspring being involved in the baseline study. Recall of the offspring's (or the pregnancy relating to it) involvement was accepted if the woman could remember the fieldworkers arriving at her home to complete the questionnaires or to weigh the newborn. Once the identification survey for a village was complete, questionnaires were entered on a special database.

The next step involved establishment of the identity of the index participant by matching the offspring data from the baseline study and follow up identification surveys. The doctor in the study team, who was not involved in the identification survey (and therefore `blind' to it), carried out this procedure. The database allowed the person doing the matching to bring up, simultaneously from both the baseline and identification surveys, the data relating to all the offspring born to the woman. Using the algorithm shown in Figure 4.1, the medical officer was able to accept or reject the identity of each child. Those accepted and `flagged' became the index participants eligible for inclusion in the clinical survey.

Figure 4.1: Algorithm used to trace children



Clinical examination

Clinics were held in the villages, generally at a central point such as the village hall or the health centre. In all but one large sized village (Maheswaram, where three separate clinic sites had to be used), a single clinic site was used to avoid measurement error arising from differences between sites. Clinics were conducted in the morning with 10-15 children invited to each clinic. Mothers (or another guardian) were asked to accompany their children, and did so in the majority of the cases. Children were asked to come to the clinic in fasting state (explained as no food or drink after midnight), wearing light and loose fitting clothes.

The study team arrived one hour before the participants to make clinic arrangements and to calibrate their instruments. The two social workers (one male and one female) greeted the participants on arrival and confirmed their identity. The children (and their mothers) were then sent to the consent station, where the nutritionist checked their fasting status (by oral questioning) and took their consent. If the child was not fasting (any food or drink other than plain water in the last 9 hours), he/she was sent back and asked to come the next day. If the child appeared reluctant to return, but had not eaten or drank anything for at least 4 hours, they were invited to participate in the study on the same day.

The children were next sent to the venepuncture station where the biochemist (or in difficult cases, the medical officer) collected their blood samples. Before venepuncture, the biochemist again checked their fasting status and enquired about recent illnesses or events (such as religious fasts) involving any major changes in the dietary patterns over the preceding three days. Date and time of the last meal was recorded. The children were offered anaesthetic cream to numb the venepuncture site but nearly all the children refused this option. Alcohol swab was used for cleaning the skin, and alcohol was allowed to dry off before

venepuncture. Samples were collected in the sitting position, generally with a 21-guage needle, and a tourniquet used throughout. If the blood could not be obtained at the first attempt, a single further attempt was made on the opposite arm with the child's consent. From each child, 10-12 mls blood was drawn and transferred to four different types of vacutainer tubes. The identification labels were applied to the tubes and inadequate or missing samples recorded on a datasheet. Any problems in taking blood samples were recorded. After venepuncture, the children were offered a light snack before proceeding to other stations for questionnaire completion and clinical examination.

The two social workers administered the questionnaires to the children, while the nutritionist conducted all the anthropometric assessments. The medical officer did the supine assessments, which included arterial stiffness and BP. The children interchanged between the stations until all their assessments were complete. They finally returned to the social workers, who verified the completion of examination, inviting them back as necessary for any outstanding assessments or reproducibility studies. The children and their mothers were thanked and a small gift (a pen to school going children and a glass to non-school going ones) was given on departure.

Major deviations from the protocol

There was only one major deviation from the protocol and this was in relation to the arterial stiffness assessments. As mentioned in the literature review, several indices for quantifying arterial stiffness exist, which differ in how they assess or express arterial stiffness.[98;104] Their interchangeable use in existing literature has made it difficult to compare findings from different studies. To make findings from this study more broadly comparable, the initial plan was to measure two of the most frequently used indices (augmentation index and pulse wave velocity) on each participant. However, soon after the study began, it became clear that this was that this was not going to be feasible because:

1. The majority of the villages did not have electricity supply for the best part of the day. This meant that the team had to transport a source of electricity for the arterial stiffness equipment. Generators can provide several hours power supply but the poor quality of current from these can damage precision electronic equipment. Systems based around rechargeable batteries provide better quality current but generally last for 2-3 hours; those that last longer are available but go up in weight markedly (in excess of 50 kg) making them unfeasible for field operations. Arterial stiffness assessments therefore had to be planned on the basis of around three hours of electricity supply that could be made available through a small rechargeable battery that the team carried with them to the field.

2. The space available for the clinics was often inadequate. As a result, participant preparation took far longer to complete. This was more of an issue for pulse wave velocity assessments which, unlike augmentation index that can be measured fully clothed (by applying the probe at the wrist), requires exposure of the chest (for application of chest leads) and groin area (for femoral artery). To avoid embarrassment to the participants, the procedure had to be conducted in an unhurried manner.

Because of the above reasons, a pragmatic decision was made to attempt measurement of one index only (augmentation index) on all the participants.

4.3.4. Questionnaires

The questionnaire was developed using, as far as possible, questions already tested and used in other studies. Preference was given to questions used in surveys conducted in local settings. Where necessary, I also attempted to contact the researchers to discuss the performance of existing questions. The important sources of questions included the Indian National Family and Health Survey[227]

and the Census of India,[228] the Parthenon[229] and Mysore[230] studies in India (provided by Dr Caroline Fall), and the Ten Town[231] (provided by Professor Peter Whincup) and the Boyd-Orr cohort follow up[232] (provided by Dr Richard Martin) studies in the UK. With the help of the communications officer of the collaborating institute, local language version of the questionnaire was produced to ensure correctness and consistency of language among those administering the questionnaires. The social workers who administered the questionnaires were trained and standardised against each other. The questionnaire was piloted in one of the study villages prior to its use. Interviews were conducted in private to encourage full disclosure. The main sections of the questionnaire were:

1. Demographic details
2. Education and employment
3. Household circumstances
4. Socio-economic position of the family
5. Lifestyle (smoking and alcohol consumption) and general health

Abstracts of the questionnaire and clinical datasheets relating to the data used in this thesis are reproduced in Appendix C. A short questionnaire was also completed with the head of each village to ascertain characteristics reflective of urbanisation of the village.

4.3.5. Anthropometric measurements

The methodology for anthropometric assessments was adapted from standard reference texts on the subject.[141;233]

General principles

Observer issues

The same member (i.e. nutritionist) of the team carried out anthropometric measurements on all the participants to eliminate inter-observer bias. I also trained other members of the fieldwork team as backup cover, and performed inter-observer reliability studies (at the outset and mid-way through the study). However, the backup cover did not have to be used. The observers were trained with the help of a detailed protocol that I prepared for this study. I carried out visual assessments of adherence to the protocol every couple of months.

Instrumentation used

A digital weighing machine (Model HD 305; Tanita, Japan) with an accuracy of 100 gms was used to measure weight. Height was measured by a portable plastic stadiometer with a base plate, accurate to 1 mm (Leicester height measure; Chasmors Ltd, London, UK). A skinfold caliper calibrated to 0.2 mm (Holtain skinfold caliper; supplied by Chasmors Ltd, London, UK) was used to measure the thickness of the skinfolds. Lengths and circumferences were measured with a non-stretch metallic tape (accurate to 1 mm) that had a narrow blade and a blank lead-in (Chasmors metallic tape; Chasmors Ltd, London, UK).

Measurement side

The arm measurements (including circumferences and skinfolds) were made on the non-dominant side of the participant, which was checked before taking the readings. The side of the writing hand for the literate participants, and the eating hand for those illiterate, was regarded as the dominant side.

Measurement recording

Weight was recorded to the nearest 100 gms. Heights, lengths, and circumferences were recorded to the last completed millimetre. Although, the dial of the Holtain caliper is calibrated to 0.2 mms, the skinfold measurements were recorded to the last completed 0.1 mm. Weight, height, and lengths were recorded once. Circumferences were recorded twice and skinfolds measurements were taken three times. Care was taken to avoid digit preference when taking the readings. Inadequacy of measurements (such as those due to physical deformities or postural problems) or any problems at the time of measurement were recorded.

Participant preparation

All measurements were conducted in light, minimal clothing. If the participant was not wearing appropriate clothing, he/she was asked to change into those carried by the study team for this purpose (a gown with tie backs for the girls and baggy shorts for the boys). The participants were asked to take off any hair bands or jewellery, if worn, and to release their hair if it was tied up at the back. Examinations were conducted in bare feet.

Weight

The weighing machine was placed on the most level part of the floor and calibrated at the start of every clinic. At the time of each reading, the scale was first turned on to ensure that the monitor read `zero'. The participant was asked to stand on the scale reasonably straight and to look ahead. Weight was recorded only when the reading on the monitor had settled. Attention was paid to avoid taking the reading as the participant came off the weighing machine.

Height

The stadiometer was set up on the most level part of the floor and calibrated at the start of every clinic. The participant was asked to stand on the stadiometer, while the observer checked for the following points: (a) feet flat on the centre of the base plate with ankles together and heels resting on the bar at the back; (b) back as straight as possible, preferably against the stadiometer but not leaning on it; (c) arms resting by sides, not behind or in front; and (d) head in the Frankfort plane (eyes looking straight ahead such that the lower edge of orbit was in line with external auditory meatus i.e. ear hole). The participant was instructed to keep his/her eyes focused on a point straight ahead, to breath deeply and to stretch to the fullest height. The observer assisted this stretching by applying gentle upwards pressure beneath the mastoid, and the headrest was lowered while checking at the same time that the participant did not stand on tiptoe. The observer tried to get level with the scale at the time of reading to avoid errors due to parallax.

Waist circumference

The participant was asked to stand straight, with feet close together and weight evenly balanced on both feet. The arms hung loosely at the sides. The measurement was carried out on the bare skin. The waist was identified as the mid point between the iliac crest below and the lower edge of the ribs (costal margin) above, measured at the sides. To locate the levels of the costal margin and the iliac crest, the fingers of the right hand were held straight and pointing in front of the participant and slid upward over the iliac crest. The right costal margin and the iliac crest were located, and the tape measure run from upper to lower point mark to mark the mid point (by measuring the distance between the two points and dividing in half). The same process was repeated on the left side. The tape was passed around the waist checking that it was horizontal. The participant was asked to breathe out gently and to look straight ahead. The

observer made sure that the tape was not pulled too tight; it rested on the skin but did not indent it.

Hip circumference

The measurement was carried out with the participant wearing a single layer of light clothing. The tape was applied to the widest part, usually between the greater trochanter (top of thigh bone) and the lower buttock level, with the legs together. The observer paid attention that the tape was horizontal and the hip muscles of the participant were not contracted.

Upper arm length and mid-arm circumference

The participant stood with his/her back to the observer with the arm flexed at 90 degrees. The tip of the acromion (the point of the shoulder) was palpated and marked. With the participant's arm flexed at 90 degrees, the olecranon (tip of the elbow) was palpated. The tape measure was put on the mark on the shoulder and dropped down to the tip of the elbow by the side of the arm. The exact distance was read as if an imaginary horizontal line had been drawn from the bottom most point of the elbow to the tape measure. The length of the upper arm was measured and recorded, and a point halfway between the acromion and the olecranon marked. This marked the vertical level at which the circumference was measured. The participant was then asked to relax, with the arm hanging by the side. The tape was placed around the upper arm such that its upper border was at the level of the marking. The tape was horizontal all round, resting firmly on the skin but not pulled too tight.

Skinfold thickness

A training video produced by the MRC Unit in Southampton was used to train staff in measuring skinfold thicknesses (permission kindly granted by Dr

Caroline Fall, MRC Southampton, UK), which in turn was adapted from standard texts on the subject.[141;233]

Triceps skinfold thickness

The tape was placed round the upper arm at the level of the mark made for measuring mid-arm circumference. With the tape in position, a horizontal line was drawn on the skin posteriorly at the level of the mark. Then, a vertical line was drawn on the most dorsal part of the upper arm, which was determined by `eyeballing' the mid-point (the part that stuck out furthest posteriorly). Where it was not obvious, a pen held vertically, with one end on the olecranon process and the other pointing towards the acromion, was used to make the vertical mark. The cross resulting from the horizontal and the vertical lines marked the point at which the skinfold was to be measured. The skinfold was picked up in a vertical tube at least 1 cm above the cross over the posterior surface of triceps muscle, on a vertical line passing upward from the olecranon to the acromion. The calipers were applied below the fingers such that the marked cross was at the apex of the fold. The readings were taken five seconds after the application of the calipers jaws.

Biceps skinfold thickness

The participant faced the observer with arms hanging by the sides and palms facing forwards. The horizontal line marked for the measurement of mid-arm circumference (and the triceps skinfold) was extended anteriorly. Then, as with the triceps skinfold, the point along the line where the arm bulged forward the most (the mid-point of the belly of the biceps muscle) was eyeballed, and a vertical line drawn to form a cross with the horizontal line. The skinfold was picked vertically and the calipers applied at the level of the cross, with the cross on the apex of the fold.

Subscapular skinfold thickness

The participant stood with the shoulders and arms relaxed. The lowermost tip of the scapula was identified. Where it was difficult to appreciate, the participant was asked to place the back of his/her hand on the lumbar region to make it more prominent. The medial border of the scapula was followed downwards until the inferior angle was felt. Once it was identified, the participant was asked to relax, with arms hanging by the sides, and a mark was applied to the skin immediately below the lower most tip (angle) of the scapula. The skinfold was picked obliquely above the mark, inclined slightly downward and laterally, in the natural cleavage of the skin. The caliper jaws were applied below the fingers, such that the marked cross was at the apex of the fold. Readings were taken five seconds after that.

Upper suprailiac skinfold thickness

The observer stood behind the participant. The participant stood straight and relaxed with arms folded in front. The iliac crest (the large curving bone, just below the waist) was located and a horizontal line drawn just above the crest at the side. Then, the participant was asked to lift the arm, and an imaginary vertical line (mid-axillary line) dropped from the apex of the axilla (the lowest point of the axillary hollow, just behind the thick fold made by the pectoral muscle). A line was drawn where this imaginary vertical line met the horizontal line to form a cross. The fold in the natural creases of the skin was picked up (with the cross on the apex of the fold) and the calipers applied at the level of the cross.

4.3.6. Vascular assessments

Blood pressure and arterial stiffness (as augmentation index) were assessed in the supine position, using protocols prepared on the basis of suggested guidelines.[234-236] In preparing the protocols for arterial stiffness assessments, I

was helped by the training I received at the Welsh Heart Institute from Professor John Cockcroft and colleagues, who are highly experienced in this technique.

General principles

Observer issues

A single observer (medical officer) carried out all the assessments according to the protocol. As the instrumentation used for arterial stiffness assessments is relatively novel, I invited an expert (Dr Ravikumar, Diabetes Centre, Chennai) to visit and observe the technique of the study observer.

Instrumentation used

BP was measured with an automated oscillometric device (OMRON HEM 705 CP; Omron, Matsusaka Co, Japan). This instrument meets the accuracy criteria laid down by the British Hypertension Society (BHS) and the American Association for the Advancement of Medical Instrumentation (AAMI), and was one of the two automated devices recommended in a recent review.[237] As the study population was known to be lean and generally small, only two cuff sizes (OMRON, UK) were made available: small (for arm circumference 15-22 cm) and medium (for arm circumference 22-32 cm). A digital thermometer (Weather Statc WS7013; Multitronics, Amsterdam, The Netherlands) was used to measure the ambient room temperature. Arterial stiffness was measured using the Sphygmocor apparatus (Sphygmocor Vx system supplied with MM3 electronics module and Millar pressure tonometer; Atcor (PWV) Medical, Sydney, Australia).

Measurement side

All measurements were carried out on the right side. This was done because it was the naturally convenient side for the observer. BP was recorded over the brachial artery and the AIx was recorded over the radial artery. If for some

reason the measurement could not be carried out on the right side, it was completed on the other side and the change recorded.

Measurement recording

Two readings each were taken for BP and arterial stiffness assessments. BP was recorded in mmHg units, heart rate in beats per minute, and augmentation index in percent. Any problems at the time of recording were noted.

Participant preparation

Participants were either fasting or had taken the light snack served at the clinic following venepuncture; no coffee or tea was served with the snack. The participants were not allowed to undertake strenuous exercise, or eat or drink anything other than water half hour before assessment. They wore loose, comfortable clothing for the examination. They were asked to lie on the floor (with a thin bedding underneath), and were covered with a sheet to put them at ease. History of any recent infection, cardiovascular disease (e.g. congenital and rheumatic heart disease) or medication was noted and the procedure to follow was explained to them. Participants were encouraged to keep still and not talk or sleep during the measurements.

Blood pressure

An appropriate size BP cuff was applied. The widest cuff practicable was used, with the lowest edge of the cuff about 2 cm above the cubital fossa (elbow crease). The cuff was placed around the right upper arm, with the centre of the bladder over the brachial artery. The cuff was applied not too tightly or too loosely; it fitted snugly so that it was just possible to put two fingers between the cuff and the arm. After applying the cuff, the participant was allowed to rest for five minutes before taking the first of the two BP recordings. During this time the participant details were entered into the laptop for arterial stiffness assessments, and room

temperature was recorded. With the inflation pressure set at 170mmHg, the `on' button of the sphygmomanometer was pressed to take the first recording. If the reading was unusually high, the participant was reassured and another reading taken after a further five minutes, which was then recorded as the first reading. After the first reading, the cuff was allowed to deflate and the arterial stiffness assessment was carried out. The second BP recording was taken after completion of the arterial stiffness assessments.

Augmentation index

The first BP reading was entered into the Sphygmocor software, which was set to pulse wave analysis to obtain AIx. With the participant still in supine position, the site of strongest impulse of the radial artery was located by palpation over the wrist. Then, the radial artery was gently flattened (but not occluded) at this site with the tonometer probe applied vertically. The augmentation index was recorded after checking that the quality of waveforms was good (see below). If the waveform was not good, further recordings were taken until the two requisite good quality recordings were obtained.

Decision to include or exclude readings was based on quality control criteria (the quality index was set at 90 percent), as well as the visual inspection of the overlayed waveform data (as little variability in the pulses as possible). If the pulse height variation or diastolic variation was outside the limits (i.e. displayed in red), the waveform traces were examined to ensure that no transients (temporary abnormalities of rhythm) occurred during the averaging period, which would make the averaging process invalid. To cover a complete respiratory cycle, the average of at least 10 successive measurements was used for analysis. Measurements were accepted as valid only when standard deviation of beat-to-beat data did not exceed 10 percent of the mean.

Up to thirty minutes were spent on each participant initially. If appropriate measurement was not obtained for some reason, the participant was asked to wait and the test repeated after 1-2 hours. If it was still not satisfactory, the participant was asked to come on another day to have the test repeated.

4.3.7. Pubertal assessment

Pubertal status was assessed on the basis of time since the onset of menstruation (girls) and testicular volume (boys).[238;239] The data on menstruation was collected through interview with the participant. Only female social worker elicited this data, and in private, to reduce embarrassment to the participant and encourage accurate disclosure. The boys were asked to self-report on their testicular volume with the help of an orchidometer.[239] An orchidometer consisting of a chain of 12 wooden testes, with defined volumetric capacities ranging from 1 - 25 mls (Prader orchidometer; supplied by Chasmors Ltd, Camden, London, UK), was used for this. The boys were asked to go behind a screen with the orchidometer, compare the volume of their right testes against the orchidometer, and to report which of the wooden testes was closest in size to their own. The examination was conducted in private and only the male social worker instructed the child in this examination.

4.3.8. Blood samples

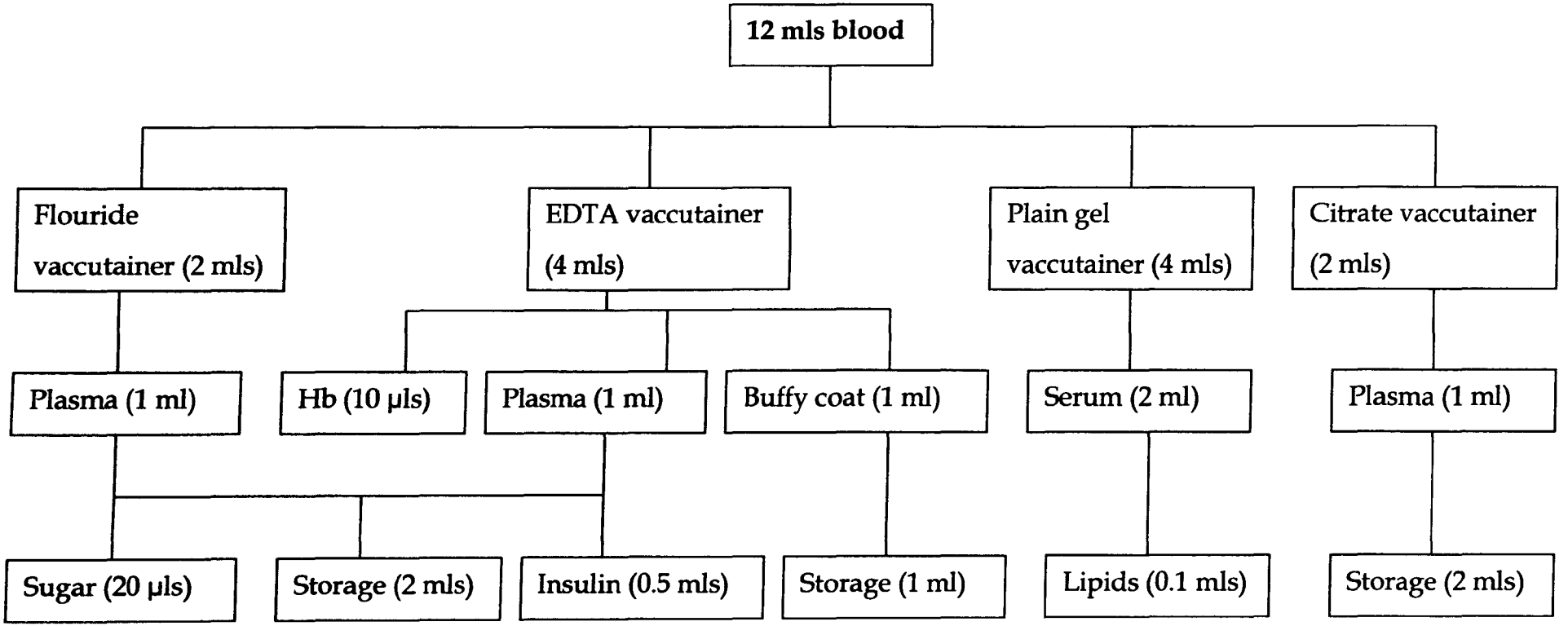
The processing of blood samples and the biochemical assays were done by the team biochemist, in the laboratory of the collaborating institute and under supervision of an experienced member of staff. The overall standards for the biochemical component of the study were ensured by a senior biochemist at the collaborating institute (Dr Ghafoorunissa), who was also a collaborator on this project. The team biochemist was already experienced in carrying out the

relevant assays when he joined the project; however, he underwent additional training to familiarise himself with the protocols prepared for this study.

The blood collected by the biochemist on venepuncture was immediately transferred to four vacutainers (fluoride, EDTA, plain gel and citrate), and mixed by gently inverting the vacutainers several times. The vacutainers were placed in racks and brought back to the collaborating institute in an icebox, which maintained temperature at 4-8 degree Celsius. Care was taken to avoid haemolysis during travel. The samples were transferred within 1-2 hours of collection and processed within 4 hours.

On arriving back at the laboratory, the biochemist transferred 0.5 ml of EDTA blood into another tube for haemoglobin (Hb) and packed cell volume (PCV) estimation. The remaining samples were then centrifuged at 3,000 revolutions per minute (rpm) for 10 minutes at room temperature to separate serum/plasma. After centrifugation, the samples were aliquoted according to the flow diagram shown in Figure 4.2. Those aliquots not required for assays immediately were stored in the freezer. The temperature of the freezer was maintained at minus 80 degrees celsius. Special freeze-resistant labels (Cryobabies; Scientific Laboratory Supplies, Nottingham, UK) stable at these low temperatures were applied on the stored aliquots.

Figure 4.2: **Blood processing plan**



Biochemical assays

Haemoglobin

Haemoglobin was estimated by the cyanmethaemoglobin method, using a commercially available kit (Haemoglobin estimation kit; Dr Reddy's Laboratories, Hyderabad, India). This method involves treatment of haemoglobin with a reagent that contains potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. The ferricyanide converts haemoglobin to methaemoglobin, which is further converted to cyanmethaemoglobin by the cyanide. The cyanmethaemoglobin has an absorbance that is proportional to the haemoglobin concentration. The absorbance of the test and the cyanmethaemoglobin standard is then measured against distilled water on spectrophotometer at 540 nm.

PCV (haematocrit)

On high centrifugation of blood, all the cells get aggregated and settle down, which allows the volume of packed cells to be estimated. Blood in a heparinised capillary tube was spun in a high-speed microhaematocrit centrifuge at 12,000 rpm for 10 minutes. The tube was then read with a haematocrit reader and PCV recorded in per cent.

Glucose, Triglycerides, Total and HDL cholesterol

Glucose, triglycerides, total and HDL cholesterol were estimated with an auto analyser (ACE Clinical Chemistry System; Schiapparelli biosystems, New Jersey, US). Reagent kits recommended for this system were used for the assays (Glucose, triglycerides, cholesterol and HDL precipitating kits; Alfa Wasserman, New Jersey, US).

In the ACE glucose method, glucose in serum is determined after enzymatic oxidation in the presence of glucose oxidase.[240] The formed hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminophenazone to form a red-violet quinoneime dye, which is used as an indicator. The absorbance of the reaction is biochromatically measured at 505-692 nanometers (nm).

The ACE triglycerides assay is a fully enzymatic procedure. In this method, the serum triglycerides are first hydrolysed by microbial lipase to form glycerol. The glycerol kinase in the reagent initiates a series of enzymatically coupled reactions that ultimately produce quinoneimine. The absorbance of quinoneimine is measured biochromatically at 505-692 nm, and is directly proportional to the concentration of the triglycerides.

The ACE cholesterol reagent contains pancreatic cholesterol esterase that hydrolyses the cholesterol esters in the serum to free cholesterol and free fatty acids. The cholesterol liberated by esterase and any free cholesterol originally present in the serum are both oxidised by cholesterol oxidase. The liberated peroxide reacts with phenol and 4-aminoantipyrine in a peroxide catalysed reaction to form a quinoneime dye which absorbs at 500nm. The change in absorbance is measured biochromatically at 505-692 nm, and is directly proportional to the amount of cholesterol present in the sample.

For HDL-cholesterol, serum is added to a mixture of magnesium chloride and phosphotungstate acid. The low density lipoproteins (LDL) and very low density lipoproteins (VLDL) are precipitated, leaving the HDL fraction in the solution. The HDL fraction is then assayed for cholesterol. The procedure involves mixing the serum with the precipitant into a centrifuge tube. After allowing the tube to sit at room temperature for 10 minutes, it is spun for a further 10 minutes at 4,000

rpm. The clear supernatant, which contains the HDL fraction, is carefully removed and assayed for cholesterol as described above.

Insulin

Insulin in plasma was estimated by radioimmunoassay.[241] The radioimmunoassay method is based on the competition of unlabelled antigen in the sample or the standard and the radio-iodinated (I-125) insulin, for the limited binding sites on a specific antibody. A second antibody separates the antibody-bound and the free insulin (antibody polyethylene glycol aided separation method) at the end of the incubation. Insulin concentration of samples is quantified by measuring the radioactivity associated with the bound fraction of the sample and the standards. A standard radioimmunoassay kit (DAKO Insulin; DAKO Ltd, Cambridgeshire, UK) was used. The reagents were mixed with the samples and incubated overnight, then centrifuged at 1500 rpm for 20 minutes. After centrifugation, the precipitate was decanted and radioactivity counted. The standard curve was plotted and sample value determined from the graph.

4.3.9. Data management

Data was stored in a Microsoft Access database (version Office 2000) that was created especially for this study by the departmental database expert. Data entry was done by two trained technicians. Thorough measures were taken to ensure the accuracy of data processing (described in detail in the following section on quality control).

4.3.10. Quality control

I took several steps to ensure that the collected data was of high quality. These are outlined below.

Fieldwork training

I developed a detailed training protocol that covered all elements of the study. The fieldworkers were trained in the use of this protocol over a 6-week period. I was assisted in this training by experts at the collaborating institute, especially in the anthropometry techniques and processing of blood samples. Training sessions were run at the collaborating institute's outpatient clinic (using staff as volunteers), and at schools, both locally and from one of the study villages (using school children from the relevant age group). The observers were standardised against each other, according to the protocol. Detailed inter-observer reliability studies were carried out, once at the beginning and then again mid-way through the study period. I formally evaluated the fieldwork every couple of months to check adherence to the protocol.

Calibration of equipment

All equipment was calibrated at the start of the study and once mid-way through the course of the study. The stadiometer was checked against a standard 1-meter ruler each time it was assembled, and the weighing machine checked against known weight at the start of each clinic. Spare back-ups were available for all instruments (except Sphygmocor) used in the study. The instruments were marked to allow identification. Where back up instruments were used, this had to be recorded.

Reproducibility

Reproducibility of measurements can be affected by differences in the technique of the measurers (inter-observer) or underlying natural variation in the outcome itself (intra-observer).[242] Measurement errors arising from inter-observer variability were eliminated in this study by using the same observer for any given measurement. To estimate the errors arising from intra-observer

variability, anthropometric and vascular assessments were repeated in a sub-sample of study participants. Five percent of the children were selected from each village with the help of a random numbers table, and invited to return for repeat measurements. Reproducibility studies are best conducted 1-3 weeks apart. However, it was difficult to manage this length of interval in the study, as the fieldwork was conducted on a village-to-village basis, and returning to the same village after a time gap to organise further clinics was inefficient. Participants selected for reproducibility studies were therefore invited back on the last clinic day at each village. Since a village survey could last from a few days to a few weeks, the time interval between two readings varied accordingly. The same observer repeated the assessments. The observers did not have access to the first readings at the time of conducting the repeat measurements.

Biochemical assay quality control

Both internal and external quality control arrangements were put in place. Internal quality control assays were run each time the samples were processed according to the recommendations of the manufacturers. External quality control was established with the cardiac biochemistry laboratory at the All India Institute of Medical Sciences, Delhi, which itself is part of the UK external quality control system (UKNEQAS; Newcastle, UK). Samples for external quality control of blood sugar, lipids and insulin were sent to the team biochemist every two months. Split assays were also performed in 5% sub-sample. Collected blood samples were divided into two sets of vacutainers by the medical officer and assigned a unique identification number. The medical officer maintained a codebook for the split samples and the biochemist conducting the assays was unaware of the identity of these samples. The code was broken on completion of the study and results from the split samples were compared.

Accuracy of data processing

At the end of each clinic, the fieldwork team reviewed the questionnaires and data entry forms to check for missing items, illegible responses and logical inconsistencies among responses. At the end of each village survey, a summary sheet for the village was produced to allow the fieldwork team to pick out any missing items. Automated range and logic checks were built into the Access database to filter out inadmissible values during data entry. After the questionnaires were entered, the data entry technicians went through every questionnaire on the computer screen for the second time to check and correct any errors. Finally, the fieldwork team counter-checked the data entered by the technicians in five questionnaires picked at random from each village. Any errors or problems identified were resolved by checking back with the original record, the fieldworker or the participant, as appropriate.

4.3.11. Validity studies

As far as possible, previously validated measurement tools and techniques were used. However, two of the measurements (arterial stiffness and self-assessed testicular volume) were relatively novel and these were validated.

Arterial stiffness assessments

Assessment of arterial stiffness using tonometry is a relatively novel technique, and although it has gained popularity rapidly in the recent years, I personally was inexperienced in its use at the start of the study. My concerns were twofold: firstly, whether the use of the instrument was valid in this population (especially with respect to the transfer function used in the software for calculation of various indices)[100-103] and secondly, whether our use of the equipment was appropriate. At the outset, I explored the option of conducting a separate study to validate the transfer function in a relevant population. However, discussions with the experts informed me that a) this would require invasive procedures, and

b) would be very expensive and beyond the scope of the study budget. I therefore did not pursue this further, reassured somewhat by the thought that even if the absolute values of transfer function were not completely valid, relative differences between the groups of comparison may be. For my second concern (about correctness of the measurement technique), however, I was able to conduct a small-scale validation study by inviting an independent expert to conduct repeat measurements on a sub-group of participants. Dr Ravikumar (Dr Mohan's Diabetes Centre, Chennai) is a cardiac radiologist, who is highly experienced in the use of Sphygmocor. He works in a laboratory dedicated to carrying out arterial stiffness assessments, and has also published research on it. In the validation study conducted within the main study, all the participants attending the clinic over two consecutive days were invited to undergo arterial stiffness assessments twice (at the same visit) - once each by the study observer and the `gold standard' (Dr Ravikumar). The two observers alternated in conducting the first assessment, to reduce any systematic bias arising from the order of the examination. The observers were not `blinded' to each other, as one of the aims of this exercise was to allow the expert to visually assess the technique of the study observer. In line with the study protocol, both observers took multiple readings until two satisfactory readings were obtained, the mean of which was used for analyses.

Self-assessed testicular volume

This was a completely novel technique and needed to be validated, especially as it may be prone to systematic bias (e.g. young boys may over-report the size of their testes). The original plan was to validate the self-reported testicular volume against that directly observed by a trained observer, within a subgroup of volunteer study participants. Due to space and time constraints, it proved practically difficult to conduct this sub-study in the field. Therefore, an independent study was conducted in a local school, among boys of a similar age. All children from a small, boys-only secondary school (ages 12 and over) were

invited to participate. Those who agreed, were asked to go behind a curtain with the Prader's orchidometer, and assess their own testicular size. Self-reported testicular size was noted down by one of observers. A male fieldworker, who was trained in the procedure (serving as the `gold standard') and unaware of the self-report, then assessed the testicular volume of the participant using the same orchidometer. The resulting pairs of observations on each participant were analysed to assess the validity of self-assessment technique.

4.3.12. My role in the fieldwork

I was responsible for planning and managing the study. I prepared the study protocol. I hired the fieldwork staff, and trained and supervised them according to the protocol. I regularly evaluated and monitored the fieldwork and managed any problems that arose. The fieldwork lasted for one and a half years during which I was in the field for about half the time. At the onset of the study, I spent six weeks in the field to train the fieldwork staff and pilot the protocols. Subsequently, I visited the field for 3-4 weeks at a time, every 6-8 weeks. Although I went to the field with the fieldworkers, I did not carry out any measurements myself (to limit the inter-observer bias). The same observer conducted each of the measurements for the duration of the study, and it was not possible for me to be in the field for the entire period due to work commitments in Bristol. During the time I was back in Bristol, I maintained regular contact with the fieldwork team through telephone calls, electronic mail and mobile phone text messaging. The last one proved to be particular useful due to its ease of use and its inexpensiveness. I was fortunate to have my collaborator on site in India (Dr K V Rameshwar Sarma) for trouble shooting problems when I was away.

4.3.13. Miscellaneous considerations

First-aid and abnormal results

The team carried a first aid box to the field. The protocol contained procedures for handling fainting participants, those who were HIV or Hepatitis B positive, and for needle stick injuries. Examination was to be discontinued if the participant appeared faint, and invited to come again if willing. Blood samples were not to be taken if the participant volunteered that they were HIV or Hepatitis B positive; however, this information was not actively sought. The protocol also contained suggested guidelines for further management of any important abnormalities detected during clinical examination. Basic medical advice was to be given, and appropriate referral made to the local government hospital (with which there was prior agreement); medical care in government hospitals is free in India. Written reports of the biochemical investigations were given to all the participants.

4.4. Analytical strategy

This section describes the statistical methods used in this thesis and the justification for their use, where appropriate. All analyses were carried out using STATA statistical package.[243]

4.4.1. Calculations and derived data

Pubertal stage

Pubertal stage in girls was defined on the basis of onset of regular menstruation, and the duration since onset (if applicable). In boys, pubertal stage was defined on the basis of testicular volume. Testicular volume was self-assessed using Prader's orchidometer, as previously described. Although Tanner's staging is the gold standard, it is not a feasible option for large field-based epidemiological

studies.[244] Both the timing of menstruation and testicular size are regarded as highly valid and reliable measures of pubertal status.[238;239] Self-assessment of testicular size has not been done before and was separately validated. The classification of pubertal stages used in the study is shown in Table 4.1. While this system of classification is relatively crude in comparison to a full Tanner staging,[244] it was considered adequate for the purposes of classifying children broadly into the main stages of maturation and delineating those who were undergoing peak growth spurt from those before and after, thus allowing measures of body composition and CVD risk factors to be adjusted for maturational age (along with chronological age).

Puberty was classified into early, middle, late and post-pubertal stages.[244] Early stage would correspond to Tanner stage II (upward limb of maturational spurt), mid-puberty corresponds to Tanner stages III and IV (peak maturational spurt), late puberty corresponds to Tanner stage V (downward limb of maturational spurt), and post-pubertal stage suggests completion of pubertal maturation. In devising this classification, certain assumptions were made. The median age at menarche in most populations is around 13 years, and a girl would typically be in mid-puberty for about two years prior to onset of menarche.[245;246] Since the youngest child in the study was 13 years old, it was assumed that all girls in whom menstruation hadn't started were somewhere in mid-puberty. Similarly, once menstruation had commenced, most girls would complete their maturation over the next 2 - 2.5 years (i.e. be in late puberty).[245;246] In boys, testicular sizes corresponding to mid-puberty are recognised as between 9 and 15 mls.[239;245-247] Accordingly, sizes below 9 mls and above 15 mls were classified as early and late puberty, respectively. Since the oldest child in the study was 18 years old, it was assumed that no boy had completed pubertal development. The lack of any girls in the early puberty stage, and any boys in the post-pubertal stage is consistent with the fact that the onset of puberty in girls precedes that in boys by about 1.5 years, and each stage lasts for approximately the same length of time.[239;245;246]

Table 4.1. *Classification of pubertal stages*

	Girls	Boys
Early puberty	None	Testicular size ≤9 mls
Mid-puberty	Menstruation not started	Testicular size >9 mls & ≤15 mls
Late puberty	Menstruation started <2.5 years ago	Testicular size >15 mls
Post-pubertal	Menstruation started ≥2.5 years ago	None

Anthropometric data

Body mass index (BMI) was defined as weight (kg) / height $(m)^2$.[248] Although BMI is a good surrogate marker of adiposity in western populations, it tends to be less specific for this purpose in others.[238;249-252] Its use in adolescent populations is also made less robust by difference in the timings of onset of maturational growth spurts of the two components (height preceding fat mass by about six months), causing the weight/height relationship (as a measure of adiposity) to vary over time.[239;249]

Carefully measured skinfold thicknesses offer a more direct measure of subcutaneous body fat, and can also be used to estimate total body fat and fat free mass using appropriate formulae.[238;239;249] Skinfolds were measured in triplicate (mm) over four recommended sites (triceps, biceps, subscapular and suprailiac). The means of the readings at each of the four sites were added and the resultant value converted to the log scale (to the base 10). The log of the sum of four skinfolds was used to calculate the body density by the equation of Durnin and Wormsley:[253]

For boys, body density=1.1620 - 0.0630 X log sum of four skinfolds

For girls, body density=1.1549 - 0.0678 X log sum of four skinfolds

Several equations were available, but this was used because it had been previously validated in a lean Indian population.[254]

The body density was then converted to body fat % by Siri's equation:[255]

Body fat % = (4.95/body density - 4.50) X 100

Body fat % was converted to total body fat and fat free mass in kg using body weight,

Total body fat (kg) = Body fat % X weight (kg) X 0.01

Total body fat free mass (kg) = Weight (kg) - body fat (kg)

Finally, body fat and fat free mass were converted into indices, on dividing by square of the height in meters,[256]

Fat mass index (kg/m^2) = Fat mass (kg) / height (m^2)

Fat free mass index (kg/m^2) = Fat free mass (kg) / height (m^2)

Conversion of fat and fat free mass into indices is recommended as it makes them independent of height, and also makes interpretation of their independent effects on disease outcome easier by putting all three measures (i.e. fat mass index, fat free mass index and BMI) on the same scale.[256]

Mid-arm, waist and hip circumferences were measured twice and then averaged. Waist-hip ratio (WHR) was calculated by dividing the mean waist circumference (mm) by the mean hip circumference (mm). Along with absolute waist circumference and WHR, a third measure of central adiposity was derived using skinfold measurements: central-peripheral skinfolds ratio (ratio of the sum of two central skinfolds (subscapular and suprailiac) by the sum of the two

peripheral skinfolds (triceps and biceps)). This additional index of central adiposity was calculated, as the waist circumference may not be very specific in some ethnic groups and lean populations.[238;250;257] Furthermore, there is some evidence to suggest a particular role for truncal fat (measured by truncal skinfolds, and distinct from central adiposity) in increasing CVD risk in Indian populations.[258]

Anthropometric data are often standardised into z-scores.[259] The z-score for an item indicates, how far and in what direction, that item deviates from its distribution's mean, and is expressed in units of its distribution's standard deviation. Standardisation of anthropometric data is generally done within categories of age and sex, to account for differences in variance across ages and between the two sexes. Since a wide range of anthropometric measures exist, standardisation also helps by expressing different measures on the same scale (i.e. z-scores instead of various units of measurements), making their effects on a given outcome directly comparable. While standardisation works well in multivariable models that include only anthropometric data, its use can be problematic in models which still require age and sex as covariates, because they may be potential confounders for other variables in the model. Introduction of age and sex in such multivariable models may distort the effect estimates of some of the variables by breaking down the age-sex dependent relationship among the covariates, as some are already age-sex standardised while others are not. It can also complicate the interpretation of such models.

Age-sex standardisation of the disease outcome as well is a potential solution to this problem, but such models are not very user friendly, as the effect estimates expressed in z-scores are not as meaningful as they are when expressed in their own natural units, say mmHg. The issue of standardisation is further compounded in adolescence where anthropometric variation is determined not only by age and sex, but also by pubertal stage. Standardisation by pubertal

stage, in addition to age and sex, is possible, but the resulting loss of power from the dramatic increase in number of sub-groups employed for standardisation, makes the z-scores less robust.

In view of all this, I decided not to age-sex standardise anthropometric data. Instead, I opted for what I considered to be the more flexible approach: incorporating age, sex and pubertal stage as covariates in all the models. Only in selected models where the components of body composition were introduced simultaneously to assess their relative impact, anthropometric data were converted to standard deviation scores (SDS). These standard deviation scores were calculated as:[259]

SDS = (observed value - mean value) / standard deviation

The means and standard deviations used were for the entire population rather than age-sex specific categories. This placed all the anthropometric measurements on the same scale (allowing direct comparison of impact) without changing the actual effect estimates in any way.

Socio-economic position

In rural India, there tends to be considerable homogeneity in the levels of education (e.g. illiteracy) and occupation (e.g. farming). Furthermore, indirect, informal sources of income can have a greater impact on the actual living conditions than direct occupational income. The standard of living index (SLI) was used as a measure of socio-economic position, as it tries to capture the actual living conditions of the person.[227] It is a summary measure of household asset ownership, which is useful in rural India where the joint family structure of the households renders individual's own socio-economic position less important. Asset ownership is considered to be a reliable and valid surrogate measure for wealth and standard of living.[260;261] The SLI was produced for use in the main

national survey of India (i.e. the National Family Health Survey),[227] and has also been used by other researchers,[262;263] thus allowing comparability. Table 4.2 provides the list of items included in the SLI. The total score for each household is added to classify a household as low (0-14), middle (15-24) or high (25-67) SLI .

Village urbanisation

Village population was used as the primary index of urbanisation. Population is the most frequently used criterion for urbanisation worldwide, and is one of the criteria used by the Indian Government to classify a place as urban or rural.[228] Although it is generally recognised that urbanisation is a function of population, the level above which important differences in lifestyle become manifest is not known. Consequently, most countries use arbitrarily defined cut-offs (the Indian Government cut-off is 5,000 persons).[228] This dichotomous rural/urban classification has been criticised for ignoring influences that may be operative at lower population levels. To control for the effects of village urbanisation in this study, I divided the villages into three groups: less than 2,000; between 2,000 and 5,000; and greater than 5,000 persons. Percentage of male population engaged in non-agricultural activities is another criterion used by the Indian Government,[228] but this has often been criticised for not being meaningful; the data was also not available in this study. The third criterion used by the Indian Government is population density (>400 persons per square mile in an urban area).[228] This data was available and I explored the effect of this variable on CVD risk by introducing it as three categories (number of persons per square mile): less than 200; between 200 and 400; and more than 400. The data for village level variables was collected from the village head and can be regarded as reasonably accurate.

Table 4.2: *Standard of living index score*

Category	Item score
House type	Pucca = 4; Semi-pucca = 2; Kutcha = 0
Toilet facility	Own flush toilet = 4; Public or shared flush toilet, or own pit toilet = 2; No facility = 0
Source of lighting	Electricity = 2; Kerosene, gas or oil = 1; Other source of lighting = 0
Main fuel for cooking	Electricity, liquid petroleum gas, or biogas = 2; Coal, charcoal or kerosene = 1; Other fuel = 0
Source of drinking water	Pipe, hand pump or well in residence/yard/plot = 2; Public tap, hand pump, or well = 1; Other water source = 0
Separate room for cooking	Yes = 1; No = 0
Ownership of house	Yes = 2; No = 0
Ownership of agricultural land	5 acres or more = 4; 2.0 to 4.9 acres = 3; Less than 2 acres or acreage not known = 2; No agricultural land = 0
Ownership of irrigated land	At least some irrigated land = 2; No irrigated land = 0
Ownership of livestock	Yes = 2; No = 0
Ownership of durable goods	Car or tractor = 4 (each); Moped/scooter/motorcycle, telephone, refrigerator, or colour television = 3 (each); Bicycle, electric fan, radio/transistor, sewing machine, black and white television, water pump, bullock cart, or thresher = 2 (each); Mattress, pressure cooker, chair, cot/bed, table, or clock/watch = 1.

Main outcome measures

Systolic and diastolic blood pressures, and augmentation index values were based on a mean of two readings. Total and HDL cholesterol, triglycerides and glucose were reported in units of mg%. These were converted into mmol/L by multiplying the reported values with relevant conversion factors: 0.0259 (total and HDL cholesterol), 0.01 (triglycerides) and 0.0555 (glucose). LDL cholesterol (mmol/L) concentration was estimated using the Friedewald-Fredrickson formula: LDL cholesterol = total cholesterol – (HDL cholesterol + triglycerides/2.19).[264] LDL values were not calculated for participants with triglyceride values above 4.5 mmol/L, as they may be invalid. Insulin resistance was estimated according to the homeostasis model assessment (HOMA), as the product of fasting glucose (mmol/L) and insulin (mU/mL), divided by the constant 22.5.[265] HOMA score was not calculated for participants with a fasting glucose ≥ 7 mmol/L, as the results are inaccurate in this group. Participants who had not fasted for at least eight hours were also excluded.

Insulin resistance syndrome (IRS) was defined by the modified criteria proposed for use in Indian adolescents.[266] These criteria include insulin resistance, as its high levels can exist in Indian populations, in isolation from the other components of IRS.[267] Similar to the World Health Organisation definition, waist circumference is replaced by BMI (using Indian cut-offs i.e. >23 kg/m^2, corresponding to the 85th centile),[251] as waist circumference does not adequately capture obesity in this population.[257;267] The diagnosis of IRS requires at least three of the following four components to co-exist:

1) Hypertension (systolic or diastolic blood pressure ≥ 90th centile for age and sex)
2) Obesity (BMI ≥ 85th centile, for age and sex)
3) Dyslipidaemia (triglycerides ≥ 90th centile or HDL cholesterol ≤ 10th centile for age and sex)

4) Abnormal glucose homeostasis (fasting insulin ≥ 30 mU/L in pubertal[268] and ≥ 20 mU/L in post-pubertal children, or fasting glucose ≥ 6.1 mmol/L)[269]

Age-sex specific centiles for the outcomes were calculated by dividing age into five annual bands and applying those separately to the two sexes.

4.4.2. Data exploration

Distribution of the continuous variables was assessed by visually assessing their distributions (using histograms), and by examining the skewness and kurtosis values. Ranges were inspected to detect values that exceeded the expected upper and lower normal limits, and to assess how far away they were (in units of standard deviation) from the sample mean. Categorical variables were tabulated and the frequency distributions inspected. Where some categories were too small for meaningful analyses, they were grouped with other appropriate categories.

4.4.3. Preliminary analyses

Prior to conducting any statistical analyses, suitable transformations were applied to outcome variables whose distribution deviated markedly from the normal curve. In the baseline study analyses, linear regression models were fitted to estimate the crude and sex-adjusted differences in birth weight between the intervention and control areas. The proportion of families and children included from each village, and at each stage of the follow up, were inspected to assess the possibility of selection bias by village-level factors. Baseline data collected in the identification survey were used to compare the characteristics of children participating in the clinic to those who did not participate. The proportion of missing values for each of the variables was inspected, and where appropriate, the baseline characteristics of individuals who were excluded from analyses

because of missing values were compared to those with complete data, to assess the possibility of selection bias in the sample used for the main analyses.

Unadjusted associations of intervention area with potential confounding factors were investigated through inspection of data, and formal statistical testing using unpaired t-tests, the Wilcoxon ranksum (Mann-Whitney) test or the χ^2-test for heterogeneity, as appropriate. Since children from the same village may be more similar to each other than those from another village, the impact of data clustering by village was assessed by calculating the intraclass correlation coefficients for each of the outcomes.[270] Intraclass correlation coefficient is defined as the ratio of the between-cluster variance to the total variance (combination of the between- and within-cluster variance), and can be calculated using the one-way analysis of variance (ANOVA).

Reproducibility of clinic measurements and biochemical assays was assessed using pairs of measurements collected on each participant. The pairs of readings can be treated as clusters (with the participant as the unit of clustering), and the within-subject standard deviations and intraclass correlation coefficients calculated using ANOVA.[271;272] The intraclass correlation coefficient serves as a measure of reproducibility. For validity studies involving assessments of arterial stiffness and testicular volume, the method-comparison analyses recommended by Bland and Altman was used.[273] The mean difference between pairs of measurements (one taken by study observer and the other by the `gold standard') was calculated, along with the reference range for the difference (limits of agreement), to assess whether the level of difference was within an acceptable range. In addition, Bland-Altman plots (graph of mean difference between the two readings plotted against the mean of the two readings) were used to search for any evidence of a systematic bias (with increasing or decreasing values) in the measurements.

4.4.4. Analysis of the determinants of CVD risk factors

Cross-sectional analyses were carried out to investigate the determinants of CVD risk factors. The study population from the intervention and control areas was taken as a whole, ignoring the presence of intervention. Linear regression modelling was used to examine the associations. Since participants who share a village may be more similar than those who don't, the assumption of independence of observations may be violated. To take this into account, robust standard errors were used in all the models (with village as the level of cluster).[270] Robust standard errors use cluster-level residuals to take account of the similarity of individuals in the same cluster. In the presence of clustering, they are larger than standard errors obtained from the usual regression models that ignore clustering. This technique does not allow the parameter estimate to vary, unlike certain other statistical techniques that do (such as multilevel models). However, the use of robust standard errors alone was considered adequate, as the main objective was to adjust for the confounding effect of differences between villages, rather than to estimate them. In addition, the differences between these relatively similar villages were not anticipated to be large.

Another potential level of clustering was the household, as more than one child from each household could participate in the study. To conduct meaningful cluster analyses, a reasonable proportion of participants have to be clustered (a robust estimate of the effect of clustering cannot be made if such a proportion is too low). Since the proportion of children from the same household taking part in the study turned out to be very low, the effect of household clustering in the primary analyses was ignored. Instead, their impact on the final models was estimated by comparing these models to alternative models that excluded the second child from the same household.

To assess the effect of village urbanisation, the village level indicators (such as village population) were applied to all children residing in the village. Some children did not live at the same village at the time of the baseline study. Where a child had moved to one of the other villages in the study area, the indicators of the new village were used in the analyses, as the aim was to capture current lifestyle differences. A very small number of children who participated were now living outside the study area; for these children, the current village indicators were not available. These children were retained in the analyses using the indicators for the village of origin, since most of these children had moved fairly recently for work or education. Their impact on the effect estimates was also examined by excluding them from the final models.

Data was available on a large number of potential determinants. Examining a large number of variables increases the likelihood of finding spurious associations by chance. Therefore, a small number of most relevant determinants were selected for analyses. As far as possible, the importance of determinants was judged a-priori on the basis of existing literature and their relevance to the local setting, rather than using data driven modelling strategies. Furthermore, the determinants were modelled within a framework of domains. Determinants within a common domain were modelled together, and the same models were fitted for all the outcomes. The exceptions to this rule were a few variables that were relevant only to certain outcomes, but not to others. The rationale for such an approach was to keep the models comparable, and to develop a cohesive picture of the role of certain key determinants in CVD risk. This was especially important since multiple outcomes were being examined.

Three main domains were considered: (a) physiological determinants, (b) current socio-economic factors, and (c) body size and composition. Tobacco and alcohol consumption were non-existent in this population; so these were not included in the analyses. In the physiological category, age, sex and pubertal stage were

considered for all outcomes. In addition, the effects of ambient room temperature on blood pressure[234] and heart rate on augmentation index[117;274-276] were also investigated, as these are known to influence the observed readings. The effect of the time of day (when the measurement was carried out) was investigated for blood pressure and anthropometry measurements.

In the current socio-economic factors, two types of influences were considered: (a) personal material circumstances of the participant, and (b) material circumstances of the area of residence i.e. urbanisation of the village. These were considered together as they are likely to be mediated through a common pathway i.e. behavioural and lifestyle differences, specifically diet, physical activity and tobacco use.[91] The standard of living index was regarded as the most appropriate measure of personal material circumstance, as explained earlier.[227] Maternal education and child's own occupation were considered as alternatives, and their additional contribution (if any) towards CVD risk of the participant was examined by adding them sequentially to models including SLI. Similarly, village population was modelled as the primary index of urbanisation, while the role of population density was examined by adding it to models already containing a term for population size. Models with different variables were compared subjectively on the basis of the variance explained by the models (R^2), and in cases of uncertainty by formal statistical tests (likelihood ratio tests).

In the body composition domain, height was included in all models as a measure of skeletal size. Since it has been postulated that the effects of early nutritional differences on later CVD risk may be mediated through changes in body composition,[277-284] specifically alterations to relative distribution of fat and fat free mass and also central adiposity, I tried to select (for this population) the most specific measure for each of these components. Ideally, one would establish the specificity of available indices by comparing their performance against a gold standard such as dual-energy X-ray absorptiometry (DXA).[285] Since this was not

available, I tried to assess the performance of various indices by correlating them with each other and with the CVD risk factors (using Pearson's correlation coefficients with Bonnferoni correction for multiple testing), and also fitted linear regression models. The rationale behind these analyses was that the most specific group of indices would be poorly correlated with each other, and possibly be correlated differentially across the main categories of CVD risk factors (such as lipids or blood pressure). Moreover, the ideal combination of indices would together explain maximal variation in the CVD risk factors, since an important purpose of assessing body composition was to control for their effect as potential confounders/intermediaries in the next stage of the analyses (effect of supplemental nutrition).

4.4.5. Analysis of supplemental nutrition and CVD risk factors

Association of supplemental nutrition with CVD risk factors was investigated using linear regression models. Area of birth (intervention or control) was used as a proxy for supplemental nutrition (primary exposure). Multivariable models were developed that controlled additionally for potential confounders. As explained in the previous section, a shortlist of potential confounders was determined a-priori and their role in this population confirmed by cross-sectional analyses. Where the choice of the most appropriate variable was not clear-cut (such as the most suitable indices for components of body composition), cross-sectional analyses allowed the most appropriate variables to be identified. Only selected variables were introduced at this stage of analyses.

Prior to studying the association of supplemental nutrition with CVD risk factors, I examined its association with puberty and anthropometry. The association of nutrition supplementation with pubertal stage was examined using ordinal logistic regression (since pubertal stage is an ordered categorical variable), and also by binary logistic regression (estimating the odds of participant being in mid-puberty, the stage of peak growth spurt). It is possible

that supplemental nutrition may influence CVD risk by long-term changes in body size and composition or by altering the timing of onset of puberty (directly or through changes in body size and composition).[286;287] Since pubertal stage was not associated with supplemental nutrition, it could be included in all the models, without concerns about appreciable distortion of the effect estimates (of direct association between supplemental nutrition and CVD risk). Self-reported chronological age in this setting is not very reliable; introduction of an additional age-related variable (which was objective, albeit in broad categories) in the analytical models was expected to provide more robust effect estimates (by reducing the `noise' from errors in age estimation).

Four pre-defined models were fitted incrementally for each CVD risk factor as the outcome. The basic model contained covariates deemed to be physiological determinants of the outcome. These included age, sex and pubertal stage for all the outcomes. In addition, ambient room temperature and heart rate were included in models with blood pressure and augmentation index as outcomes, respectively. In the next model, covariates related to the current socio-economic circumstances were added to the previous model. These included the standard of living index (of the household) and the village population size. In the final two models, height and body composition (combination of variables) were incrementally added to the previous models. As mentioned earlier, separate models were fitted for height and body composition, in view of their role as potential intermediaries.

Although several interactions were potentially possible, the study was not powered to detect these (see section on sample size and study power). The sample sizes required to detect interactions are typically four times of that required to detect the main effects.[288] Probability of chance findings is also increased on routine testing for multiple interactions. Therefore, I tested for only one pre-specified interaction, which had the strongest basis in existing literature:

between sex of the child and supplemental nutrition. There is evidence to suggest that rural families in India preferentially feed male children.[289;290] Conversely, there is also evidence that nutritional programming of CVD risk may be more prominent in males, as compared to females.[165;291] Interaction was tested in the final models, using the likelihood ratio test and where appropriate, separate models were fitted for boys and girls to examine the effect estimates for the intervention.

Insulin resistance is believed by many to be the central pathway by which effects of early nutrition on CVD risk are mediated.[11;18;292] To investigate this, I examined the association between supplemental nutrition and CVD risk factors, with and without adjustment for HOMA scores. The basic model (with physiological variables only) and the fully adjusted models (basic model, socio-economic variables, height and body composition) were assessed, with and without adjustment for HOMA scores, for each of the other CVD risk factors. To further investigate the role of insulin resistance, I analysed the determinants of Insulin Resistance Syndrome and assessed its association with supplemental nutrition, using the same set of covariates as described for other CVD risk factors.

As described in the previous section, regression models were fitted using robust standard errors to account for clustering of the data at the village level. Similar to the analyses of CVD risk factor determinants, clustering at household level (in case of siblings) and potential misclassification of current village environment (for those who were living away from home) was deemed to be small and ignored in the primary analyses. Their effects on the estimates in the final models, however, were examined by fitting models that excluded these children (second child among the siblings, and those living away from home). The assumptions underlying linear regression models (linearity, normality and

constant variance) were checked for the fitted models using appropriate regression diagnostic plots, and the influence of extreme values was examined.

4.5. Study power

Prior to conducting the follow-up, sample size calculations were performed to estimate the size of difference in selected risk factors that could be obtained. At the start of the study, family identifiers were available for 2,012 participants. From the baseline data, I expected around 20% of these women to contribute more than one child (mostly two children) to this study, resulting in 2,414 potential study participants. A small pilot study was conducted prior to the start of the main study to estimate the likely trace rate. Over 80% (49 out of 60) of the families could be traced, and almost all appeared willing to participate in the study. Making more conservative assumptions, I expected to trace 1,690 (70%) children, of which I expected 1,268 (75%) to participate in the clinics.

The above number would represent the actual sample size if all the participants were truly independent.[288] However, since the children from the same village are likely to be more similar to each other than those from a different village, the effective sample size would be smaller. The effective sample size can be calculated by dividing the sample size by the design effect, which depends on the intraclass correlation coefficient of the outcome, and is determined by the formula:[288]

Design effect (Deff) = 1 + (n-1) X ICC

Where n = average cluster size, and ICC = Intraclass correlation coefficient

Dividing the expected sample size (n=1,268) by the number of villages (n=29) gave the average cluster size (n=44). Published papers don't tend to report the

intraclass correlation coefficients; in addition, they may not be applicable to a rural Indian village cluster. To address this, I calculated the design effect for one high (0.1) and one low value (0.01) for the ICC, and applied those to all the outcomes.[288] I expected the ICC for most outcomes to fall within this range. This yielded design effects of 1.4 and 5.3 for low and high assumptions, respectively. Dividing the expected sample size by the design effects gave the range of effective sample size, i.e. 239 to 906 participants. I then used these new sample sizes to calculate the mean detectable differences (5% significance, 80% power) using a normogram by Altman.[293] This normogram gives the value of the standardised difference that can be detected for a given sample size, assuming exposure groups are dichotomised at the midpoint. The low and high detectable standardised differences were read as 0.19 and 0.36, respectively. Multiplying the standardised difference with the standard deviation of the variable (taken from the literature) gave the mean detectable difference that one could expect to find in the study. Table 4.3 shows the mean detectable differences for the main study outcomes thus calculated.

There were no existing nutrition intervention studies to allow comparison with reported differences in study outcomes. Estimates from birthweight studies suggested that the study was underpowered to detect important differences in the main outcomes. For example, systematic reviews have suggested effect sizes of 1.5 - 2 mmHg for systolic BP[21;160;161] and 0.04 mmol/L for total cholesterol,[167] for each kg difference in birthweight. Given the 61 g birthweight difference between the intervention and control arms of the Hyderabad study, these would translate into an expected difference of 0.1 mmHg for systolic BP and 0.002 mmol/L for total cholesterol, which are much lower than the mean detectable differences in this study sample. These estimates however would be meaningful only if all the effect of nutrition in pregnancy and early childhood was accurately reflected in birthweight, which is clearly unlikely.

Table 4.3: *Mean differences detectable at p = 0.05 with 80% power*

Outcome	SD of variable from literature	Mean detectable difference	
		ICC = 0.1	ICC = 0.01
Systolic BP (mmHg)	9.3 - 15.3 [294;295 296]	3.3 - 5.5	1.8 - 2.9
Diastolic BP (mmHg)	6 - 12.1 [294;295 296]	2.2 - 4.4	1.1 - 2.3
Augmentation index (%)	7 - 8 [128]	2.5 - 2.9	1.5
Height (mm)	6 - 8 [297]	2.2 - 2.9	1.1 - 1.5
BMI (kg/m^2)	2.6 - 4 [294;295;298 296]	0.9 - 1.4	0.5 - 1.4
Total cholesterol (mmol/L)	0.6 - 0.9 [296 299;300]	0.2 - 0.3	0.1 - 0.2
LDL cholesterol (mmol/L)	0.5 - 0.9 [299;300]	0.2 - 0.3	0.1 - 0.2
HDL cholesterol (mmol/L)	0.2 - 0.5 [299;300]	0.07 - 0.2	0.04 - 0.1
Triglycerides (mmol/L)	0.3 - 0.5 [296;299;300]	0.1 - 0.2	0.06 - 0.1
Glucose (mmol/L)	0.6 - 0.7 [296]	0.2 - 0.3	0.1
Insulin (mU/mL)	9 - 17 [297;301]	3.2 - 6.1	1.7 - 3.2

ICC = 0.1 corresponds to an effective sample size of 239 and detectable standardised difference of 0.36
ICC = 0.01 corresponds to an effective sample size of 906 and detectable standardised difference of 0.19

CHAPTER 5. DESCRIPTIVE ANALYSES

The results of this study are presented in the next three chapters. This chapter describes the results of the analyses conducted on the data from baseline study, and the characteristics of the participants included in the current follow up. The representativeness of the participants and the reproducibility of the clinic measurements are presented. The two chapters that follow report on analyses of the determinants of CVD risk factors in this population (chapter 6) and the association of supplemental nutrition with CVD risk factors (chapter 7).

5.1. Baseline study

Of the 4,338 pregnancies recorded in the trial, birth weights (measured within 48 hours of birth) were available for 2,964 children (68%), relatively fewer in control as compared to the experimental area (Table 5.1). The remaining pregnancies without corresponding birthweights are likely to be a combination of pregnancies not resulting in live birth, births taking place outside the study area, and children whom the fieldwork team could not weigh in time. The actual reasons for the missing birthweights were not recorded.

The mean birthweight of children born in the two areas combined was 2629g (SD 427g; Range: 1,100 to 4,300). The distribution of birthweights was normal. Mean birthweight of children born in the intervention area was 61g higher (95% CI: 18 to 104; p=0.007) than controls (Table 5.1). Data on sex of the child was missing for six children; for the remaining, adjustment for sex made little difference to this estimate (60g; 95% CI: 18 to 102; p=0.007). Data on other important confounders such as birth order and gestation was available for far fewer children (less than 50%) to be usefully included in the analyses. Among the 1,782 women (992 with birthweights) for whom the history of smoking was available, none smoked.

Table 5.1: *Birthweights of children in the intervention and control areas*

	N	Intervention area (N=2,421)	Control area (N=1,917)	P-value
Birthweight recorded, N (%)	2,964	1,701 (70)	1,263 (66)	0.002
Male sex, N (%)	2,958	1,277 (53)	955 (50)	0.051
Mean (SD) birthweight (gms)	2,964	2655 (424)	2594 (430)	<0.001

P-values for difference between the two areas are based on t-test (birthweight) and χ^2 tests (proportion of birthweights recorded and male sex)

5.2. Current follow up

The flow diagram for recruitment of participants is shown in Figure 5.1. Although historical records were available for 4,338 pregnancies, not all had linked personal identifiers. Personal identifiers in the form of village name, family name (surname), woman's first name, and husband's first name were available for 2,756 families. These were used to trace the families in the first instance. A total of 1,963 (71%) families could be contacted personally: 1,001 (75%) in the intervention area and 962 (67%) in the control area. The status of a further 326 (12%) families could be confirmed with the help of the villagers: 81 (3%) were temporarily resident at the time of the baseline study (all of them were daughters of the villagers who had married and moved away, but were visiting their parents at the time of the baseline study); 234 (8%) were originally resident, but had since migrated out of the area; and 11 were away from their homes at the time of the field team's visit (despite several visits) (Table 5.2).

Four hundred and sixty-seven families (17%) were unknown to the residents of their respective villages (Table 5.2). Keeping in mind the close-knit village life in these areas, these are almost all likely to be temporary migrant workers at the time of baseline study. Influx of such workers is common in this area, especially during the harvest season when additional help is required. The higher number

of baseline family identifiers available in the control area, along with a corresponding higher number of unknown families also supports this view, as the control area traditionally has higher influx of such seasonal workers. In the baseline study, such temporary residents were eligible for inclusion to the study. The other main category of temporary residents in this study, common to intervention and control areas, was that of visiting daughters. This reflects a local custom in the area, whereby married daughters will often return to their own parent's home for delivery of their child (typically arriving a few weeks before the expected date of delivery, and departing a few weeks after childbirth).

Table 5.2: *Reasons for failure to contact families*

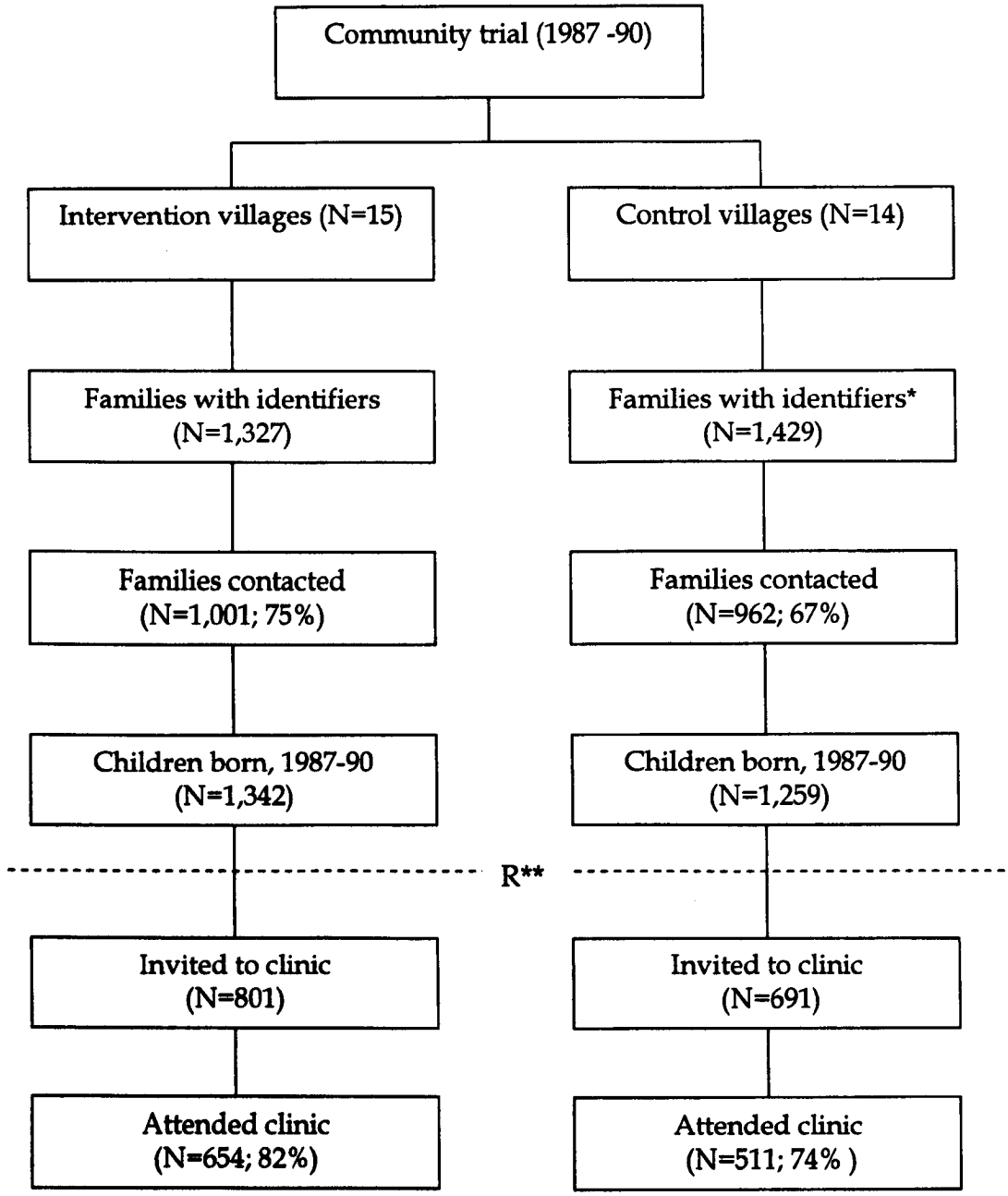
	Intervention area	**Control area**
Unknown*, N (%)	168 (13)	299 (21)
Temporary residents during baseline study**, N (%)	40 (3)	41 (3)
Migrated out of area since baseline study, N (%)	111 (8)	123 (9)
Away from home, N (%)	7 (0)	4 (0)
Total not contacted, N (%)	*326 (25)*	*467 (33)*

Percentages are of the total number of families in each area

**Unknown group were most likely temporary migrant workers at the time of the baseline study*

***Temporary residents during baseline study were all daughters of the villagers who had married and move away, but were visiting their parents at the time of the baseline study*

Figure 5.1: Flow chart of participant recruitment



Notes:

* Higher figure most likely reflects greater influx of temporary migrant workers during harvest season in the control area

**R = Restricted to those with historical records

Of the 1,963 families thus contacted, an interviewer-administered questionnaire was completed with the woman who had participated in the baseline study. In that questionnaire, the woman was asked to provide information on all the children she had given birth to. At the time of current follow up, these 1,963 women had delivered a total of 8,246 children, of which 952 had died. The child birth and death rates, in the intervention and control areas, are compared to the national Indian figures in Table 5.3. The comparability of the figures to the national figures suggests that the child listing process was fairly accurate. The lower fertility and mortality rates in the intervention area are consistent with some of the other objectives of the intervention, suggesting effective implementation.

Table 5.3: *Fertility and mortality in the intervention and control areas*

	Intervention area	**Control area**	**India**
Total children born to participating women till date, N (per woman)	4,121 (4.1)	4,125 (4.3)	4.0*
Of the above, children not alive now, N (%)	423 (10)	529 (13)	14.2**
Remaining children currently alive (and born 1987-90), N (%)	1,342 (33)	1,259 (31)	NA

**Figure relates to the completed family size while not all women in the study had completed their families*
***Figure is for under-five mortality (deaths under the age of five years) while the study area figures are for child death at any age*

Of these 8,246 children, 2,601 were born during 1987 - 1990 and were still alive at the time of the follow up. These children were all potential study participants. However, because of limited resources and to make maximal use of historical data, I decided to invite only those children who could be linked to existing historical records. On the basis of the screening strategy explained in the methods section, 1,492 (57%) children were linked and these were invited to participate in the study clinics.

Of the 1,492 children invited, 1,165 appeared for the clinical examination, giving an overall response rate of 78% (intervention area: 82%; control area: 74%). These represented 49% and 41% of all eligible children (total 1987-90 births) in the intervention and control areas, respectively. The main reason for the lower response rate in the control area was the poor cooperation from the village heads of a few villages, due to political reasons; the participation rates in the villages were dependent on support from the village heads. The number of children recruited ranged from 2 to 82 in the intervention villages and 15 to 122 in the control villages (Tables 5.4 and 5.5; Figures 5.2 and 5.3).

Table 5.4: *Recruited families and children in the intervention villages*

	Number (%)				
	Families		Children		
Village name (ID)	**Total with identifiers**	**Contacted**	**Total born 1987-90**	**Invited to clinic**	**Attended clinic***
Ibrahimpatnam (1)	169 (100)	120 (71)	155 (100)	95 (61)	72 (76; 47)
Sheriguda (2)	51 (100)	47 (92)	62 (100)	40 (65)	38 (95; 61)
Patelguda (3)	122 (100)	107 (88)	163 (100)	95 (58)	73 (77; 45)
Mangalpally (4)	34 (100)	29 (85)	37 (100)	25 (68)	19 (76; 51)
Uppariguda (5)	90 (100)	67 (74)	104 (100)	56 (54)	53 (95; 51)
Pocharam (6)	61 (100)	52 (85)	73 (100)	30 (41)	29 (97; 40)
Ramireddygudam (7)	12 (100)	10 (83)	11 (100)	4 (36)	2 (50; 18)
Seetharampet (8)	47 (100)	31 (66)	43 (100)	25 (58)	21 (84; 49)
Nomula (9)	76 (100)	69 (91)	95 (100)	63 (66)	52 (83; 55)
Lingampally (10)	121 (100)	75 (62)	93 (100)	61 (66)	49 (80; 53)
Engalguda (11)	37 (100)	33 (89)	44 (100)	27 (61)	22 (82; 50)
Polkampally (12)	71 (100)	64 (90)	83 (100)	55 (66)	46 (84; 55)
Nainampally (13)	81 (100)	67 (83)	85 (100)	53 (62)	36 (68; 42)
Raipole (14)	224 (100)	139 (62)	178 (100)	105 (59)	82 (78; 46)
Dandumylaram (15)	131 (100)	91 (70)	116 (100)	67 (58)	60 (90; 52)
Total	1,327 (100)	1,001 (75)	1,342 (100)	801 (60)	654 (82; 49)

**The two proportions relate to those invited to the clinic, and the total number born in 1987-90, as the respective denominators*

Table 5.5: *Recruited families and children in the control villages*

	Number (%)				
	Families		Children		
Village name (ID)	**Total with identifiers**	**Contacted**	**Total born 1987-90**	**Invited to clinic**	**Attended clinic***
Rachloor (1)	97 (100)	66 (68)	79 (100)	37 (47)	21 (57; 27)
Lemur (2)	52 (100)	40 (77)	57 (100)	24 (42)	17 (71; 30)
Mankhal (3)	94 (100)	54 (57)	66 (100)	34 (52)	21 (62; 32)
Thummalur (4)	189 (100)	123 (65)	156 (100)	91 (58)	77 (85; 49)
Maheswaram (5)	325 (100)	209 (64)	297 (100)	157 (53)	122 (78; 41)
Mansanpally (6)	58 (100)	39 (67)	51 (100)	27 (53)	17 (63; 33)
Gudur (7)	72 (100)	56 (78)	73 (100)	46 (63)	31 (68; 43)
Kandukur (8)	61 (100)	41 (67)	49 (100)	36 (74)	27 (75; 55)
Kummadivally (9)	89 (100)	61 (69)	78 (100)	40 (51)	27 (68; 35)
Thimmapur (10)	56 (100)	45 (80)	66 (100)	33 (50)	19 (58; 29)
Meerkhanpet (11)	50 (100)	35 (70)	38 (100)	26 (68)	24 (92; 63)
Sardarnagar (12)	35 (100)	29 (83)	42 (100)	17 (41)	15 (88; 36)
Aakulamylaram (13)	108 (100)	69 (64)	80 (100)	46 (58)	37 (80; 46)
Nedunur (14)	143 (100)	95 (66)	127 (100)	77 (61)	56 (73; 44)
Total	1,429 (100)	962 (67)	1,259 (100)	691 (55)	511 (74; 41)

**The two proportions relate to those invited to the clinic, and the total number born in 1987-90, as the respective denominators*

Figure 5.2: Children recruited from intervention villages

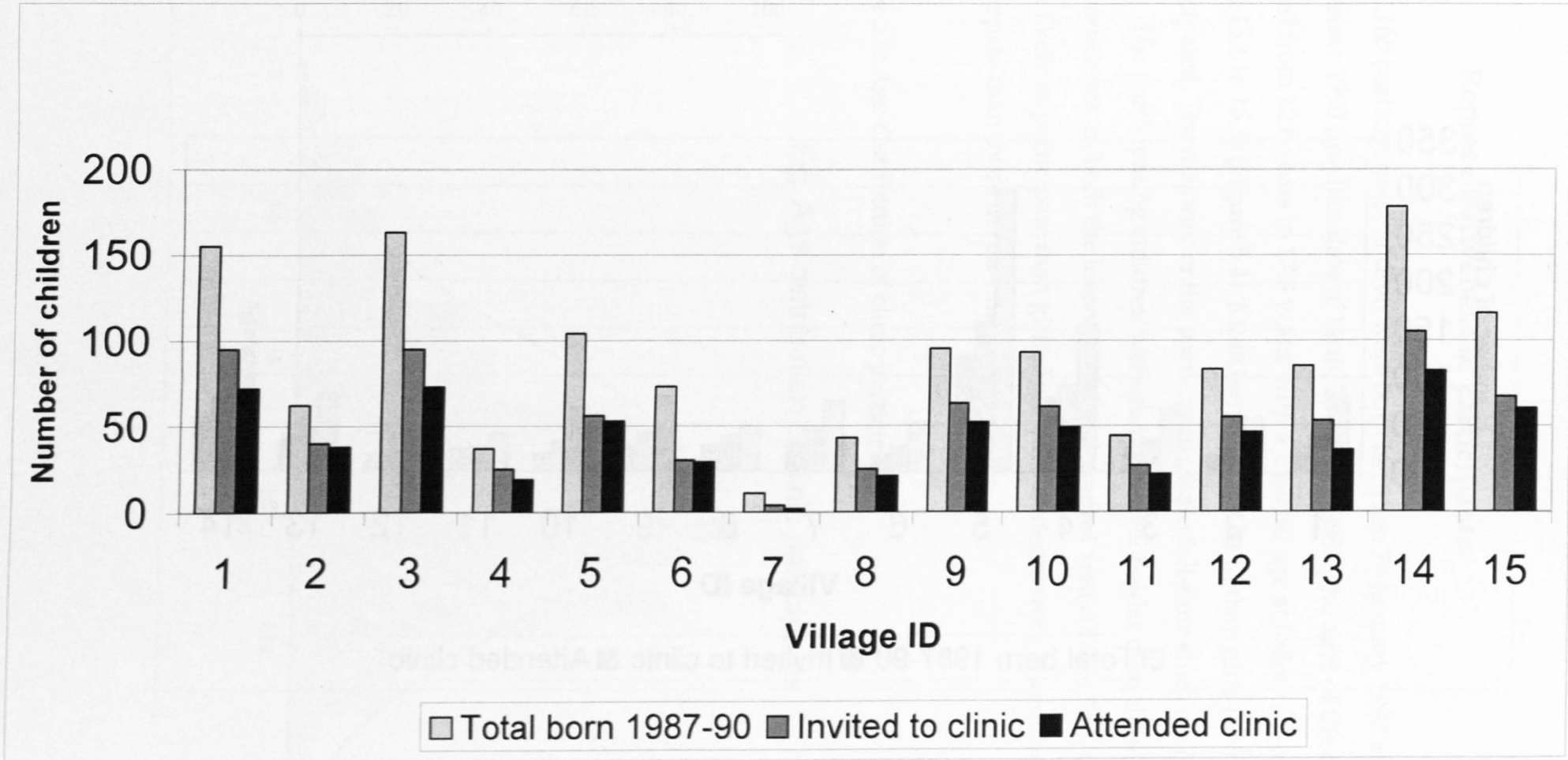
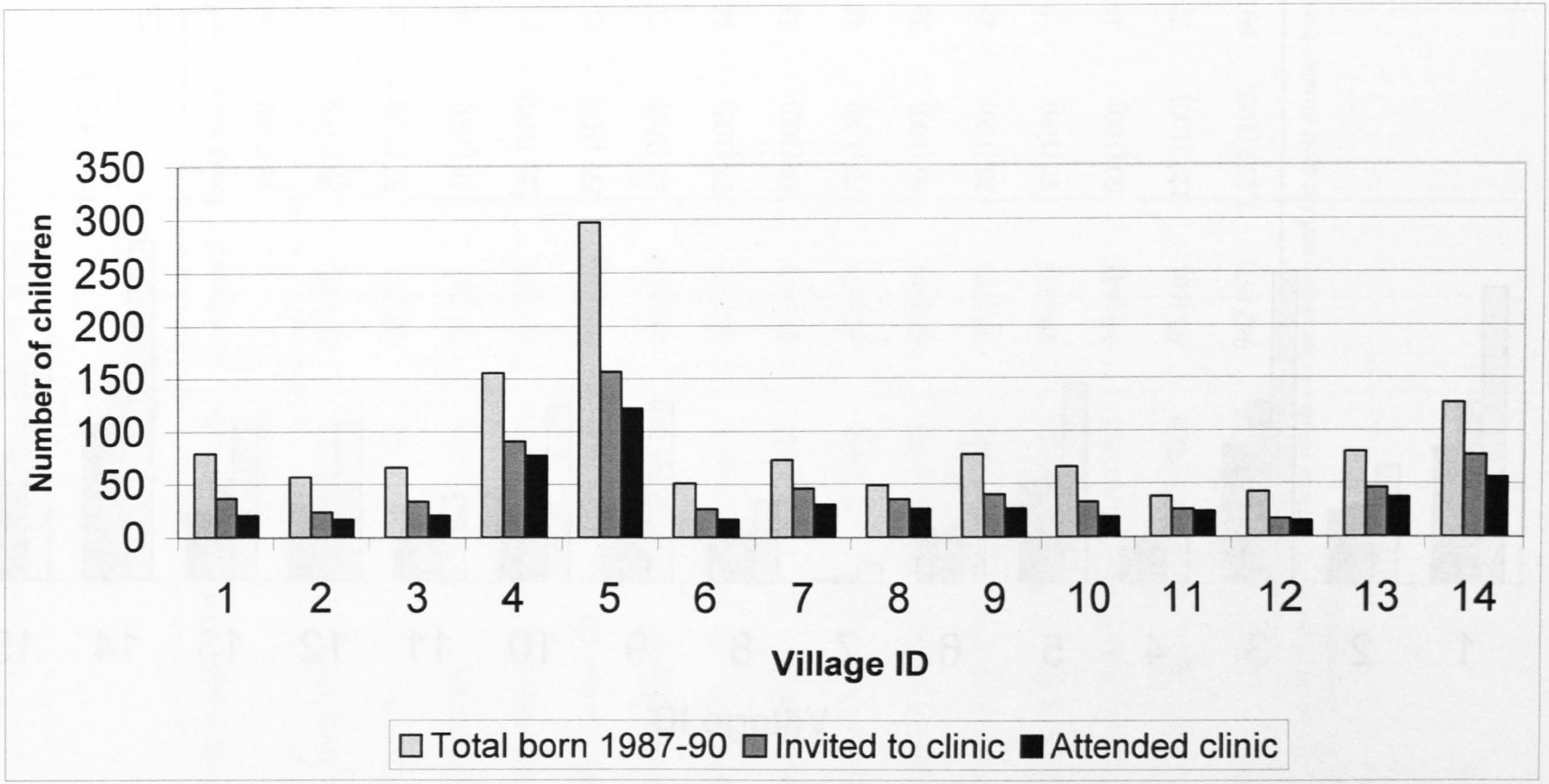


Figure 5.3: Children recruited from control villages



5.3. Representativeness of participants

The 1,165 participating children were born between 7th January 1987 and 15th December 1990 (median date of birth: 23rd June 1988). The ages of the children ranged from 12.8 years to 17.8 years, with a median age at follow up of 15.7 years (IQR: 15.1 to 16.3) (Figure 5.4). More boys (n=628; 54%) than girls (n=537; 46%) participated. The majority of the participants were full-time students (n=901; 77%). The participating children were comparatively older than those who did not participate, in both the intervention and control areas (Table 5.6). Boys were more likely to participate than girls. Full-time students were also more likely to participate than those in full-time employment.

Figure 5.4: Age distribution of clinic participants

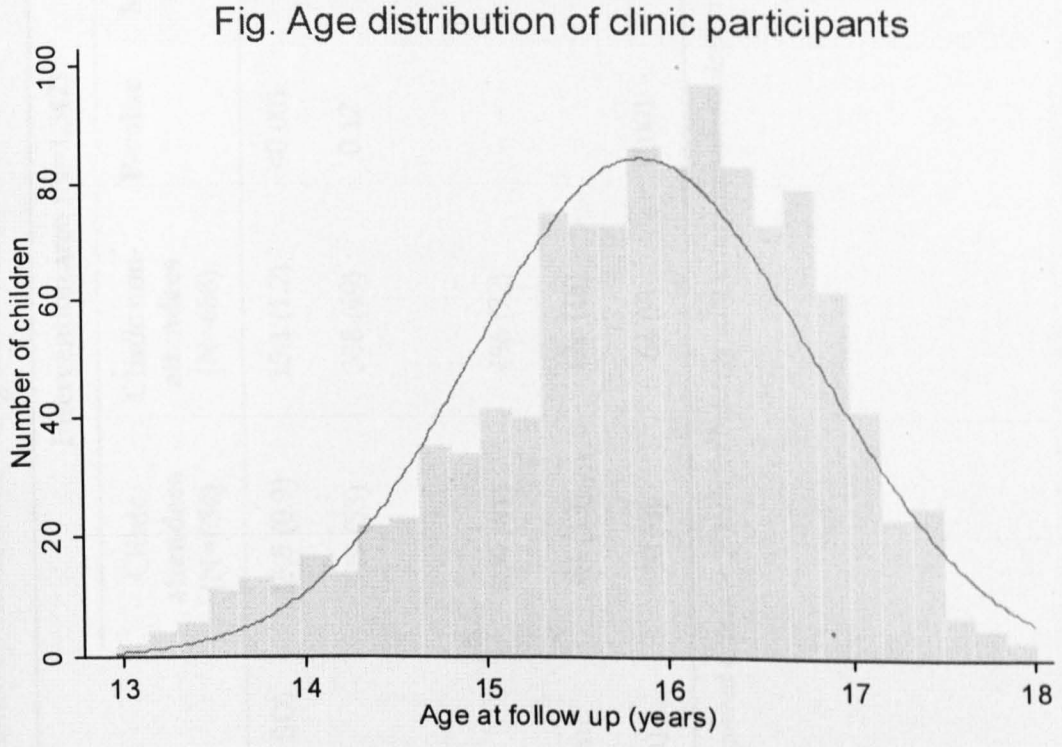


Table 5.6: *Characteristics of children attending clinical examination*

	Intervention area (N=1,342)				Control area (N=1,259)			
	Clinic attendees (N=654)	**Clinic non-attendees (N=688)**	**P-value**	**Missing values**	**Clinic attendees (N=511)**	**Clinic non-attendees (N=748)**	**P-value**	**Missing values**
Age in years (Mean, SD)	15.5 (0.9)	15.1 (1.2)	<0.001	0	15.7 (0.9)	15.3 (1.2)	<0.001	0
Male sex (N, %)	349 (53)	338 (49)	0.12	0	279 (55)	355 (47)	0.013	0
Occupation (N, %)								
Full-time student	529 (81)	496 (72)			372 (73)	507 (68)		
Full-time employment	84 (13)	126 (18)			96 (19)	194 (26)		
Others (neither, both)	40 (6)	64 (9)	0.001	3	43 (8)	44 (6)	0.012	3

P-values are based on unpaired t-tests or χ^2 tests for heterogeneity using appropriate degrees of freedom

5.4. Complete datasets used for analyses

Of the 1,165 children who attended the clinics, three girls reported being pregnant and they were excluded from the analyses at the outset. Among the remaining 1,162 children, I excluded those with missing data on key confounders i.e. socio-economic position (standard of living index variables), pubertal stage (testicular volume for boys and status of menstruation for girls), and body composition (height and weight; waist, hip, and arm circumferences; and four skinfolds) (Table 5.7). Eight children had fasted for less than eight hours and they were excluded from analyses of biochemical parameters only. In addition, those with fasting glucose above 7 mmol/L were excluded from analyses involving HOMA scores (n=5), as these are not considered to be reliable. Finally, each of the outcome measures was considered in turn, and after excluding those with missing data on relevant outcomes, complete datasets for analyses were available for the following numbers of children:

- Any outcome (N=1,120; 96%)
- Blood pressure (N=1,118; 96%)
- Lipid profile (N=1,050; 90%)
- Glucose/insulin (N=1,008; 87%)
- Arterial stiffness (N=862; 74%)

There were two main sources of missing data. Thirty-one (3%) children arrived at the clinic and provided a blood sample, but could not stay long enough to complete the questionnaire and physical examination. On the other hand, blood samples were unavailable for sixty-three (5%) children, either because they were unwilling or the venepuncture attempt was unsuccessful. Other than these reasons, there were two other reasons relating to specific data items. Insulin assay could not be carried out on the last batch of samples

(n=48; 5%), as there was a delay in receiving the reagents, and the team biochemist had moved on to a new position at the end of the study. Arterial stiffness measurements could not be carried out on 111 (25%) children. As explained in the methods section, the lack of continuous electricity supply in most of the villages meant that these assessments could not be completed successfully during the course of a clinic day. Some, but not all, of the participants returned subsequently to have their assessments completed.

Table 5.7: *Missing data among the 1,162 children considered for analyses*

Data item	Missing values (N, %)
Potential confounders	
Age	0 (0)
Sex	0 (0)
Testicular volume for boys (n=627)	23 (2)
Menstruation status for girls (n=535)	15 (1)
Standard of living index	34 (3)
Height, weight	31 (3)
Circumferences (waist, hip, mid-arm)	32 (3)
Skinfolds	32 (3)
Main outcome measures	
Blood pressure	33 (3)
Lipid profile	63 (5)
Glucose	63 (5)
Insulin	111 (10)
Arterial stiffness	292 (25)

5.5. Distributions of main variables

5.5.1. Village characteristics

Table 5.8 shows the key characteristics of the villages in the intervention and the control areas. These analyses were based on the data provided by the village heads. There were no important differences in indicators, which could be regarded as indices of development and urbanisation. There were comparatively more villages in the intervention area that had television sets in at least 50% of the households. The interpretation of this difference is not straightforward though, as the propensity to buy television may reflect not only material affluence, but also the quality of the signal reception (which can be quite variable in rural India).

Table 5.8: *Characteristics of the villages*

	N (%)		P-value
	Intervention area (N=15)	**Control area (N=14)**	
Population size (persons)			
<2,000	5 (33)	3 (21)	
2,000 - 5,000	7 (47)	7 (50)	
>5,000	3 (20)	4 (29)	0.7
Population density (persons per square mile)			
<200	4 (27)	4 (29)	
200 - 400	5 (33)	5 (36)	
>400	6 (40)	5 (36)	0.9
Any industry in village (yes)	6 (55)	5 (45)	0.8
Regular electricity in village (yes)	5 (33)	5 (36)	0.9
Bank in village (yes)	5 (33)	6 (43)	0.6
Health facility in village (yes)	5 (33)	8 (57)	0.2
At least 50% households have television (yes)	9 (60)	5 (36)	0.2

P-values are based on unpaired t-tests or χ^2 tests for heterogeneity using appropriate degrees of freedom

5.5.2. Distribution of key exposures

Table 5.9 shows the distribution of key exposures by area. The children in the control area were slightly older and less likely to be students. In all other respects, the exposure distribution was very similar. The performance of SLI in this setting appeared to be good, with the pattern of the SLI categories in-between the reported figures for rural and urban India, consistent with the peri-urban situation of the study villages.[227] Maternal literacy was low and most participants were students in both areas. Only two children consumed tobacco and both used the oral form; none smoked. One child reported currently consuming alcohol (local brand), while one other had consumed alcohol in the past.

There were 36 sib-pairs in the study sample, of which 23 sib-pairs were in the intervention area. Five of these sib-pairs were twins, four of whom were in the control area. Of the children participating in the study, 32 had moved from their village at the time of the baseline study (11 from the intervention area, and 21 from the control area). Of these, nine had moved to another village in the study area. All except one (who moved from control to the intervention village) had moved within their own category of area (i.e. intervention or control). Nine children had moved to Hyderabad city and 14 had moved to villages/towns outside the area. Of the 32 children had who moved, age at which they had moved was available for 24, of which 19 had moved after the age of 10 years. The majority of these were staying away for educational or employment reasons, but returned home frequently. Thirty of these children were in the final study sample used for analyses.

Table 5.9: *Distribution of key exposures in those completing clinic questionnaire*

	Intervention area (N=633)	Control area (N=498)	P-value*	Missing values
Mean (SD) age (years)	15.8 (0.9)	15.9 (0.9)	0.024	0
Male (N, %)	333 (53)	274 (55)	0.4	0
Pubertal stage (N, %)				
Early puberty	88 (14)	83 (17)		
Middle puberty	209 (33)	156 (31)		
Late puberty	184 (29)	132 (27)		
Post puberty	147 (23)	125 (25)	0.4	7
Standard of living index (SLI) (N, %)				
High SLI (25 - 67)	228 (36)	158 (32)		
Medium SLI (15 - 24)	291 (46)	239 (48)		
Low SLI (0 - 14)	111 (18)	101 (20)	0.2	3
*Own occupation (N, %)***				
Student/ vocational training	534 (84)	391 (79)		
Employed	66 (10)	53 (11)		
Unemployed/housework	33 (5)	53 (11)	0.002	1
Literate mother (N, %)†	58 (9)	57 (11)	0.2	0
Lifestyle (N, %)				
Consumed tobacco (ever)	0 (0)	2 (0)	0.2	1
Consume alcohol (ever)	0 (0)	2 (0)	0.2	1

N=1,131 (intervention area = 633; control area = 498)

** P-values are based on unpaired t-tests or χ^2 tests for heterogeneity using appropriate degrees of freedom*

*** Vocational training (n=15) and unemployed (n=36); † Literate/primary school (n=45), middle school (n=43); high school (n=16); intermediate level (n=9), and graduate (n=2)*

5.5.3. Distribution of outcome measures

Tables 5.10 to 5.13 show the sex-specific distribution of body composition and CVD risk factors, allowing assessment of the range of values observed and comparison of their average values. The normality of the data was assessed by computing histograms for each variable. Where data departed greatly from normal distribution, the histograms are shown, together with their distribution after appropriate transformation (Figures 5.5 and 5.6).

Examination of body composition data (Tables 5.10 and 5.11) showed that boys were, on average, seven centimetres taller than the girls. The mean BMI was greater in girls, although the waist circumference, waist-hip ratio and the central-peripheral skinfold ratio were all greater in boys. A much greater proportion of BMI in the girls was made of fat mass index, as expected. All values were in the plausible range, and generally within three to four standard deviations of the mean. Barring a few overweight children, the body composition data on the whole were suggestive of an extremely lean population.

Distributions of most body composition variables were good approximations of the normal distribution. For one measurement (fat mass index), distribution was not entirely normal, but it was not transformed in the interests of interpretability (Figure 5.5). The primary purpose of expressing fat mass as an index was to allow direct comparison with fat free mass index, and that advantage would have been lost after transformation. Moreover, the deviation from normality was not too great and fat mass index was used mainly as a covariate in regression models, rather than as an outcome. For models with fat mass index, regression diagnostic plots were carefully examined to confirm that the residuals were normally distributed.

Table 5.10: *Distribution of body size and composition in boys (N=607)*

	Mean (SD)	Median (IQR)	Range
Measured variables			
Standing height (mm)	1588 (85)	1597 (1534 to 1652)	1341 to 1794
Weight (Kg)	42.4 (8.0)	42.6 (36.7 to 47.5)	24.4 to 80.1
Waist circumference (mm)	620 (58)	599 (569 to 635)	486 to 983
Hip circumference (mm)	746 (63)	748 (708 to 785)	556 to 1018
Mid-arm circumference (mm)	217 (25)	217 (201 to 232)	157 to 337
Triceps skinfold (mm)	5.7 (2.0)	5.3 (4.5 to 6.4)	3.1 to 21.7
Biceps skinfold (mm)	3.3 (1.0)	3.1 (2.7 to 3.5)	2.0 to 13.9
Subscapular skinfold (mm)	6.7 (2.0)	6.4 (5.5 to 7.5)	2.4 to 22.6
Suprailiac skinfold (mm)	6.9 (2.6)	6.3 (5.5 to 7.7)	3.1 to 22.9
Derived variables			
BMI (kg/m^2)	16.7 (2.1)	16.4 (15.4 to 17.7)	11.6 to 29.3
Waist-hip ratio	0.83 (0.05)	0.83 (0.80 to 0.86)	0.63 to 1.01
Fat mass index (kg/m^2)	1.6 (0.7)	1.5 (1.2 to 1.9)	0.4 to 6.7
Fat free mass index (kg/m^2)	15.1 (1.5)	15.0 (14.1 to 16.0)	11.0 to 22.6
Central-peripheral skinfold ratio	1.53 (0.24)	1.52 (1.35 to 1.68)	0.95 to 2.45

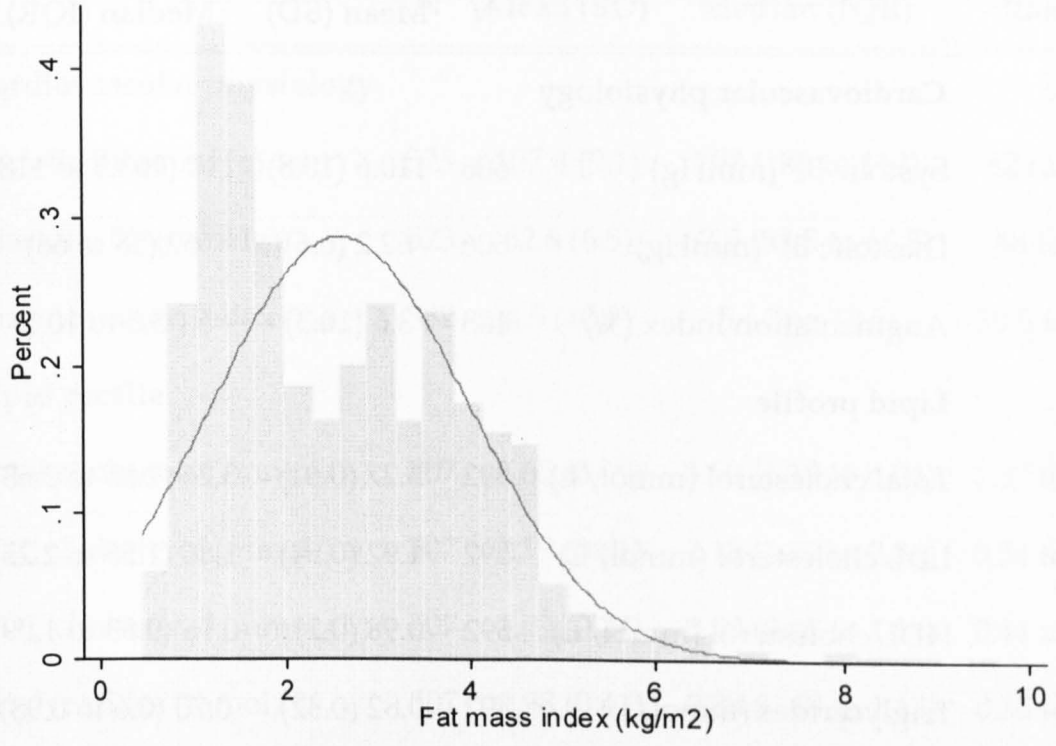
BMI = weight/height²; Fat mass index=fat mass/height²; Fat free mass index= fat free mass/height²
Fat mass and fat free mass values were calculated from four skinfolds using appropriate formulae
Central-peripheral skinfold ratio is the ratio of central (subscapular+suprailiac) to peripheral (biceps+triceps) skinfolds

Table 5.11: *Distribution of body size and composition in girls (N=523)*

	Mean (SD)	Median (IQR)	Range
Measured variables			
Standing height (mm)	1515 (57)	1516 (1476 to 1554)	1303 to 1670
Weight (Kg)	41.0 (6.1)	40.5 (37.1 to 43.8)	24.8 to 73.1
Waist circumference (mm)	606 (58)	599 (569 to 635)	486 to 983
Hip circumference (mm)	781 (53)	781 (750 to 810)	586 to 1055
Mid-arm circumference (mm)	222 (22)	221 (209 to 236)	162 to 340
Triceps skinfold (mm)	9.5 (2.7)	9.1 (7.5 to 11.1)	4.1 to 23.5
Biceps skinfold (mm)	4.9 (1.7)	4.5 (3.9 to 5.5)	2.4 to 18.8
Subscapular skinfold (mm)	10.3 (2.8)	9.9 (8.1 to 11.8)	4.2 to 22.1
Suprailiac skinfold (mm)	9.9 (3.0)	9.5 (7.7 to 11.7)	4.1 to 20.9
Derived variables			
BMI (kg/m^2)	17.8 (2.2)	17.6 (16.4 to 18.9)	12.5 to 29.4
Waist-hip ratio	0.78 (0.05)	0.77 (0.74 to 0.80)	0.65 to 1.03
Fat mass index (kg/m^2)	3.8 (1.1)	3.6 (3.0 to 4.3)	1.3 to 9.6
Fat free mass index (kg/m^2)	14.0 (1.3)	14.0 (13.2 to 14.8)	10.4 to 19.8
Central-peripheral skinfold ratio	1.42 (0.24)	1.40 (1.24 to 1.54)	0.88 to 2.17

BMI = weight/height²; Fat mass index=fat mass/height²; Fat free mass index= fat free mass/height²
Fat mass and fat free mass values were calculated from four skinfolds using appropriate formulae
Central-peripheral skinfold ratio is the ratio of central (subscapular+suprailiac) to peripheral (biceps+triceps) skinfolds

Figure 5.5: Histogram showing frequency distribution of fat mass index



Tables 5.12 and 5.13 show the sex-specific distribution of CVD risk factors. The mean systolic blood pressure was higher in the boys, while the augmentation index was higher in girls. The levels of lipids were higher in the girls. Fasting insulin and HOMA score were also slightly higher in the girls, as compared to the boys.

The distributions of triglycerides, insulin and HOMA score were positively skewed, and were therefore transformed using natural logarithms (Figure 5.6). The log-transformed data were reasonable approximations of the normal distribution (Figure 5.6). In the presentation of subsequent results for these logged data, geometric means are presented and regression coefficients refer to the logged data.

Table 5.12: *Distribution of CVD risk factors in boys*

	N	Mean (SD)	Median (IQR)	Range
Cardiovascular physiology				
Systolic BP (mmHg)	606	110.6 (10.8)	110 (103.5 to 118)	80 to 143.5
Diastolic BP (mmHg)	606	62.2 (6.6)	62 (58 to 66)	45 to 85.5
Augmentation index (%)	488	3.6 (10.3)	5 (-3.5 to 10.5)	-40.5 to 31.5
Lipid profile				
Total cholesterol (mmol/L)	592	3.27 (0.61)	3.24 (2.85 to 3.63)	1.92 to 5.80
LDL cholesterol (mmol/L)	592	1.92 (0.54)	1.86 (1.55 to 2.25)	0.42 to 4.26
HDL cholesterol (mmol/L)	592	0.98 (0.21)	0.96 (0.83 to 1.09)	0.54 to 1.94
Triglycerides (mmol/L)	592	0.82 (0.32)	0.75 (0.6 to 0.98)	0.31 to 2.82
Glucose homeostasis				
Glucose (mmol/L)	592	4.7 (0.6)	4.7 (4.4 to 5.1)	1.4 to 8.4
Insulin (mU/L)	563	18.8 (10.8)	16.8 (10.6 to 25.2)	2 to 89.2
Insulin resistance (HOMA)	561	4.0 (2.5)	3.5 (2.2 to 5.1)	0.4 to 22.0

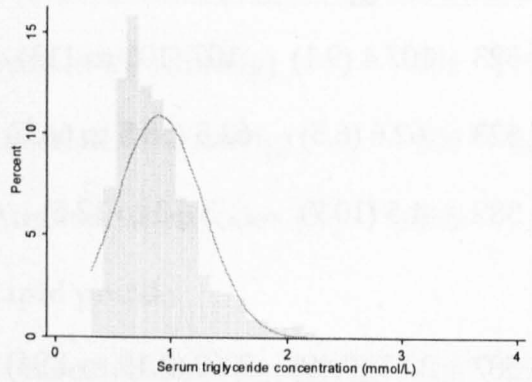
Cardiovascular physiology measures are means of two measurements
Lipid profile and glucose homeostasis measurements are on fasting (over 8 hours) blood samples
LDL is low density lipoprotein and HDL is high density lipoprotein

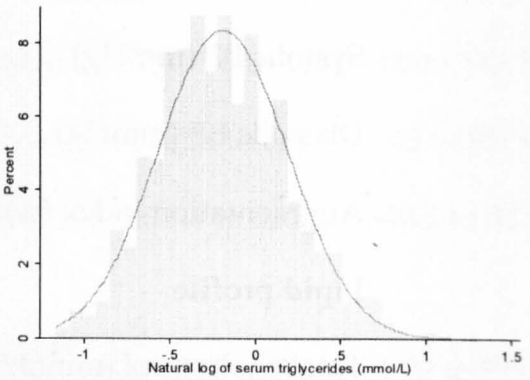
Table 5.13: *Distribution of CVD risk factors in girls*

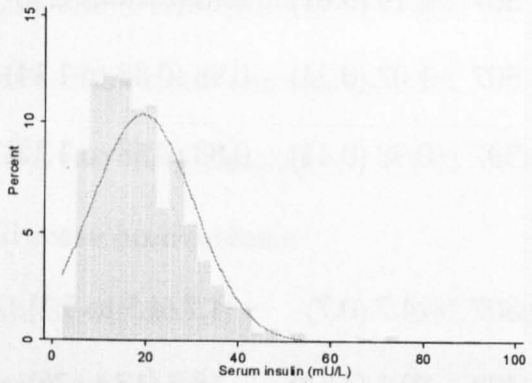
	N	Mean (SD)	Median (IQR)	Range
Cardiovascular physiology				
Systolic BP (mmHg)	523	107.4 (9.1)	107 (101 to 113)	82 to 133
Diastolic BP (mmHg)	523	62.6 (6.5)	62.5 (58.5 to 66.5)	48 to 84
Augmentation index (%)	382	4.5 (10.7)	5 (-3 to 12.5)	-30.5 to 31.5
Lipid profile				
Total cholesterol (mmol/L)	507	3.65 (0.69)	3.60 (3.19 to 4.04)	1.37 to 6.84
LDL cholesterol (mmol/L)	507	2.19 (0.61)	2.15 (1.77 to 2.56)	0.54 to 4.44
HDL cholesterol (mmol/L)	507	1.02 (0.24)	0.98 (0.85 to 1.14)	0.44 to 2.14
Triglycerides (mmol/L)	507	0.98 (0.41)	0.88 (0.68 to 1.15)	0.35 to 3.36
Glucose homeostasis				
Glucose (mmol/L)	507	4.7 (0.7)	4.7 (4.3 to 5.1)	2.1 to 8.8
Insulin (mU/L)	488	20.1 (11.4)	18.3 (12 to 26)	2.3 to 84
Insulin resistance (HOMA)	485	4.2 (2.7)	3.7 (2.4 to 5.3)	0.5 to 24.9

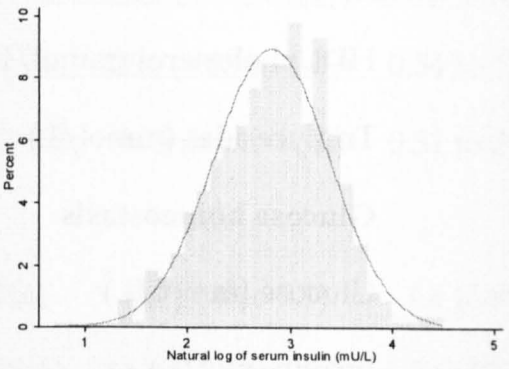
Cardiovascular physiology measures are means of two measurements
Lipid profile and glucose homeostasis measurements are on fasting (over 8 hours) blood samples
LDL is low density lipoprotein and HDL is high density lipoprotein

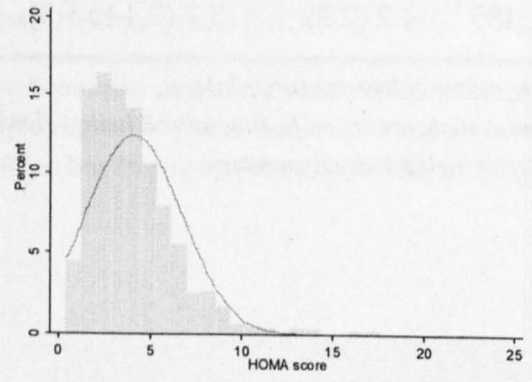
Figure 5.6: Histograms showing frequency distributions of the values of serum triglycerides, insulin and HOMA, before and after log transformation

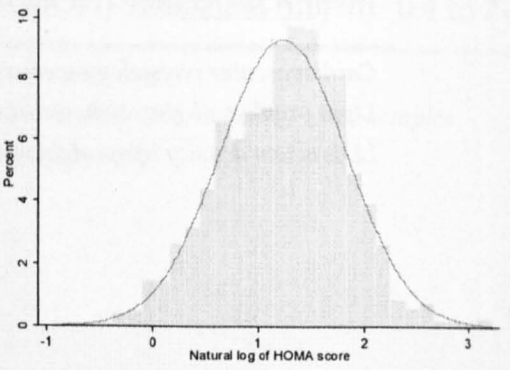












5.6. Clustering of outcomes within villages

Since participants from the same village are likely to be more similar to each other than those from another village, clustering of variables within villages was examined. Intraclass correlation coefficient (ICC), estimated by one-way analysis of variance, was used as a measure of clustering. Table 5.14 shows that clustering for the majority of the variables was low (ICC < 0.05) to moderate (ICC: 0.05 - 0.1).[302] ICCs for all the outcomes except glucose were within the range used for sample size calculations (i.e. 0.01 to 0.1).

Table 5.14: *Village level clustering of outcomes (29 clusters)*

	N	ICC (95% CI)
Body size and composition		
Standing height (mm)	1,130	0 (0 to 0.01)
BMI (kg/m^2)	1,130	0 (0 to 0.02)
Mid-arm circumference (mm)	1,130	0.01 (0 to 0.03)
Waist circumference (mm)	1,130	0 (0 to 0.02)
Hip circumference (mm)	1,130	0.01 (0 to 0.02)
Waist hip ratio	1,130	0.01 (0 to 0.03)
Fat mass index (kg/m^2)	1,130	0.01 (0 to 0.03)
Fat free mass index (kg/m^2)	1,130	0.03 (0 to 0.06)
Central-peripheral skinfold ratio	1,130	0.07 (0.02 to 0.13)
Cardiovascular physiology		
Systolic BP (mmHg)	1,129	0.05 (0.01 to 0.10)
Diastolic BP (mmHg)	1,129	0.06 (0.01 to 0.10)
Augmentation index (%)	870	0.08 (0.02 to 0.14)
Lipid profile		
Total cholesterol (mmol/L)	1,099	0.03 (0 to 0.06)
LDL cholesterol (mmol/L)	1,099	0.04 (0 to 0.07)
HDL cholesterol (mmol/L)	1,099	0.09 (0.03 to 0.15)
Triglycerides (mmol/L)	1,099	0.02 (0 to 0.04)
Glucose homeostasis		
Glucose (mmol/L)	1,099	0.14 (0.06 to 0.23)
Insulin (mU/L)	1,051	0.07 (0.02 to 0.12)
HOMA score	1,046	0.06 (0.01 to 0.10)

ICC is intraclass correlation coefficient and was estimated by one-way analysis of variance

5.7. Reproducibility of clinic measurements

The reproducibility of clinic examinations was assessed by repeating the measurements on the same participant 1-3 weeks apart. The same observer carried out the repeat measurements. Results shown in Table 5.15 suggest that the reproducibility of measurements was high. Among anthropometry, hip circumference had low reproducibility. As adequate privacy could not be arranged at all the clinics, children were often reluctant to change into the loose garments provided for the study. Consequently, measurements had to be carried out sometimes over the normal dress of the participant. Due to the low reproducibility of hip circumference, I decided not to use waist-hip circumference in further analyses.

Blood pressure reproducibility in this study appears to be low when compared to studies conducted in adults, but is higher than what is generally achieved in studies in children.[234;303] The reproducibility of blood pressure in children tends to be much lower because of the relatively higher day-to-day physiological variation.[303] Reproducibility of radial augmentation index measurements was also comparable to other studies, and improved further on exclusion of two extreme values.[123;304]

The reproducibility of biochemical assays was tested by splitting the collected blood sample into two (with the analyst 'blind' to the identity of the split sample). The results are presented in Table 5.16, and show high levels of reproducibility for all the parameters. Quality control for biochemical assays was also ensured by running internal quality control assays each time the samples were processed and by external quality control that was maintained for the duration of the study. Samples for external quality control of blood sugar, lipids and insulin were sent to the team biochemist every two months, and the quality of reporting was compared to other laboratories participating in the

arrangement. The reports were consistently high. Elimination of inter-observer sources of error by use of a single observer for each of the measurements in this study may be a reason why reproducibility was generally quite high.

Table 5.15: *Reproducibility of clinic examinations*

	N	Within-subject standard deviation	Intraclass correlation coefficient
Anthropometry			
Height (mm)	38	11.5	0.982 (0.971, 0.993)
Weight (kg)	38	0.34	0.998 (0.997, 0.999)
Waist circumference (mm)	38	0.72	0.999 (0.999, 0.999)
Hip circumference (mm)	38	22.8	0.866 (0.786, 0.946)
Triceps skinfold (mm)	38	0.08	0.999 (0.999, 0.999)
Biceps skinfold (mm)	38	0.06	0.999 (0.998, 0.999)
Subscapular skinfold (mm)	38	0.11	0.999 (0.998, 0.999)
Suprailiac skinfold (mm)	38	0.12	0.999 (0.998, 0.999)
Mid-arm circumference (mm)	38	2.1	0.991 (0.985, 0.997)
Cardiovascular physiology			
Systolic BP (mmHg)	36	3.0	0.880 (0.0807, 0.954)
Diastolic BP (mmHg)	36	2.7	0.857 (0.769, 0.944)
Radial augmentation index (%)	80	3.8	0.854 (0.795, 0.914)
Radial augmentation index (%)*	78	3.1	0.900 (0.858, 0.943)

Intraclass correlation coefficient estimated by one-way analysis of variance by taking two readings on the same participant 1-3 weeks apart; same observer took the first and second readings

Values are means of multiple readings for the following: two readings (waist and hip circumference, systolic and diastolic blood pressure and augmentation index), and three readings (skinfolds)

**Excluding two participants with extreme values*

Table 5.16: *Reproducibility of biochemical assays*

	N	Within-subject standard deviation	Intraclass correlation coefficient
Total cholesterol (mmol/L)	36	0.05	0.995 (0.991, 0.998)
LDL cholesterol (mmol/L)	36	0.07	0.985 (0.975, 0.995)
HDL cholesterol (mmol/L)	36	0.05	0.943 (0.908, 0.980)
Triglycerides (mmol/L)	36	0.03	0.992 (0.987, 0.997)
Glucose (mmol/L)	36	0.11	0.971 (0.953, 0.990)
Insulin (mU/L)	27	2.57	0.966 (0.941, 0.992)

Intraclass correlation coefficient estimated by one-way analysis of variance by splitting the collected blood sample in two; both assays conducted by same analyst on the same day but blind to the identity

5.8. Validity of clinic assessments

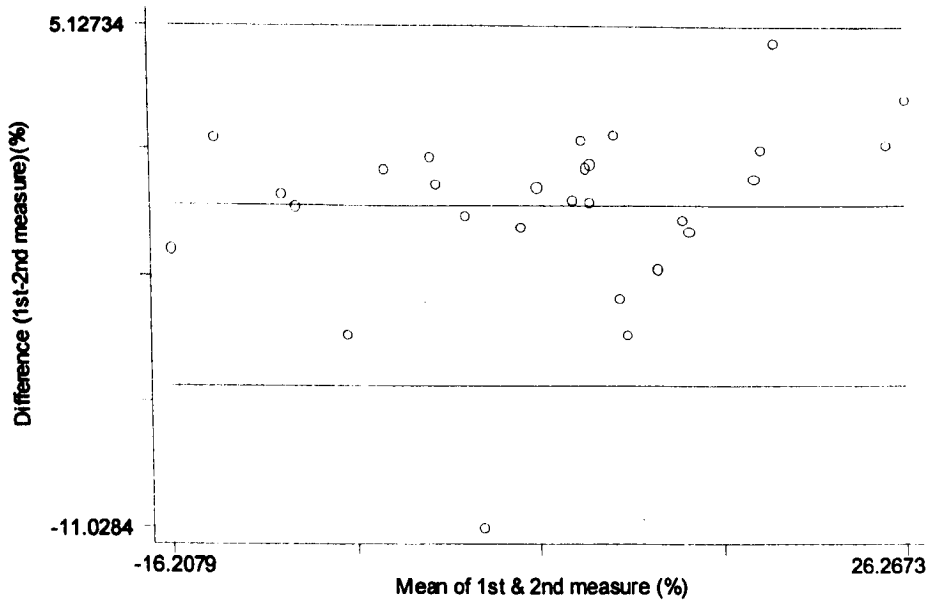
As explained in the methods section, validity (method comparison) studies were conducted for arterial stiffness measurement and testicular self-assessment, and the results for these studies are presented below.

5.8.1. Arterial stiffness study

Augmentation index was measured twice in all children (n=28) appearing for examination over two consecutive days. The measurements were taken once by the study observer and once by an independent expert (the `gold standard'). The observers alternated in taking the first measurement to eliminate bias that may arise from the order of measurement. The mean of two best readings for each observer was used for comparison. The pairs of assessments were conducted within few minutes of each other, and the observers were not blinded to each other's measurements.

The within-subject standard deviation was 2.08, with an intraclass correlation coefficient (ICC) of 0.964. The within-subject standard deviation is lower, and the ICC higher, as compared to the figures from the reproducibility study (see Table 5.15). This may be because unlike the readings for this study (taken within few minutes of each other), the readings for the reproducibility study were taken 1-3 weeks apart. Greater differences in measurement conditions, and consequently greater physiological variation, could have resulted in greater within-subject standard deviation. These data also suggest that error arising from differences in the observer technique was low (possibly because recordings were automated).

Figure 5.7: Difference in readings of radial augmentation index plotted against the average (1st measure=study observer; 2nd measure=gold standard)



The mean difference between the measurements taken by the two observers was -0.69% (95% CI: -1.82 to 0.44), with limits of agreement ranging from -6.50% to 5.13%. On dropping one extreme value, mean difference was reduced to -0.31% (95% CI: -1.15% to 0.54%), with limits of agreement from -4.55 to 3.94. The limits

of agreement (the 95% reference range for the mean difference) suggest that, in the majority of the cases, the readings taken by the two observers were within 4 - 5% of each other. The Bland-Altman plot (differences between pairs of measurements plotted against their means) did not show any evidence of a systematic increase or decrease in difference according to the magnitude of the reading (Figure 5.7).[273]

5.8.2. Testicular self-assessment study

This study was conducted in an all-boys, secondary school (ages 12 years and over). All the children were invited to participate, and everybody agreed (N=101; 100% response rate). The boys were asked to go behind a curtain with Prader's orchidometer and assess their testicular volume. The self-reports of the boys were compared to the repeat measurements conducted by the trained observer ('gold standard'), who was unaware of the earlier findings.

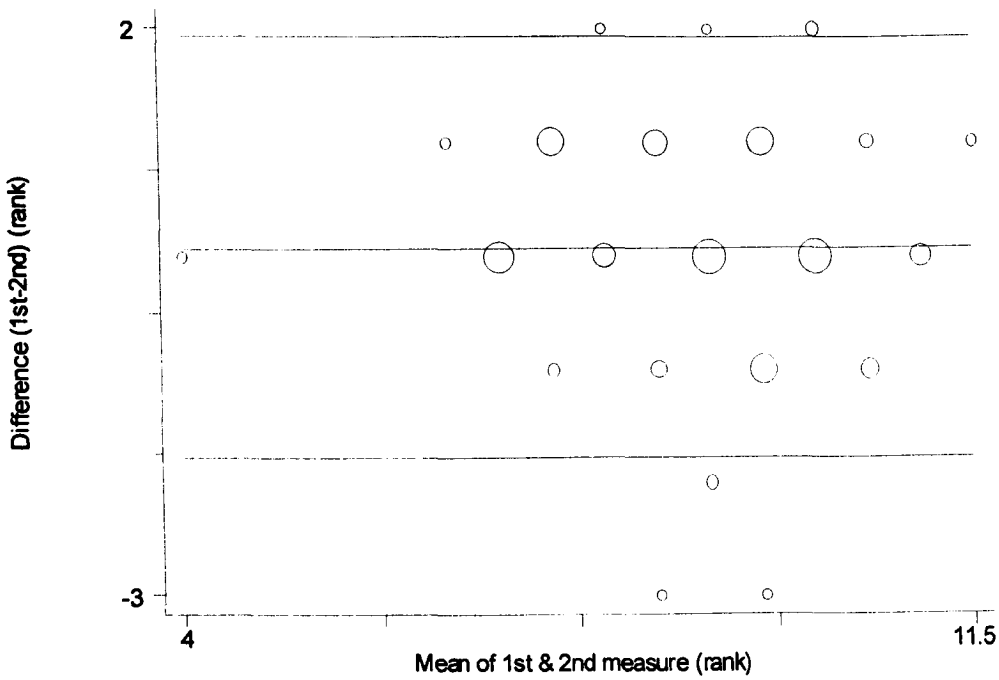
Forty-eight percent of the ranks agreed completely, while a further 45% were within one rank of each other (limits of agreement: -1.8 to 1.9) (Table 5.17). The means of self- and observer-reported Prader's model ranks were identical (8.9), with a mean difference of 0.07 (95% CI: -0.11 to 0.25). The Bland-Altman plot (differences between pairs of measurements plotted against their means) did not show any evidence of a systematic bias in self-reporting of testicular size (Figure 5.8).[273] As the children were in a narrow maturational age range, most of the observations were clustered within a few ranks only. Kappa statistic, normally a useful test for reproducibility of categorical variables, does not perform well under such circumstances (since it estimates agreement over and above that expected by chance) and hence is not reported.[305]

Table 5.17: *Difference in self-reported and directly observed ranks of testicular model*

Difference in ranks (observed minus self-reported)	**N (%)**
-2	4 (4)
-1	27 (27)
0	48 (48)
1	18 (18)
2	2 (2)
3	2 (2)

N=101

Figure 5.8: Difference in rank of Prader's orchidometer testicular model plotted against the average (1st measure=self-reported; 2nd measure=directly observed)



5.9. Summary and implications

The results presented in this chapter had several implications for further analyses.

Firstly, the analyses of the baseline study data confirmed that the intervention had been effective. The 61g difference in birthweight was consistent with other estimates in the literature. The analysis was conducted at an area level; since the uptake of nutrition supplement in ICDS programme typically ranges from 50-60%, the true increase in birthweight (among those actually taking up the supplement) is likely to be higher.

Secondly, since the selection of villages was on a geographical (rather than randomised) basis, it was important to ensure that the villages were similar in all other respects. Analyses of the village level data confirmed that the villages in the intervention and control areas were very similar in their indices of development and urbanisation. This implied that if any important differences in CVD risk factors were found, they could reasonably be attributed to the earlier intervention rather than differences in current environment. Despite lack of obvious differences, adjustment for indices of village urbanisation were made in analyses, to account for unmeasured confounding and thereby obtain more robust estimates of the effect of intervention.

Thirdly, the recruitment process appeared to have been fairly successful in obtaining a representative sample of the trial population from the two areas. The comparisons between participants and non-participants, at each of the study stages and across villages, did not show any evidence that the recruited sample was biased, either in relation to the baseline study sample or between the two arms of the study. The data items were also fairly complete. The standard deviations of the outcome variables and their village level intraclass correlation

coefficients were comparable to the estimates used in the sample size calculations. On that basis and the final sample size achieved in the two arms, the study appeared to be adequately powered to detect important differences in the main outcomes (if present).

Fourth, power of the study depends not only on the sample size but also on the degree of measurement error. Reproducibility studies showed that clinic measurements were highly reliable. Inter-observer error was non-existent in this study and intra-observer error appeared mostly to be a function of the physiological variability in the outcome. The only exception to this was the low reproducibility of hip circumference, which could not be measured consistently due to practical reasons (dress changing). Consequently, hip measurements (i.e. waist-hip ratio) were dropped from further analyses. The two novel techniques (arterial stiffness and self-assessed testicular volume) were also validated in this setting.

Fifth, the distribution of potential confounders was fairly equal between the two arms of the study. There were no important differences in the distribution of age, sex or pubertal stage of the participants. Only two children reported consuming tobacco or alcohol, which meant that these exposures could be ignored in the analyses. Although the levels of SLI were also similar in the two areas, it was controlled for in further analyses to take account of unmeasured social confounding. The distribution of SLI was consistent with the national data (NFHS) and evenly spread across the population (unlike maternal education or child occupation that were heavily skewed), confirming it as the most suitable measure of socio-economic position in further analyses.

Sixth, most clinic measurements were normally distributed. Values of triglyceride levels, fasting insulin and HOMA score were positively skewed, but

were natural lognormal. Further analyses were therefore carried out with logged data and geometric means presented. Fat mass index was only approximately normal, but was left untransformed in favour of ease of interpretation. In further analyses involving fat mass index, regression diagnostic plots were checked to ensure that residuals were normally distributed and that the models were robust. Data exploration showed some extreme values, for a few of the variables, that could not be attributed to data entry errors. Since none of these values were implausible, they were retained in the analyses and their influence on the effect estimates in the final models was assessed.

Seventh, the data confirmed that the study population, on the whole, was very lean. Since the validity of commonly used measures such as BMI in lean populations is questionable, further analyses to select the most appropriate body composition variables in this population was important.

Finally, several variables showed important levels of village level clustering. Since this violated the assumption of independence of the participants, use of analytical techniques that take account of clustering (such as use of robust standard errors) was warranted in further analyses. The proportion of participants who were siblings was too small for meaningful cluster analyses (with household as cluster). A small number of participating children lived outside their baseline village. While the urbanisation status of current village was used for those who migrated within the study area, urbanisation status of the baseline village was used for those very few children who were residing outside the study villages (current data were not available). The effect of potential misclassification of current environment, and that of inclusion of sib-pairs, was estimated by excluding data on these children from the final models, and comparing the effect estimates.

CHAPTER 6. DETERMINANTS OF CVD RISK FACTORS

6.1. Introduction and aims

This chapter presents the results of analyses conducted to establish the determinants of CVD risk factors in this population. The purpose of these analyses was twofold. Firstly, although the determinants of CVD risk factors in high-risk populations (populations from high-income countries, and to some extent, urban populations from low-income countries) are well known, much less is known about low-risk populations (rural populations from low-income countries).[3] Secondly, the establishment of these determinants would allow selection of a reasonable number of appropriate covariates that could be taken forward to the main analyses (association of supplemental nutrition with CVD risk) to fit the most parsimonious models.

Cross-sectional analyses were carried out to investigate the association between potential determinants and CVD risk factors. The study population from the intervention and control areas was taken as a whole, ignoring the presence of intervention. As explained in the methods section, the determinants were selected on the basis of existing literature and relevance to the setting, and examined together in logically constructed domains. Three domains were considered: (a) physiological determinants, (b) current socio-economic factors, and (c) body size and composition. The socio-economic factors were examined separately from the body composition variables, since any association between socio-economic factors and CVD risk factors could be mediated, at least in part, through changes in body composition. However, in order to study the relationship of CVD risk factors with components of body composition, it was important to clarify the specificity of available body composition indices, since their performance in lean adolescent populations is not well established.[238;249;250]

6.2. Body size and composition

The aim of this part of analyses was to identify the most specific indices for various components of body composition (i.e. fat mass, fat free mass and central adiposity). It was anticipated that such indices would correlate less strongly with each other, and possibly correlate differentially with different categories of CVD risk factors e.g. blood pressure or serum lipids (suggesting specificity).

Table 6.1 shows that pairwise correlation coefficients between various indices of body size and composition. The indices, on the whole, appear to be highly correlated with each other. Fat mass index was correlated, relatively less strongly, with fat free mass index. BMI and mid-arm circumference were correlated equally strongly with fat mass index and fat free mass index, suggesting their role as composite indices of fat and fat free mass in this population. Surprisingly both waist circumference and central-peripheral skinfold ratio were correlated more strongly with fat free mass index, rather than fat mass index. If these were indices of central adiposity, one would expect them to correlate more with overall adiposity. A possible explanation for this observation is that in this extremely lean population, both these indices were surrogate measures of body size rather than central adiposity.

Table 6.2 shows that height was positively correlated with blood pressure and inversely correlated with augmentation index. Both these associations are well established.[117;274;275;306;307] Fat mass index correlated more strongly with blood lipids, while fat free mass index correlated more strongly with blood pressure. The lack of association between fat mass and blood pressure in this generally lean population is not altogether surprising. The correlation between fat free mass and blood pressure has also been noted by others, and may suggest a need

for a relatively higher blood pressure to maintain satisfactory perfusion in individuals with greater amount of fat free mass.[277;306;308;309]

Table 6.1: *Pairwise correlation coefficients for indices of body size and composition*

	Ht	BMI	MAC	WC	FMI	FFMI	CPSR
Ht	1						
BMI	0.09*	1					
MAC	0.34	0.89	1				
WC	0.40	0.76	0.75	1			
FMI	-0.24	0.74	0.60	0.43	1		
FFMI	0.35	0.79	0.75	0.73	0.17	1	
CPSR	0.39	0.12	0.23	0.25	-0.10*	0.27	1

Ht – height (mm); BMI – body mass index (kg/m²); MAC – midarm circumference (mm); WC – waist circumference (mm); FMI – fat mass index (kg/m²); FFMI – fat free mass index (kg/m²); CPSR – Central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

*All p- values (with Bonnferoni correction) <0.001 except those marked *: BMI-Height (p=0.067) and Fat mass index-skinfold ratio (p=0.019)*

HOMA score and insulin levels were moderately correlated with both fat mass and fat free mass. HOMA score and insulin levels were not strongly correlated with central-peripheral skinfold ratio, suggesting that anthropometry may be a crude method of assessing central adiposity in some populations.[238;250;257;277] The relatively stronger association of waist circumference with fasting insulin probably arose from its strong association with both fat and fat free mass indices (which were in turn correlated with fasting insulin), rather than its specificity for central adiposity.

On the basis of these investigations and criteria outlined earlier, fat mass index and fat free mass index were chosen as the more specific indices of body composition in this population. BMI and mid-arm circumference were rejected for being non-specific composite measures. The choice between waist circumference and central-peripheral skinfolds (as the better measure for central adiposity) was less clear-cut, as both appeared to be non-specific in this

population. To resolve this issue, I fitted linear regression models for CVD risk factors that contained height, fat mass and fat free mass index as explanatory variables, along with age, sex and pubertal status. I then added waist circumference and central-peripheral skinfold ratio to the models, separately. For the majority of outcomes, central-peripheral skinfold ratio explained additional variation (as assessed by R^2 values), and even appeared to be an important independent predictor of outcome for some variables, while waist circumference was non-contributory. The lack of an independent effect of waist circumference may be attributable to its high correlation with fat and fat free mass indices. However, it was unclear whether central-skinfold ratio was acting as an index of central adiposity or capturing residual variation in fat and fat free mass. With this limitation in mind, the central-peripheral skinfold ratio was chosen over waist circumference as an additional measure of body composition. Subsequent models were therefore adjusted for height and fat mass index, fat free mass index and central-peripheral skinfold ratio (as measures of body size and composition).

Table 6.2: *Pairwise correlation coefficients between anthropometric measures and cardiovascular disease risk factors*

	SBP	DBP	AIx	T-chol	L-chol	H-chol	Trigs	Glu	Ins	HOMA
Ht	0.36**	0.14**	-0.23**	-0.16**	-0.11	-0.06	-0.17**	-0.02	0.07	0.06
BMI	0.29**	0.22**	-0.08	0.17**	0.14**	-0.01	0.17**	-0.13**	0.18**	0.15**
MAC	0.40**	0.26**	-0.13*	0.08	0.08	-0.04	0.11*	-0.10	0.19**	0.16**
WC	0.33**	0.19**	-0.13*	0.05	0.05	-0.06	0.07	-0.11	0.14**	0.11
FMI	0.04	0.14**	-0.03	0.28**	0.24**	0.03	0.24**	-0.10	0.14**	0.10
FFMI	0.40**	0.18**	-0.08	-0.02	-0.01	-0.04	0.02	-0.11	0.15**	0.12*
CPSR	0.30**	0.14**	-0.09	-0.12*	-0.11	-0.08	0.01	-0.00	0.11	0.10

Ht – height (mm); BMI – body mass index (kg/m^2); MAC – midarm circumference (mm); WC – waist circumference (mm); FMI – fat mass index (kg/m^2); FFMI – fat free mass index (kg/m^2); CPSR – Central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds); SBP – systolic blood pressure (mmHg); DBP – diastolic blood pressure (mmHg); AIx – radial augmentation index (%); T-chol – total cholesterol (mmol/L); L-chol – low density lipoprotein cholesterol (mmol/L); H-chol – high density lipoprotein cholesterol (mmol/L); Trigs – triglycerides (mmol/L); Glu – fasting glucose (mmol/L); Ins – fasting insulin (mU/L); and HOMA – HOMA score for insulin resistance

*P values are indicated by ** (<0.001) and * (<0.01) and incorporate bonferroni correction*

6.3. Determinants of body size and composition

Table 6.3 shows the results of regression analyses for each of the body size and composition outcomes. Age and pubertal stage were positively and independently associated with all the outcomes, highlighting the importance of adjusting for pubertal stage in adolescence. Boys had higher levels of all the indices except fat mass index, which was higher in girls. Standard of living index was positively associated with the majority of indices, including fat mass index. Unlike high-income countries, affluence and fat mass index tend to be positively associated in low-income countries.[140;310;311] There was a weak inverse association between SLI and central-peripheral skinfold ratio, which could be a chance finding or reflect the non-specificity of central adiposity measures in this population. Village urbanisation (as assessed by the size of the population) had little effect on the body composition indices, suggesting relative homogeneity in the economic development of these villages.

Table 6.3: *Determinants of body size and composition*

	Height (cm)	BMI (kg/m²)	Mid-arm circumference (mm)	Waist circumference (mm)	Fat mass index (kg/m²)	Fat free mass index (kg/m²)	Central-peripheral skinfolds ratio
Age (per year)	1.90 (1.26, 2.54); p<0.001	0.38 (0.21, 0.54); p<0.001	6.09 (4.32, 7.86); p<0.001	9.95 (4.70, 15.20); p=0.001	0.11 (0.04, 0.18); p=0.003	0.27 (0.16, 0.38); p<0.001	0.06 (0.04, 0.78); p<0.001
Sex (female *vs* male)	-10.72 (-12.1, -9.4); p<0.001	0.10 (-0.34, 0.54); p=0.7	-5.90 (-10.62, -1.19); p=0.016	-33.39 (-45.4, -21.4); p<0.001	1.83 (1.64, 2.01); p<0.001	-1.74 (-2.02, -1.44); p<0.001	-0.15 (-0.20, -0.09); p<0.001
Pubertal stage (per stage)	2.02 (1.48, 2.56); p<0.001	0.64 (0.44, 0.84); p<0.001	7.31 (5.02, 9.59); p<0.001	11.10 (5.39, 16.80); p<0.001	0.17 (0.08, 0.27); p=0.001	0.47 (0.35, 0.58); p<0.001	0.03 (0.01, 0.06); p=0.01
SLI (per category)	1.28 (0.59, 1.98); p=0.001	0.31 (0.10, 0.52); p=0.006	2.65 (0.15, 5.14); p=0.038	9.88 (4.80, 14.96); p<0.001	0.15 (0.05, 0.25); p=0.004	0.16 (0.02, 0.29); p=0.023	-0.03 (-0.06, -01); p=0.015
Urbanisation (per category)	-0.09 (-0.88, 0.70); p=0.8	-0.07 (-0.25, 0.13); p=0.5	-0.59 (-2.80, 1.62); p=0.6	-3.32 (-9.91, 3.26); p=0.3	-0.09 (-0.19, 0.02); p=0.093	0.02 (-0.12, 0.16); p=0.8	0.02 (-0.02, 0.05); p=0.3

Linear regression models were used with robust standard errors to account for village level clustering; the determinants (age, sex, pubertal stage, SLI and urbanisation) are adjusted for each other

Central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds); Pubertal stage (4 stages: early puberty, mid-puberty, late puberty, and post-pubertal); SLI – standard of living index (3 categories: high, medium and low); Urbanisation – total village population (3 categories: <2,000, 2,000-5,000, >5,000)

6.4. Determinants of CVD risk factors

6.4.1. Cardiovascular physiology

Table 6.4 shows the determinants of outcomes related to cardiovascular physiology. Age and pubertal stage were important determinants of blood pressure, but not augmentation index. The weak inverse association observed with age was, at least in part, due to the inverse association of height with augmentation index (in model adjusted for height, coefficient for age was: 0.59 (95% CI: -1.26 to 1.42; p=0.2)). Room temperature was inversely associated with blood pressure, and heart rate was inversely associated with augmentation index. Time of the day at which the examination was carried out did not affect blood pressure, and was therefore not included in the final models.

Standard of living index was positively associated with diastolic blood pressure and augmentation index, while systolic blood pressure showed a weak positive association with urbanisation. Height was positively associated with blood pressure and negatively with augmentation index, as noted in other studies.[117;274;275;306;307] Fat free mass index was an important determinant of systolic and diastolic blood pressure, while fat mass index showed a weak association with diastolic blood pressure only. Systolic (and to some extent diastolic) blood pressure was positively associated with central-peripheral skinfold ratio, although the relevance of this association was unclear. Augmentation index was not associated with any of the body composition measures.

Table 6.4: *Determinants of cardiovascular physiology*

Variable group	Systolic BP (mmHg)	Diastolic BP (mmHg)	Augmentation index (%)
	(N=1,118)	(N=1,118)	(N=862)
Physiological			
Age (per year)	2.17 (1.50, 2.85); p<0.001	0.88 (0.43, 1.33); p<0.001	-0.30 (-1.26, 0.66); p=0.5
Sex (female *vs* male)	-5.82 (-8.10, -3.54); p<0.001	-0.91 (-2.37, 0.54); p=0.2	5.14 (1.00, 9.28); p=0.017
Pubertal stage (per stage)	1.94 (1.12, 2.76); p<0.001	1.13 (0.48, 1.79); p=0.001	-1.46 (-3.33, 0.41); p=0.1
Room temperature (per degree celsius)	-0.25 (-0.49, -0.00); p=0.046	-0.41 (-0.55, -0.26); p<0.001	NA
Heart rate (per beat/minute)	NA	NA	-0.29 (-0.34, -0.23); p<0.001
Socio-economic			
SLI (per category)	0.63 (-0.13, 1.34); p=0.1	0.58 (0.12, 1.04); p=0.015	-1.34 (-2.54, -0.13); p=0.031
Urbanisation (per category)	0.94 (-0.06, 1.94); p=0.063	0.43 (-0.33, 1.20); p=0.3	-0.07 (-2.28, 2.13); p=0.9
Body composition			
Height (SDS)	2.08 (1.42, 2.74); p<0.001	0.53 (0.00, 1.05); p=0.049	-3.42 (-4.10, -2.73); p<0.001
Fat mass index (SDS)	0.09 (-1.42, 1.60); p=0.9	0.97 (0.00, 1.93); p=0.049	-0.91 (-2.14, 0.32); p=0.1
Fat free mass index (SDS)	2.88 (2.01, 3.74); p<0.001	0.55 (0.01, 1.10); p=0.047	-0.10 (-1.17, 0.98); p=0.9
Central-peripheral skinfolds ratio (SDS)	1.29 (0.54, 2.04); p=0.001	0.38 (-0.07, 0.82); p=0.094	-0.00 (-0.91, 0.89); p=1.0

Linear regression models with robust standard errors were used to account for village level clustering; variables in the physiological group are adjusted for other variables in the group; variables in the socio-economic and body size and composition groups are adjusted for other variables within their own group and all variables in the physiological group

Central-peripheral skinfolds ratio (subscapular+suprailiac/biceps+triceps skinfolds); Pubertal stage (4 stages: early puberty, mid-puberty, late puberty, and post-pubertal); SLI – standard of living index (3 categories: high, medium and low); Urbanisation – total village population (3 categories: <2,000, 2,000-5,000, >5,000); SDS (standard deviation scores) for body composition variables were calculated using the variable mean and standard deviation for the study population

6.4.2. Lipid profile

Blood lipids showed a weak association with age, and no association with pubertal stage (Table 6.5). However, for the same age and pubertal stage, girls were likely to have higher levels of lipids than boys. One possible explanation for this could be the higher levels of adiposity in girls. In models that were adjusted for body size and composition, the association of lipids with sex was attenuated. In models adjusted for age, pubertal stage, height, fat mass index, fat free mass index and central-peripheral skinfolds ratio, the coefficients for association of sex with lipids (in mmol/L, with logged coefficients for triglycerides) were as follows: (a) total cholesterol 0.30 (95% CI: 0.05 to 0.55; p=0.02), (b) LDL-cholesterol 0.20 (95% CI: 0.02 to 0.40; p=0.042), (c) HDL-cholesterol 0.08 (95% CI: -0.01 to 0.17; p=0.073), and (d) triglycerides 0.07 (95% CI: -0.05 to 0.19); p=0.3).

Cholesterols but not triglycerides were higher in those with a higher standard of living index, and in those living in an urbanised environment. Further adjustment for body size and composition made little difference to these associations, suggesting that smaller changes in diet associated with affluence may result in altered cholesterol levels, without manifesting a change in body size and composition, at least in early stages.

Higher fat mass index was associated with higher levels of total and LDL cholesterol, and triglycerides, although the effect sizes were modest. Similarly, higher central-peripheral skinfold ratio showed modest associations with HDL cholesterol and triglycerides. The overall picture was consistent with an association between adiposity and blood lipid levels in this population.

Table 6.5: *Determinants of lipid metabolism (N=1,050)*

Variable group	Total cholesterol (mmol/L)	LDL cholesterol (mmol/L)	HDL cholesterol (mmol/L)	Triglycerides (mmol/L)
Physiological				
Age (per year)	0.02 (-0.02, 0.07); p=0.4	0.02 (-0.03, 0.06); p=0.2	0.02 (-0.00, 0.04); p=0.082	-0.03 (-0.05, -0.00); p=0.019
Sex (female *vs* male)	0.48 (-0.30, 0.60); p<0.001	0.31 (0.21, 0.42); p<0.001	0.01 (-0.01, 0.14); p=0.075	0.15 (0.09, 0.21); p<0.001
Pubertal stage (per stage)	-0.04 (-0.10, 0.03); p=0.3	-0.02 (-0.08, 0.03); p=0.4	-0.01 (-0.05, 0.02); p=0.4	0.01 (-0.02, 0.04); p=0.7
Socio-economic				
SLI (per category)	0.07 (-0.00, 0.13); p=0.054	0.02 (-0.01, 0.1); p=0.078	0.02 (0.00, 0.04); p=0.044	-0.02 (-0.06, 0.03); p=0.5
Urbanisation (per category)	0.10 (0.05, 0.16); p=0.001	0.03 (-0.00, 0.13); p=0.051	0.04 (0.00, 0.08); p=0.033	-0.02 (-0.06, 0.02); p=0.4
Body composition				
Height (SDS)	-0.04 (-0.10, 0.03); p=0.3	-0.01 (-0.06, 0.05); p=0.7	-0.01 (-0.02, 0.01); p=0.4	-0.05 (-0.09, -0.01); p=0.007
Fat mass index (SDS)	0.08 (-0.01, 0.17); p=0.069	0.04 (-0.01, 0.15); p=0.086	-0.02 (-0.05, 0.01); p=0.1	0.06 (0.00, 0.11); p=0.039
Fat free mass index (SDS)	0.05 (-0.02, 0.09); p=0.2	0.02 (-0.04, 0.08); p=0.5	0.01 (-0.02, 0.03); p=0.6	0.03 (-0.00, 0.06); p=0.088
Central-peripheral skinfolds ratio (SDS)	-0.05 (-0.10, 0.01); p=0.1	-0.05 (-0.10, 0.00); p=0.066	-0.02 (-0.03, -0.00); 0.048	0.04 (0.00, 0.08); p=0.03

Linear regression models with robust standard errors were used to account for village level clustering; variables in the physiological group are adjusted for other variables in the group; variables in the socio-economic and body size and composition groups are adjusted for other variables within their own group and all variables in the physiological group

Central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds); Pubertal stage (4 stages: early puberty, mid-puberty, late puberty, and post-pubertal); SLI – standard of living index (3 categories: high, medium and low); Urbanisation – total village population (3 categories: <2,000, 2,000-5,000, >5,000)

SDS (standard deviation scores) for body composition variables were calculated using the variable mean and standard deviation for the study population

6.4.3. Glucose homeostasis

The measures of glucose homeostasis (fasting glucose, insulin and HOMA score) showed little change with age, sex or pubertal stage (Table 6.6). The inverse association between glucose and pubertal stage was probably a chance finding, owing to the multiplicity of outcomes tested in these models. Socio-economic indicators also showed no association with these outcomes. Among body composition variables, insulin and HOMA score were more likely to be associated with higher fat free mass index and, to some extent, central-peripheral skinfold ratio, rather than with fat mass index.

6.4.4. Additional analyses

In analyses of all the above outcomes, certain pre-specified additional models were examined. Among physiological covariates, the effect of the time of day when the examination was carried out was assessed (as a continuous variable and in tertiles). Maternal education (literate or not) and child's own occupation (student or otherwise) were examined as additional indices of socio-economic position, and village population density (<200, 200-400, >400 persons per square mile) and percentage of households with television sets (>50% or not) were examined as additional indices of village urbanisation. In body composition, models with BMI, mid-arm circumference and waist circumference were compared to models with selected variables (fat mass index, fat free mass index and central-peripheral skinfold ratio). Final models were also compared to models which excluded second child of the sib-pairs (n=36), and those with village urbanisation indices for baseline rather than current village of residence (n=23). None of the above models altered the final effect estimates to any appreciable degree, and so are not presented.

Table 6.6: *Determinants of glucose homeostasis*

Variable group	Glucose (mmol/L)	Insulin (mU/L)	HOMA score
	(N=1,008)	(N=1,008)	(N=1,003)
Physiological			
Age (per year)	-0.01 (-0.06, 0.35); p=0.5	0.04 (-0.03, 0.10); p=0.2	0.03 (-0.03, 0.09); p=0.3
Sex (female *vs* male)	0.09 (-0.01, 0.19); p=0.085	0.03 (-0.10, 0.16); p=0.7	0.04 (-0.09, 0.17); p=0.6
Pubertal stage (per stage)	-0.09 (-0.14, -0.03); p=0.004	0.03 (-0.04, 0.10); p=0.5	0.01 (-0.06, 0.08); p=0.8
Socio-economic			
SLI (per category)	-0.04 (-0.12, 0.03); p=0.2	0.05 (0.00, 0.10); p=0.7	0.05 (-0.00, 0.10); p=0.08
Urbanisation (per category)	0.03 (-0.16, 0.21); p=0.8	0.02 (-0.10, 0.15); p=0.7	0.02 (-0.10, 0.15); p=0.7
Body size and composition			
Height (SDS)	0.0 (-0.05, 0.06); p=0.9	0.03 (-0.00, 0.08); p=0.1	0.03 (-0.01, 0.09); p=0.1
Fat mass index (SDS)	-0.06 (-0.16, 0.03); p=0.2	0.03 (-0.06, 0.12); p=0.5	0.02 (-0.08, 0.12); p=0.7
Fat free mass index (SDS)	-0.04 (-0.11, 0.02); p=0.1	0.08 (0.00, 0.15); p=0.04	0.07 (-0.00, 0.15); p=0.074
Central-peripheral skinfolds ratio (SDS)	0.02 (-0.03, 0.07); p=0.5	0.05 (-0.02, 0.12); p=0.2	0.04 (-0.02, 0.12); p=0.1

Linear regression models with robust standard errors were used to account for village level clustering; variables in the physiological group are adjusted for other variables in the group; variables in the socio-economic and body size and composition groups are adjusted for other variables within their own group and all variables in the physiological group

Central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds); Pubertal stage (4 stages: early puberty, mid-puberty, late puberty, and post-pubertal); SLI - standard of living index (3 categories: high, medium and low); Urbanisation - total village population (3 categories: <2,000, 2,000-5,000, >5,000)

SDS (standard deviation scores) for body composition variables were calculated using the variable mean and standard deviation for the study population

6.5. Summary and implications

These analyses established the key determinants of CVD risk in this lean adolescent population from rural south India. Age and pubertal stage were important independent determinants of blood pressure, but not of blood lipids, fasting glucose or insulin. Blood pressure was lower in girls, but lipids were higher, as compared to the boys. Room temperature was inversely associated with blood pressure, and heart rate was inversely associated with augmentation index, as expected.

Socio-economic position, as assessed by the standard of living index, was only weakly associated with blood pressure, lipids and insulin resistance. The associations were mainly in the positive direction (i.e. higher socio-economic position was associated with higher blood pressure, lipids, and insulin resistance). Greater village urbanisation was also positively associated with lipids, and to a lesser extent, blood pressure.

Height was positively associated with blood pressure, and inversely associated with augmentation index, as expected. Height was not associated with lipids or insulin resistance. Blood pressure and, to a smaller extent, insulin resistance were positively associated with fat free mass index, while lipids were positively associated with fat mass index. Central-peripheral skinfold ratio was weakly and variably associated with CVD risk factors.

Alternative personal and village level socio-economic determinants of CVD risk (maternal education, child's own occupation, population density and television coverage in the village) did not explain any additional variation in CVD risk, as compared to the pre-specified indices (standard of living index and village population). Fat mass index, fat free mass index and central-peripheral skinfold ratio were more specific determinants of body composition (adiposity, lean mass

and central adiposity) than BMI, mid-arm circumference and waist circumference, and between them explained maximal variation in CVD risk in this lean adolescent population.

The results of these analyses were used as the basis for choosing the covariates to fit the most parsimonious models in the next stage of the analyses (i.e. role of supplemental nutrition in CVD risk), presented in the following chapter.

CHAPTER 7. SUPPLEMENTAL NUTRITION AND CVD RISK FACTORS

7.1. Introduction and aims

The literature review suggested a role for early life nutrition in cardiovascular disease risk. The majority of evidence, however, was indirect: from animal experiments and human studies using anthropometry (especially birthweight) as a proxy for early nutrition. Direct evidence, based on dietary intake, came from few observational studies in high-income countries (where food intake is generally adequate), two extreme natural experiments of starvation (hard to generalise), and one small randomised controlled trial.[24-27] The randomised trial, conducted in a middle-income country (Guatemala), found no evidence of an effect of supplemental nutrition in pregnancy and childhood on blood pressure and fasting glucose in young adulthood.[26;27] The small sample size and the modest background prevalence of undernutrition may be potential explanations for the null finding. The Guatemala study also did not have data on potential intermediary measures of cardiovascular disease risk, important in view of the relatively young age of the participants.

The present study has attempted to address this gap in evidence by collecting data on intermediary and classical risk factors of cardiovascular disease, in a reasonably sized sample of adolescents, born within a trial of nutrition supplementation conducted in an area with high prevalence of undernutrition. The absence of maternal smoking and relative socio-economic homogeneity of the population were additional unanticipated benefits of conducting a study in this setting, affording an opportunity to obtain relatively unbiased estimates of the effects of early nutrition on CVD risk.

The previous chapter reported the results of cross-sectional analyses examining the determinants of some of the cardiovascular disease risk factors. The study population was analysed as a whole, ignoring whether the participants were born in the experimental or control area. This chapter presents the results of the analyses of the associations between area of birth (nutritional intervention or control) and current levels of cardiovascular disease risk factors. The crude associations were cumulatively adjusted for potential confounding factors to get robust estimates of the effect of intervention. Only a limited number of pre-specified models were examined to reduce the likelihood of spurious findings.

The basic model included age, sex, pubertal stage, room temperature (blood pressure only) and heart rate (augmentation index only) as covariates. In the next model, socio-economic factors (standard of living index and village population) were added to the basic model. In the third model, height was added to the preceding model. Finally, body composition variables (fat mass index, fat free mass index and central-peripheral skinfold ratio) were added to arrive at the final model. Where body composition variables were included as covariates, they were expressed as standard deviation scores, using the means and standard deviations for the study population. Only one pre-specified interaction (between intervention and sex) was examined, and this was done in the final models. The potentially central role of insulin resistance in this population was examined by assessing the prevalence and determinants of Insulin Resistance Syndrome, and by investigating the effect of insulin resistance (HOMA score) on other CVD risk factors, and their associations with supplemental nutrition. As explained earlier, linear regression modelling with robust standard errors (to take account of village level clustering) was used. The impacts of household level clustering (within sib-pairs) and potential misclassification of current environment were examined by exclusions from the final models.

7.2. Supplemental nutrition and puberty

Improved nutrition can potentially result in an earlier onset of puberty and thus be on the pathway of any association between supplemental nutrition and CVD risk factors.[286;287] Therefore, prior to introducing pubertal status as a covariate in regression models, the effect of supplemental nutrition on pubertal stage was examined. The mean pubertal stage (graded 1-4) was 2.6 (SD 1.0) in both the intervention and control areas. There was no association between nutrition intervention and pubertal stage of the participants (tested by ordinal logistic regression) or the odds of being in mid-puberty (stage 2) (tested by binary logistic regression) (Table 7.1).

Table 7.1: *Association of supplemental nutrition with puberty*

	Mean difference (95% CI) in pubertal stage (control minus intervention)	**Odds ratio (95% CI) for being in mid-puberty** (ref category, intervention=1)
Crude	-0.02 (-0.29, 0.25); p=0.9	0.91 (0.64, 1.30); p=0.6
Adjusted for age, sex	-0.01 (-0.46, 0.45); p=1	0.85 (0.55, 1.30); p=0.4

Ordinal logistic regression was used to estimate mean difference and binary logistic regression was used to estimate the odds ratio for stage 2

Puberty (4 stages): early puberty, mid-puberty, late puberty, and post-pubertal

7.3. Supplemental nutrition and body size and composition

Tables 7.2 and 7.3 show the association between supplemental nutrition and child's current anthropometry. Children born in the intervention area were 10 mm taller than children in the control area, and this difference in height was not attenuated by further adjustments for age-sex distribution, pubertal stage or socio-economic position of the households. The BMI and mid-arm circumference were greater in the control area, and the associations strengthened after adjustment for current height. There was no difference in fat mass index, but fat free mass index was greater in the control population. Adjustments for current

socio-economic position (Table 7.3) or occupation of the child (data not presented) made no difference to the strength of this association. There was no difference in the measures of central adiposity (i.e. waist circumference and central-peripheral skinfold ratio) between the two areas.

Table 7.2: *Unadjusted associations of supplemental nutrition with anthropometry*

	Mean (SD)		Mean difference (95% CI)
	Intervention (N=624)	**Control (N=496)**	**(control minus intervention)**
Height (mm)	1559 (83)	1549 (82)	-10.0 (-18.7, -1.4); p=0.024
BMI (kg/m²)	17.1 (2.0)	17.3 (2.4)	0.27 (-0.08, 0.61); p=0.1
Midarm circumference (mm)	219 (22)	220 (26)	1.87 (-2.12, 5.86); p=0.3
Waist circumference (mm)	614 (52)	613 (59)	-0.41 (-8.8, 7.98); p=0.9
Fat mass index (kg/m²)	2.6 (1.3)	2.6 (1.5)	-0.02 (-0.23, 0.19); p=0.8
Fat free mass index (kg/m²)	14.5 (1.4)	14.8 (1.6)	0.29 (0.01, 0.57); p=0.043
Central-peripheral skinfold ratio	1.48 (0.25)	1.47 (0.25)	-0.01 (-0.06, 0.05); p=0.8

Linear regression models with robust standard errors were used to estimate the mean difference
Central-peripheral skinfold ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

Table 7.3: *Multivariable association between supplemental nutrition and anthropometry*

	Mean difference (control minus intervention)		
Adjusted variables	**β-coefficient**	**95% CI**	**P-value**
Height (cm)			
Age, sex	-1.43	-2.20, -0.67	0.001
+ Pubertal stage	-1.41	-2.33, -0.49	0.004
+ Current socio-economic	-1.36	-2.31, -0.41	0.007
BMI (kg/m²)			
Age, sex	0.23	-0.07, 0.53	0.1
+ Pubertal stage	0.24	-0.08, 0.56	0.1
+ Current socio-economic	0.28	0.02, 0.54	0.033
+ Current socio-economic, height	0.34	0.11, 0.58	0.006
Midarm circumference (mm)			
Age, sex	1.09	-2.0, 4.18	0.4
+ Pubertal stage	1.17	-2.30, 4.64	0.5
+ Current socio-economic	1.54	-1.73, 4.80	0.3
+ Current socio-economic, height	3.12	0.55, 5.67	0.019
Waist circumference (mm)			
Age, sex	-2.1	-10.7, 6.53	0.6
+ Pubertal stage	-2.0	-11.3, 7.31	0.7
+ Current socio-economic	-0.54	-8.61, 7.53	0.9
+ Current socio-economic, height	2.83	-3.55, 9.21	0.4
Fat mass index (kg/m²)			
Age, sex	0.01	-0.14, 0.15	0.9
+ Pubertal stage	0.01	-0.15, 0.16	0.9
+ Current socio-economic	0.04	-0.10, 0.18	0.5
+ Current socio-economic, height	0.06	-0.07, 0.19	0.4
Fat free mass index (kg/m²)			
Age, sex	0.23	0.02, 0.43	0.03
+ Pubertal stage	0.23	0.03, 0.43	0.029
+ Current socio-economic	0.24	0.06, 0.43	0.012
+ Current socio-economic, height	0.28	0.11, 0.46	0.003
Central-peripheral skinfold ratio			
Age, sex	-0.02	-0.07, 0.03	0.5
+ Pubertal stage	-0.02	-0.06, 0.03	0.5
+ Current socio-economic	-0.02	-0.08, 0.03	0.4
+ Current socio-economic, height	-0.01	-0.07, 0.05	0.7

Linear regression models with robust standard errors were used

Variables in current socio-economic group are household's SLI (standard of living index: high, medium, low) and village urbanisation (village population: <2,000, 2,000-5,000, >5,000); Pubertal stages are early puberty, mid-puberty, late puberty, and post-pubertal; Central-peripheral skinfold ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

7.4. Supplemental nutrition and CVD risk factors

Tables 7.4 to 7.7 show the crude and multiply adjusted associations between supplemental nutrition and CVD risk factors.

Systolic blood pressure was lower in the intervention area, although there was little evidence to refute the null hypothesis (Table 7.5). Adjustment for current socio-economic circumstances attenuated the association to some extent, as did adjustment for body composition. Adjustment for height, on the other hand, strengthened the association. Diastolic blood pressure was similar across the two groups. Augmentation index showed a statistically robust association with supplemental nutrition that was not attenuated by multivariable adjustments. A greater augmentation index in the control area suggests relatively stiffer arteries and consequently greater risk of cardiovascular disease.

Lipids showed no evidence for an association with supplemental nutrition, with or without adjustment for potential confounders (Table 7.6). Fasting glucose levels were also not associated with supplemental nutrition (Table 7.7). However, fasting insulin and HOMA score were strongly associated with supplemental nutrition. The effect estimates for fasting insulin and HOMA are identical due to near perfect correlation between the two. Children in the control area were more insulin resistant, suggesting greater risk of cardiovascular diseases in the future. Adjustments for height or body composition made little difference to the effect sizes of these associations. The values presented in the tables for insulin and HOMA scores are logged regression coefficients; these equate to 20% lower levels in the intervention group for models adjusted for baseline variables and current socio-economic circumstances. Exclusion of the small numbers of sib-pairs (one of the two) and migrant children made no material difference to the results (data not presented).

Table 7.4 *Unadjusted associations of supplemental nutrition with CVD risk factors*

		Mean (SD)		Mean difference (95% CI)
	N	Intervention	Control	(control minus intervention)
Cardiovascular physiology				
Systolic BP (mmHg)	1,118	108.7 (10.3)	109.6 (10.0)	0.83 (-1.44, 3.11); p=0.5
Diastolic BP (mmHg)	1,118	62.5 (6.5)	62.2 (6.5)	-0.23 (-1.70, 1.25); p=0.8
Augmentation index (%)	862	2.5 (11.4)	5.6 (9.1)	3.16 (0.8, 5.51); p=0.011
Lipid profile				
Total cholesterol (mmol/L)	1,050	3.45 (0.69)	3.45 (0.67)	-0.00 (-0.12, 0.12); p=1.0
LDL cholesterol (mmol/L)	1,050	2.05 (0.60)	2.04 (0.59)	-0.02 (-0.13, 0.09); p=0.8
HDL cholesterol (mmol/L)	1,050	0.99 (0.23)	1.00 (0.22)	0.01 (-0.05, 0.06); p=0.7
Triglycerides* (mmol/L)	1,050	0.82 (1.46)	0.83 (1.46)	0.02 (-0.04, 0.07); p=0.6
Glucose homeostasis				
Glucose (mmol/L)	1,008	4.68 (0.58)	4.72 (0.74)	0.03 (-0.23, 0.29); p=0.8
Insulin* (mU/L)	1,008	15.36 (1.76)	18.45 (1.72)	0.18 (0.03, 0.33); p=0.02
HOMA score*	1,003	3.16 (1.80)	3.79 (1.78)	0.18 (0.04, 0.32); p=0.015

Linear regression models with robust standard errors were used to estimate the mean differences

**Values are geometric means and SDs; mean differences are logged regression coefficients*

Table 7.5: *Multivariable associations between supplemental nutrition and cardiovascular physiology measures*

	Mean difference (control minus intervention)		
	β-coefficient	**95% CI**	**P-value**
Systolic BP, mmHg (N=1,118)			
Baseline variables only	0.86	-0.72, 2.45	0.2
+ Current socio-economic	0.59	-1.11, 2.29	0.4
+ Current socio-economic, height	1.10	-0.68, 2.87	0.2
+ Current socio-economic, height, body composition	0.64	-0.99, 2.27	0.4
Diastolic BP, mmHg (N=1,118)			
Baseline variables only	0.21	-0.81, 1.23	0.7
+ Current socio-economic	0.08	-0.90, 1.07	0.8
+ Current socio-economic, height	0.21	-0.77, 1.19	0.6
+ Current socio-economic, height, body composition	0.11	-0.85, 1.07	0.8
Augmentation index, % (N=862)			
Baseline variables only	3.29	0.74, 5.84	0.013
+ Current socio-economic	3.30	0.96, 5.65	0.008
+ Current socio-economic, height	2.84	0.39, 5.30	0.025
+ Current socio-economic, height, body composition	2.96	0.55, 5.38	0.018

Linear regression models with robust standard errors were used

Baseline variables include age, sex, pubertal stage (early puberty, mid-puberty, late puberty, and post-pubertal), room temperature (blood pressure only), and heart rate (augmentation index only)

Variables in current socio-economic group are household's SLI (standard of living index: high, medium, low) and village urbanisation (village population: <2,000, 2,000-5,000, >5,000)

Body composition variables include fat mass index (kg/m²), fat free mass index (kg/m²), and central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

Table 7.6: *Multivariable associations between supplemental nutrition and lipid metabolism (N=1,050)*

	Mean difference (control minus intervention)		
	β-coefficient	95% CI	P-value
Total cholesterol (mmol/L)			
Baseline variables only	0.01	-0.11, 0.12	0.9
+ Current socio-economic	-0.02	-0.11, 0.08	0.7
+ Current socio-economic, height	-0.02	-0.12, 0.07	0.6
+ Current socio-economic, height, body composition	-0.03	-0.13, 0.06	0.5
LDL cholesterol (mmol/L)			
Baseline variables only	-0.01	-0.12, 0.09	0.8
+ Current socio-economic	-0.03	-0.13, 0.07	0.6
+ Current socio-economic, height	-0.03	-0.13, 0.07	0.5
+ Current socio-economic, height, body composition	-0.04	-0.14, 0.06	0.4
HDL cholesterol (mmol/L)			
Baseline variables only	0.01	-0.05, 0.06	0.7
+ Current socio-economic	-0.00	-0.06, 0.05	1.0
+ Current socio-economic, height	-0.00	-0.06, 0.05	0.9
+ Current socio-economic, height, body composition	-0.00	-0.06, 0.05	0.9
Triglycerides (mmol/L)*			
Baseline variables only	0.02	-0.03, 0.07	0.4
+ Current socio-economic	0.03	-0.03, 0.08	0.3
+ Current socio-economic, height	0.02	-0.03, 0.08	0.4
+ Current socio-economic, height, body composition	0.02	-0.03, 0.07	0.4

Linear regression models with robust standard errors were used

Baseline variables include age, sex and pubertal stage (early puberty, mid-puberty, late puberty, and post-pubertal)

Variables in current socio-economic group are household's SLI (standard of living index: high, medium, low) and village urbanisation (village population: <2,000, 2,000-5,000, >5,000)

Body composition variables include fat mass index (kg/m²), fat free mass index (kg/m²), and central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

**logged regression coefficients*

Table 7.7: *Multivariable associations between supplemental nutrition and glucose homeostasis*

	Mean difference (control minus intervention)		
	β-coefficient	**95% CI**	**P-value**
Glucose (mmol/L) (N=1,008)			
Baseline variables only	0.04	-0.22, 0.29	0.8
+ Current socio-economic	0.03	-0.21, 0.26	0.8
+ Current socio-economic, height	0.03	-0.21, 0.26	0.8
+ Current socio-economic, height, body composition	0.04	-0.20, 0.28	0.8
Insulin (mU/L) (N=1,008)*			
Baseline variables only	0.18	0.04, 0.32	0.016
+ Current socio-economic	0.18	0.03, 0.34	0.02
+ Current socio-economic, height	0.19	0.04, 0.35	0.016
+ Current socio-economic, height, body composition	0.19	0.03, 0.35	0.016
HOMA score (N=1,003)*			
Baseline variables only	0.18	0.04, 0.32	0.014
+ Current socio-economic	0.18	0.03, 0.33	0.021
+ Current socio-economic, height	0.19	0.04, 0.35	0.016
+ Current socio-economic, height, body composition	0.19	0.03, 0.34	0.02

Linear regression models with robust standard errors were used

Baseline variables include age, sex and pubertal stage (early puberty, mid-puberty, late puberty, post-pubertal)

Variables in current socio-economic group are household's SLI (standard of living index: high, medium, low) and village urbanisation (village population: <2,000, 2,000-5,000, >5,000)

Body composition variables include fat mass index (kg/m²), fat free mass index (kg/m²), and central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

**logged regression coefficients; note that the identical effect estimates for insulin and HOMA are due to near perfect correlation between the two*

7.5. Interaction between supplemental nutrition and sex

An interaction between supplemental nutrition and sex was pre-specified for two reasons. Firstly, male child is given preference in many parts of rural India, especially in terms of nutrition.[289;290] Secondly, it has been suggested that the effects of early undernutrition on CVD risk are stronger in males, as compared to females, possibly due to hormonal differences.[165;291] There was little evidence to support a sex-differential effect of nutrition supplementation in this population (Table 7.8). The p-value for the interaction term was relatively small for HDL cholesterol (p=0.06), but the actual effect estimates were not found to be different between the two sexes. In fully adjusted models (similar to the ones presented in Table 7.8), the coefficients for supplemental nutrition were: 0.01 (95% CI: -0.02, 0.05; p=0.5) in boys, and -0.03 (95% CI: -0.07, 0.02; p=0.2) in girls.

Table 7.8: *Interaction of supplemental nutrition with sex*

CVD risk factor	P for interaction*
Systolic BP (mmHg)	0.5
Diastolic BP (mmHg)	0.2
Augmentation index (%)	0.9
Total cholesterol (mmol/L)	0.4
LDL cholesterol (mmol/L)	0.9
HDL cholesterol (mmol/L)	0.064
Triglycerides (mmol/L)	0.3
Glucose (mmol/L)	0.7
Insulin (mU/L)	0.5
HOMA score	0.6

P-values from comparison of linear regression models using the likelihood ratio tests

All models are adjusted for age, pubertal stage (early puberty, mid-puberty, late puberty, and post-pubertal), household's SLI (standard of living index: high, medium, low), village urbanisation (village population: <2,000, 2,000-5,000, >5,000), fat mass index (kg/m²), fat free mass index (kg/m²), central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds), room temperature (blood pressure only), and heart rate (augmentation index only)

7.6. Role of insulin resistance

A central role for insulin resistance in CVD risk has been postulated, especially among Asian Indians.[134;135;267] I, therefore, examined the association of HOMA score with other CVD risk factors, and also the impact of HOMA score (as a covariate) on the association between supplemental nutrition and CVD risk. Table 7.9 shows that blood pressure and triglycerides were positively associated with HOMA score. These associations were markedly attenuated after adjustment for other variables, including body composition.

Table 7.10 presents the baseline and fully adjusted models for the association between supplemental nutrition and CVD risk factors, with and without adjustment for HOMA score. The biggest changes in the effect estimates, following adjustment for HOMA score, was seen for blood pressure. The effect estimates in the majority of the models were more than halved after adjustment for HOMA. These results could be explained by HOMA score being a confounder (if, for example, greater insulin resistance in children from the control area was a result of selection or some other form of bias, rather than the effect of intervention). However, they would also be consistent with HOMA score being an intermediary i.e. if the effect of supplemental nutrition on blood pressure was mediated, at least in part, through greater insulin resistance.

Table 7.9: *Multivariable association of HOMA score with other CVD risk factors*

Outcome	β-coefficient	95% CI	P-value
Systolic BP (mmHg)			
Baseline model	2.04	0.96, 3.13	0.001
Fully adjusted model	0.92	-0.09, 1.93	0.073
Diastolic BP (mmHg)			
Baseline model	0.87	0.35, 1.40	0.002
Fully adjusted model	0.46	-0.07, 0.99	0.086
Augmentation index (%)			
Baseline model	0.31	-1.37, 1.98	0.7
Fully adjusted model	0.95	-0.60, 2.49	0.2
Total cholesterol (mmol/L)			
Baseline model	0.05	-0.04, 0.13	0.3
Fully adjusted model	0.04	-0.04, 0.12	0.3
LDL cholesterol (mmol/L)			
Baseline model	0.03	-0.05, 0.11	0.4
Fully adjusted model	-0.02	-0.04, 0.01	0.3
HDL cholesterol (mmol/L)			
Baseline model	-0.02	-0.04, 0.13	0.3
Fully adjusted model	-0.02	0.04, 0.12	0.3
Triglycerides (mmol/L)*			
Baseline model	1.07	1.02, 1.13	0.006
Fully adjusted model	0.06	0.01, 0.11	0.02

N=1,009 (systolic and diastolic blood pressure), N=770 for augmentation index, and N=1,003 for lipids

Linear regression models with robust standard errors were used

Baseline models are adjusted for age, sex, pubertal stage (early puberty, mid-puberty, late puberty, post-pubertal), room temperature (blood pressure), and heart rate (augmentation index)

Fully adjusted models include baseline model covariates, household's SLI (standard of living index: high, medium, low), village urbanisation (village population: <2,000, 2,000-5,000, >5,000), fat mass index (kg/m²), fat free mass index (kg/m²), and central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

**logged regression coefficients*

Table 7.10: *Multivariable association of supplemental nutrition with CVD risk factors, with and without adjustment for HOMA score*

	Mean difference (control minus intervention) (95% CI)	
	Unadjusted for HOMA	**Adjusted for HOMA**
Systolic BP (mmHg)		
Baseline model	0.86 (-0.72, 2.45); p=0.2	0.44 (-1.07, 1.95); p=0.5
Fully adjusted model	0.64 (-0.99, 2.27); p=0.4	0.57 (-1.06, 2.21); p=0.5
Diastolic BP (mmHg)		
Baseline model	0.21 (-0.81, 1.23); p=0.7	0.06 (-0.99, 1.10); p=0.9
Fully adjusted model	0.11 (-0.85, 1.07); p=0.8	0.06 (-0.94, 1.05); p=0.9
Augmentation index (%)		
Baseline model	3.29 (0.74, 5.84); p=0.013	3.16 (0.54, 5.78): p=0.02
Fully adjusted model	2.96 (0.55, 5.38); p=0.018	2.72 (0.25, 5.19); p=0.032
Total cholesterol (mmol/L)		
Baseline model	0.01 (-0.11, 0.12); p=0.9	-0.00 (-0.12, 0.11); p=0.9
Fully adjusted model	-0.03 (-0.13, 0.06); p=0.5	-0.05 (-0.14, 0.05); p=0.3
LDL cholesterol (mmol/L)		
Baseline model	-0.01 (-0.12, 0.09); p=0.8	-0.02 (0.13, 0.09); p=0.7
Fully adjusted model	-0.03 (-0.13, 0.06); p=0.5	-0.05 (-0.14, 0.05); p=0.3
HDL cholesterol (mmol/L)		
Baseline model	0.01 (-0.05, 0.06); p=0.7	0.01 (-0.04, 0.07); p=0.7
Fully adjusted model	-0.00 (-0.06, 0.05); p=0.9	-0.00 (-0.06, 0.05); p=0.9
Triglycerides (mmol/L)*		
Baseline model	0.02 (-0.03, 0.07); p=0.4	-0.02 (-0.13, 0.09); p=0.7
Fully adjusted model	0.02 (-0.03, 0.07); p=0.4	-0.05 (-0.14, 0.05); p=0.3

N=1,009 (systolic and diastolic blood pressure), N=770 for augmentation index, and N=1,003 for lipids

Linear regression models with robust standard errors were used

Baseline models are adjusted for age, sex, pubertal stage (early puberty, mid-puberty, late puberty, post-pubertal), room temperature (blood pressure), and heart rate (augmentation index)

Fully adjusted models include baseline model covariates, household's SLI (standard of living index: high, medium, low), village urbanisation (village population: <2,000, 2,000-5,000, >5,000), fat mass index (kg/m²), fat free mass index (kg/m²), and central-peripheral skinfolds ratio (subscapular+suprailiac/ biceps+triceps skinfolds)

**logged regression coefficients*

To further investigate the role of insulin resistance, I examined the determinants of insulin resistance syndrome (IRS).[269] As explained in the methods section, IRS was defined using a modified classification suggested for use in Indians.[266] According to this classification, the prevalence of IRS in the control area was double that of the intervention area: 2.0% (11/552) in the intervention area and 4.36% (20/459) in the control area; a crude odds ratio of 2.24 (95% CI: 1.15, 4.37; p=0.018). Higher standard of living index and greater fat free mass index were the main determinants of IRS in this population (Table 7.11). The association of fat free mass index with IRS probably reflects its combined association with its two components, blood pressure and insulin resistance. The crude association between supplemental nutrition and IRS was not attenuated by adjustments for current socio-economic circumstances and height (Table 7.12).

Table 7.11: *Determinants of Insulin Resistance Syndrome*

Variable group	OR	95% CI	P-value
Physiological			
Age (per year)	0.73	0.51, 1.04	0.082
Sex (female *vs* male)	2.59	0.66, 10.08	0.2
Pubertal stage (per stage)	1.54	0.85, 2.78	0.2
Socio-economic			
Standard of living index (per category)	2.51	1.22, 5.18	0.013
Urbanisation (per category)	0.81	0.57, 1.15	0.2
Body composition			
Height (SDS)	1.65	0.85, 3.22	0.14
Fat mass index (SDS)	1.77	0.85, 3.67	0.1
Fat free mass index (SDS)	5.04	2.60, 9.79	<0.001
Central-peripheral skinfolds ratio (SDS)	1.55	0.91, 2.64	0.1

N=1,011; Logistic regression models with robust standard errors were used

Variables in the physiological group are mutually adjusted for other variables in the group; variables in the socio-economic and body size and composition groups are adjusted for other variables within their own group and all variables in the physiological group

Pubertal stages: early puberty, mid-puberty, late puberty, post-pubertal; Standard of living index: high, medium, low; Village urbanisation (village population): <2,000, 2,000-5,000, >5,000; Central-peripheral skinfolds ratio: subscapular+suprailiac/ biceps+triceps skinfolds

SDS (standard deviation scores) for body composition variables were calculated using the variable mean and standard deviation for the study population

Table 7.12: *Multivariable association between supplemental nutrition and insulin resistance syndrome*

	Control (reference category: intervention)		
Variable group	**Odds ratio**	**95% CI**	**P-value**
Baseline variables only	2.43	1.20, 4.91	0.014
+ Current socio-economic	2.53	1.23, 5.24	0.012
+ Current socio-economic, height	2.78	1.29, 5.98	0.009

N=1,011; Logistic regression models with robust standard errors were used

Baseline variables include age, sex, and pubertal stage (early puberty, mid-puberty, late puberty, post-pubertal)

Variables in current socio-economic group are household's standard of living index (high, medium, low) and village urbanisation (village population: <2,000, 2,000-5,000, >5,000)

7.7. Summary

This chapter presented the analyses relating to the effects of supplemental nutrition on CVD risk. There was no difference in the sexual maturation of children in the two study arms. Supplemented children were notably taller (14 mm; 0.4 to 2.3), but similar in their body composition i.e. BMI, midarm and waist circumferences, fat mass index and central-peripheral skinfold. The small difference in fat free mass index (greater in controls) was probably a chance finding.

Among CVD risk factors, there were statistically robust differences in augmentation index and insulin resistance. Augmentation index was 3.3% (1 to 5.7) greater in controls (i.e. stiffer arteries), as compared to supplemented children. HOMA score was 20% higher (3 to 39) in control, as compared to the supplemented children suggesting greater insulin resistance in the controls. These effects were largely unchanged on further adjustments for height and body composition. The regression estimates for HOMA score and fasting serum insulin were same, as expected.

There was a modest difference of 0.6 mmHg (-1.1 to 2.3) in systolic blood pressure (greater in controls), which was not statistically robust. Diastolic blood pressures were similar, as were the levels of blood lipids and fasting glucose, in the two arms of the study. There was no notable interaction between the intervention (i.e. nutrition supplementation) and sex of the participant for any of the outcomes.

The odds of having insulin resistance syndrome were 2.5 times (1.2 to 5.2) in the control area, as compared to the intervention area. Blood pressures, but not augmentation index were associated with HOMA scores, and adjustment for

HOMA scores roughly halved the association between supplementation and blood pressures, suggesting a possible intermediary role for insulin resistance.

CHAPTER 8. DISCUSSION

The aim of this chapter is to discuss the results, their validity and their implications. The chapter is organised as follows. First, the strengths and limitations of the study are discussed. Second, the key results are summarised. Third, the results are discussed in the context of existing literature. Finally, the potential public health implications and future directions for research are highlighted.

8.1. Strengths and limitations

This study has several important strengths and limitations, which need to be considered first so that their impact on the results can be anticipated. These are presented under the traditional headings of chance, bias and confounding. Reverse causality is not discussed, as the outcome could not have determined whether the participant was born in the intervention or control area.

8.1.1. Chance

There were several positive and negative findings in this study and the likelihood that these could have arisen due to chance needs to be considered i.e. Type 1 and Type 2 errors.

Type 1 (alpha error)

Type 1 error is the mistaken rejection of the null hypothesis i.e. declaring that a difference exists when it does not. Type 1 error is a particular problem in epidemiological research involving cardiovascular diseases, since a large number of statistical tests are often carried out (because of multiple risk factors). The problem is further compounded when a large number of interactions are sought.

This may account, to some degree at least, for the range of differing results in literature presented in Chapters 2 and 3. In this research, I have tried to control the Type 1 error rate by examining a limited number of variables and models that were largely pre-specified (on the basis of existing literature and relevance to the setting). Although, this kind of rigid approach reduces the chances of finding potentially novel findings, I believe that reducing Type 1 error rate is more important in the context of multifactorial disorders. To illustrate with the example of this research, the total number of p-values presented in the main results chapter (Chapter 7) alone is 153, with perhaps an equal number of p-values in data that are referred to, but not presented.

In addition to limiting the number of statistical tests, I have also based my interpretation of the results on priors and the general trend of tests. For example, isolated positive findings, especially when they have occurred at variance to others in the same domain (with which they would be expected to correlate), have been attributed to chance, irrespective of the significance of the statistical test. Conversely, weaker tests of association have been accepted as evidence against the null hypothesis, where they fitted the prior and general trend.

Type 2 (beta error)

The type 2 error is the error of failing to reject a false null hypothesis i.e. declaring that a difference does not exist when in fact it does. It depends mainly on the power of the study. In doing the power calculations, two key assumptions had to be made: the standard deviation of the outcome variables and their clustering within villages (using intraclass correlation coefficients). Although I was able to extract standard deviation values from published literature that were reasonably applicable, intraclass correlation coefficients relevant to this setting were not readily available. So I calculated a range of detectable differences using ICC values that are generally regarded as low (0.01) and high (0.1) ICC in cluster analyses.[302] I was aware that such values are typically based on analyses

conducted in distinctly different settings (e.g. classrooms or UK communities).[302] Although it was not possible for me to confidently predict whether clustering would be comparatively higher or lower within adolescents in an Indian village, I *a-priori* expected it be on the lower side due to greater homogeneity in lifestyle.

The final study sample size (n=1,165) was not too different from that expected (n=1,268). The standard deviations for virtually all the outcomes in the study were either lower or at the lower end of the range used for power calculations. However, the ICCs of several outcomes were nearer the higher end of the assumed range. This meant that the study was adequately powered to detect reasonable differences for some but not all the outcomes. For example, in the case of systolic blood pressure, the mean detectable was 1.8 - 2.9 mmHg with an ICC of 0.01, but 3.3 - 5.5 with an ICC of 0.1. The ICC for systolic BP in the study was halfway (0.05). Assuming an effect size similar to that seen in the associations between birthweight and BP i.e. 1.5 - 2 mmHg (realising that it relates to a large difference in birthweight of 1 kg),[21;160;161] the study was inadequately powered for this outcome. The final effect estimate for systolic BP in the study ranged between 0.6 to 1.1 mmHg, depending on the level of adjustment.

There are two potential explanations for higher than expected village clustering of outcome variables, assuming that it is not real. The first is that measurement conditions were more similar for participants from same villages, than between villages. This was possible as participants from the same villages were examined together, and clustering could arise due to the clinic conditions, seasonality or batch operations. One way of examining these issues is to look at the associations over time: the effect of the month of examination was investigated, but found to have no effect, independent of the room temperature, which was already adjusted for in the relevant outcomes. All biochemical assays, except insulin were done on a daily basis, so batching was not an issue.

The other explanation, which appears more likely, is that the apparently high ICCs were not due to greater similarity between participants from the same villages, but because of less variability between participants across all the villages. Since ICC is a ratio of how related individuals are within a cluster, to how related they are generally, a homogeneous population may artefactually manifest a high ICC (by influencing the denominator).[270] This hypothesis is supported by the low standard deviations found for the majority of the outcomes in this population. The high ICCs will have reduced study power but not influenced the conclusions, as all the confidence intervals presented throughout this were based on robust standard errors.[270]

8.1.2. Bias

There are two important biases that need to be considered: selection bias and measurement bias.

Selection bias

Systematic differences in participants could have arisen at the time of the baseline study or at the time of the present follow up and they are each considered in turn.

In the baseline study, all the pregnant women from the selected villages were eligible for study inclusion. Although there are no data to support the claim, the participation rate at the time of pregnancies is believed to be 100% (information provided by the baseline investigators). This appears plausible as the fieldwork team resided in the study area with the support of the villagers, and they were able to maintain a good relationship with them.

The present follow up was restricted to those with personal identifiers and historical records, which were not complete. Bias could arise if children with these data were somehow different from those without, or if there was a differential loss of data across the two areas. There was no reason to suggest that either of the two was the case. The baseline study was planned to be a one-off study. Since follow-up was not anticipated, safekeeping of the identifiers and collected data was not a priority. Discussions with the baseline study investigators did not yield any obvious explanations for the loss of data, apart from lack of care in storing it. Such a loss of data is likely (although by no means definitely) to be non-systematic. This was confirmed somewhat by roughly similar numbers of families with identifiers in the two areas (1,327 in the intervention and 1,429 in the control). Fewer personal identifiers were available for the latter two years of the study (as compared to the first two years), but this loss affected both areas to a similar extent, and was confirmed by roughly similar distributions of date of births.

Among the families with identifiers, 71% women could be contacted: 75% in the intervention area and 67% in the control area. Of those not contacted, a similar proportion (11%) in both areas were now living outside the area. The difference in the proportion of families contacted in the two areas arose mainly from the category of those that could not be identified at all: 13% in the intervention area and 21% in the control area. These are believed to be temporary migrant workers at the time of the baseline study. Influx of such workers is common in this area, especially during the harvest season when additional help is required. In the baseline study, such temporary residents were eligible for inclusion, although, unfortunately, their status as such was not recorded. The control area traditionally gets a greater influx of such seasonal workers, which explains the difference in the proportion of unidentified families. This is supported by the slightly higher number of baseline family identifiers available in the control area (despite similar populations). While the explanation of this category of

unidentified families is conjectural, it has face validity to the extent that in the close-knit community life of these small villages, it is generally not possible to live without being known by other villagers and the key persons such as the village head and the anganwadi worker (whose help was enlisted in tracing). On this basis, one would not expect the difference in tracing of families between the intervention and control areas to lead to any systematic bias.

Of the children ever born to the traced women, fewer matched to the historical records in the control area (55%), as compared to the intervention area (60%). There is no obvious explanation for this difference, which could have arisen due to chance. The response rate among those invited to attend the clinic was higher in the intervention (82%), as compared to the control area (74%). The perceived reason (according to the fieldwork team) for the lower overall response rate in the control area was the disinterest shown by the village heads of few of the villages, who were more interested in certain ongoing political activities. The participation rates in the villages were strongly dependent on support from the village heads, who were instrumental in encouraging the families to participate. If this explanation is correct, it should not have resulted in any systematic bias in the study.

As the result of the losses in the above two steps, the participating children represented 49% (intervention) and 41% (control) of all children born in the area in baseline study period. Comparison of these children to those not in the study sample, showed that the participating children were slightly older (15.6 years versus 15.2 years), and more likely to be boys (54% versus 48%) and full-time students (77% versus 70%). Boys were more likely to participate than girls, possibly due to cultural reasons (traditional households may not allow girls to participate). Full-time students were also more likely to participate than those in full-time employment. Most of those employed were on daily wage, and could not afford to take a day off work to attend the clinic. The magnitude of the

differences between the participating and non-participating children were similar across the two areas, and therefore unlikely to have introduced any systematic bias in the study sample.

The final sample size was less than ideal, but not unusual for studies of this nature involving follow up of participants after a considerable time gap. The direct comparators to this study are the Guatemala trial, and the two famine studies (Dutch winter hunger and Leningrad siege), and they all had follow up rates below 35%.[24-27] More crucially, the reasons for loss to follow up do not appear to be such as to introduce any systematic bias in the associations of interest. This was confirmed to some extent by the comparison of the baseline characteristics of participating children in the intervention and control areas, which were very similar.

Measurement bias

Exposure

The study exposure was the place of residence - intervention or control area - and could not be biased. Thirty two children had moved from their original villages, and most of these had moved after the age of 10 years i.e. beyond the age at which supplemental nutrition was offered. All analyses were repeated after excluding these children, and findings remained unchanged. Not all women in the intervention area will have taken the supplement. Data from similar programmes, and also that collected from the same area in the past suggest that uptake tends to be around 50-60%.[45;46;312] Common reasons for non-uptake of intervention include inability to get to the centre to collect the supplement (most women work in the fields during the day) and a dislike for taste of the supplement.[45;46] The affluent women may not also feel the need for supplement, but such women were very few in these villages. Potentially, bias could be introduced if both the uptake of supplement and participation in the current

follow up were socially patterned. However, the socio-economic homogeneity of this population renders such a bias less important.

An additional concern could be sharing of the supplement with other members of the household. Both the lack of supplement uptake and its sharing would serve to dilute the effect estimate. Furthermore, the effect estimates obtained from this study design would remain valid for its programmatic implications, i.e. the expected effect of intervention in the `real world' scenario.

Outcome measures

Only previously validated outcome measures were used. Where there were specific concerns about extending the validity to a modified use of the outcome (i.e. self-assessment of testicular volume) or its local application (i.e. assessment of arterial stiffness), independent sub-studies were conducted to address those concerns.

In addition to these, there were two other areas of uncertainty for which validation studies were beyond the scope of this project: a) the validity of formulae used for calculation of body composition and augmentation indices (in the study population), and b) the validity of pubertal staging system devised for this study. The Sphygmocor software uses an inbuilt transfer function to estimate the augmentation index,[102] which has been validated in several populations (and shown to be generalisable),[100;101;103] although not specifically Indians. It is unlikely that it would differ materially in Indians. Furthermore, any error arising out of inaccurate transfer function would operate equally in both arms of the study, serving only to dilute the strength of the association.

Two schools of thought exist concerning the estimation of body composition by anthropometry. While some experts argue that anthropometric measures be used

as such (without applying formulae to calculate components of body composition, because of the extra level of measurement error introduced as a result), others favour the use of formulae to improve interpretability. I opted for a mixed approach, using formulae where they had been previously validated in similar populations and their performance was supported by a large body of evidence (e.g. estimation of body fat and lean mass from skinfolds),[313] but not where there was not enough cumulative experience in their use (e.g. estimation of muscle mass from skinfolds and circumferences).[314]

Puberty was assessed using the timing of menstruation and the testicular volume. Timing of menstruation is generally accepted as a valid and reliable measure of pubertal status in girls.[238;239] Its validity will have been further improved in this study by the fact that the question related to a contemporaneous event (hence lower recall bias). In boys, the use of Prader's orchidometer to assess testicular volume is already regarded as a valid and reliable measure of pubertal status,[238;239] its validity was extended to self-assessment by conducting a independent study. While the abstracted system of pubertal staging used in this study lacked the sensitivity of a full Tanner staging,[244] it was adequate for the purpose: classifying children broadly into the main stages of maturation (delineating those who were undergoing peak growth spurt from those before and after), thereby allowing measures of body composition and CVD risk factors to be adjusted for maturational age, along with the chronological age.

The fieldworkers were aware of the intervention status of the villages. The lack of 'blinding' could have biased the assessment of outcome measures by the fieldworkers, depending on their own views of the hypothesis under study. While the potential for such bias is an important concern for anthropometric measures, it is less so for the majority of primary outcome measures used in this study because of a degree of automation involved. Biochemical assays, and the

assessment of arterial stiffness and blood pressure were all to some extent automated procedures. While automation does not completely exclude bias, it makes it less likely. Still this remains a potential but unavoidable source of bias.

Apart from validity, reproducibility of outcome measures is also very important. Reproducibility of clinic measurements was checked by recalling a proportion of randomly selected participants for repeat measurements. All measurements were found to be highly reproducible, except assessment of hip circumference (because of inconvenience of changing dress), which was consequently not used in the analyses.

Reproducibility values for blood pressure measurements (ICC) were: 0.88 for systolic BP and 0.86 for diastolic BP. Although relatively low in comparison to reproducibility values for BP typically found in adults, they compare more than favourably with values reported from epidemiological studies in children and adolescents.[234;303] Reproducibility tends to vary with age and can be particularly low in childhood and adolescence due to a high component of physiological variability.[303] For example, values (intraclass correlation coefficients) reported from a major reproducibility study in 9 to 13 year olds (using an oscillometric device, Dinamap 845XT) were 0.45, 0.55, 0.80, and 0.85, following one, two, three and four measurements per visit, respectively.[234] In the present study, only two measurements of blood pressure were taken. While some might argue the need to take more than two readings to obtain satisfactory reproducibility, the data from the present study suggest that the same end can be achieved by carefully following the guidelines, and allowing participants adequate time to relax before measurements. Adequate relaxation time is crucial, but often does not get rigidly adhered to in the busy clinics of large-scale epidemiological studies.[234] By arranging the BP measurements around the arterial stiffness assessments (which took at least 10 minutes to complete, during which the participant had to lie still), it was possible to enforce an inherent relaxation time.

The reproducibility values (ICC) for radial augmentation index were 0.85 - 0.90. Data for children are not available for comparison,[123;304] but bearing in mind its relationship to blood pressure (and consequently high levels of physiological variability), these values appear reasonable. The validity of the arterial stiffness measurements taken by the study observer was also confirmed against an independent expert. Reproducibility of biochemical assays was assessed by repeating the assays on split samples, and was found to be consistently high.

There are several reasons why the study had such high levels of reproducibility for measurements. A detailed protocol was prepared and team were thoroughly trained according to the protocol. Regular training sessions were held throughout the course of the study. However, possibly the most important reason was the policy of using the same observer for any given measurement during the course of the study. Large epidemiological studies generally involve multiple observers, and despite efforts to standardise, differences in their technique invariably reduce reproducibility. A case in point is skinfold assessments. Measurement of skinfold thickness is potentially a more valid measure of body fat than BMI, especially in lean populations and adolescents (in whom the validity of BMI is particularly low).[238;249;250] Despite this, skinfolds are infrequently used because of poor reproducibility in the field. In this study, high reproducibility for skinfold measurements was documented, and could be attributed, at least in part, to the absence of variations in technique introduced by use of multiple observers. It is appreciated that this is not always possible in large-scale epidemiological studies, and I was fortunate that none of the team members had to be absent from fieldwork for any length of time.

8.1.3. Confounding

A key strength of this study was the complete absence or severely restricted presence of several crucial confounders that typically plague epidemiological research on this topic.[4;20] Maternal smoking in pregnancy was not an issue in this study, as women do not smoke in this population. Tobacco and alcohol consumption was also almost non-existent in this population. Although it is possible that the children may have been reluctant to admit, their denial has face validity, as the children generally start experimenting with tobacco and alcohol at a relatively later age in this area. The population is remarkably homogeneous in its socio-economic circumstances, and the diet and physical activity patterns tend to be fairly similar. Importantly, even though the study design did not fully meet the rigorous standards of a randomised controlled trial, there was a reasonably robust control group that provided baseline levels of confounding exposures, thus allowing estimation of the effect of intervention in isolation.

In the baseline study, all villages within a 10 km radius of a central village were selected. Bias could not have arisen because of non-randomisation of villages, since every village within that radius was included. However, bias could have arisen if the choice of the central village itself was biased in some way (say if one of the two had better transport links), which could then have resulted in important differences between the two groups of villages; however, this scenario appears unlikely. Bias could also arise if the reason to introduce intervention in one area and not the other was based on factors that could potentially also influence the outcome. In this regard, two possibilities exist and these were explored.

First, although the ICDS programme is being rolled out universally, within it there is a political commitment to introduce it in the poorer areas first.[40;46] Secondly, the introduction of the programme can be expedited to some extent by

political influence (say for example, if one village was the birth place of an important politician). Both the above factors could operate as confounders, as poverty (linked to underdevelopment) or political influence at baseline could also determine the speed of economic development, and therefore the current level of urbanisation (important determinant of CVD risk factor prevalence). However, there was no evidence to suggest any material difference in the current level of urbanisation of the two areas, either visually or in terms of the individual and village level economic data collected. Baseline study investigators confirmed this to be the case at baseline (although data was not available), and further reassurance could be had by the fact that the control area had the programme introduced some years later (suggesting that they were similar). Despite this, all analyses were adjusted for household's socio-economic position and village urbanisation to account for any residual confounding from potential imbalances at baseline.

One potential criticism of this study could be that data on current diet and physical activity were not collected and differences in these could account for the differences in CVD risk. This appears to be unlikely as an important source of error in this study for several reasons. The villages were fairly homogeneous in their level of urbanisation. Excessive sedentariness is not common among rural Indian adolescents, where an outdoor way of life is the norm. Almost all meals are taken at home, and the variety of meals and cooking styles is extremely limited. Adiposity is a net total of energy intake (diet) and energy expenditure (physical activity). The low fat mass in this population and its narrow distribution range argue against any important variations in diet and physical activity patterns.

8.1.4. Generalisability

The study population came from 29 villages in south India. The sampling frame comprised of all children born to women in these villages over a four-year period. There were no exclusion criteria, and these villages can be regarded as typical of the villages in India. The conditions in these villages, especially the prevalence of chronic undernutrition, will be generalisable to much of the developing world.[315] The ICDS programme used a framework for this study covers 4.83 million expectant and nursing mothers and 22.9 million children under the age of 6 years.[41,42] The results from this study have direct relevance to this population, and also to several such programmes currently operational in many low-income countries.

8.1.5. Summary

This section has summarised the strengths and limitations of the study. The main strengths of the study were the study setting (malnourished population with a nutrition intervention), controlled trial design with an adequate control group, absence or severely limited presence of some key confounders, and valid and reliable outcome measurements.

The sample size proved to be adequate for many of the outcomes but not all. The main reason for this appears to have been the greater than expected homogeneity (of CVD risk factors) among the study participants. As a result, the study may have been underpowered to detect differences in some outcomes such as blood pressure. The possibility of finding positive results by chance was curtailed by limiting the number of statistical tests, but cannot be completely ruled out. Similarly, although there were no baseline differences study participants and non-participants and the losses to follow up appear to be non-differential,

selection bias cannot be completely ruled out, as only about half of the original study participants attended the clinics. Overall, the study limitations were of the level and nature that is generally acceptable in similar epidemiological studies, allowing reasonable confidence in the results of the analyses.

8.2. Summary of results

The key results are presented in Tables 8.1 and 8.2. An important point to note is that in the case of multivariably-adjusted regression models (relevant to Chapters 6 and 7), the headline results presented in these tables are for models adjusted for age, sex, pubertal stage and current socio-economic circumstances (but not body size and composition). The main conclusions from this study are also based on these models. Differences in body size and composition of children from the two areas may or may not be a direct consequence of supplemental nutrition. In view of the controlled trial design of this study, it was assumed that any observed differences in body size and composition were a result of extra nutrition. Under these circumstances, adjustment for body size and composition could be regarded as adjustment for mediators, and therefore inappropriate (in terms of estimating the net association). Models with adjustment for body size and composition have been presented in the results section mainly to better understand the impact of these mediators and explore the underlying mechanistic pathways. An exception was made for pubertal status (also potentially a mediator), which was included in these models because of the necessity for more robust adjustment for age (poorly reported in this setting). Absence of an association between supplemental nutrition and timing of puberty onset was, however, confirmed prior to its inclusion in the basic models.

In the descriptive analyses (Chapter 5), birthweight was found to be 61 g (95% CI: 18 to 104; p=0.007) higher in the supplemented area, as compared to the control area. In the two validity sub-studies carried out, arterial stiffness

assessment in the field setting by a non-specialist observer was found to be comparable to a specialist assessment. The intraclass correlation coefficient between measurements taken by the field observer and the expert was 0.964. Self-assessment of testicular volume with Prader's orchidometer compared favourably with observer-assessed methodology. 93% of measurements taken by the two methods were within one rank of each other. There was no evidence of systematic over or under-assessment on the Bland-Altman plot.

In analyses of the determinants of CVD (Chapter 6), increasing age and pubertal stage were found to predict positively all measures of body size and composition. Boys were taller, but had similar BMI as girls: girls had more fat mass, while boys had more fat free mass. Higher socio-economic position positively predicted height, fat mass and lean mass. Urbanisation was not found to influence body size and composition in this population.

Increasing age and pubertal stage positively predicted blood pressure. Systolic BP, but not diastolic BP, was higher in boys. Blood pressure was inversely associated with room temperature: a 10 degree Celsius decrease in room temperature was associated with a 2.5 mmHg (0, 4.9) increase in systolic BP. Current socio-economic circumstances and village urbanisation showed no clear association with blood pressure. Height, fat free mass index and central-peripheral skinfolds ratio (but not fat mass index) were positively associated with blood pressure, especially systolic BP.

Increasing height and heart rate inversely predicted augmentation index. Girls had higher augmentation index than boys. Age, pubertal stage, village urbanisation, and measures of body composition bore no relationship to augmentation index, although there was a modest inverse association between higher socio-economic position and augmentation index.

Girls had higher lipid concentrations compared to boys. There was no clear trend for an association between lipid concentrations and age or pubertal stage, although triglyceride concentrations fell slightly with age. There was a modest but consistent trend for higher concentrations of cholesterols (total, HDL and LDL) with increasing socio-economic position and urbanisation, but none for triglyceride concentrations. Fat mass index was weakly predictive (positively) of total and LDL cholesterol, and triglycerides, but not HDL cholesterol concentrations. Triglycerides showed a weak inverse association with height. There were weak inconsistent associations between lipid concentrations and central-peripheral skinfolds ratio.

There were no clear trends for an association between measures of glucose-insulin homeostasis and any of the determinants, except for a weakly positive association between fat free mass (but not fat mass) and insulin or HOMA score.

In the analyses of the effects of supplementation on CVD risk, no effects of supplementation on the timing of onset of puberty were found (Chapter 7). Supplemented children were clearly taller, with a mean difference in height of 14 mm (4.1, 23.1), p=0.007. There was some evidence for greater BMI and fat free mass in control children, but no difference in fat mass index and central-peripheral skinfold ratio. The mean difference in fat free mass index (control minus intervention) was 0.24 kg/m^2 (0.06, 0.43), p=0.01.

Systolic blood pressure was lower in the supplemented children (mean difference: 0.6 mmHg; -1.1, 2.3; p=0.4), but the difference was consistent with random variation. Augmentation index was lower in the supplemented children, with a mean difference (control minus intervention) of 3.3 % (1.0, 5.7), p=0.008.

There were no differences in the lipid concentrations of children from the supplemented and control areas. There was no difference in fasting glucose, but important differences in fasting insulin and HOMA scores. The mean HOMA score was 20% (3, 39; p=0.02) lower in supplemented, as compared to the control children.

No sex-specific effects of the intervention were found on any of the CVD risk factors. In investigating the central role of insulin resistance, HOMA score was found to predict blood pressure and triglycerides but not augmentation index. For each unit increase in HOMA score, systolic BP increased by 2.0 mmHg (1, 3), p=0.001. The effect of supplemental nutrition on BP appeared to be mediated at least in part by insulin resistance. The estimates for an effect of supplemental nutrition on blood pressure were roughly halved for a unit increase in HOMA score. Insulin resistance syndrome was more prevalent in the children from the control area, as compared to the supplemented area: odds ratio of 2.5 (1.2, 5.2); p=0.01.

Table 8.1: *Summary of key results from Chapter 5 (Descriptive analyses) and Chapter 6 (Determinants of CVD risk)*

Outcome	Key results
Descriptive analyses (Chapter 5)	
Birthweight	Birthweight higher in supplemented area by 61 g (18, 104); p=0.007.
Validity of arterial stiffness	Arterial stiffness assessment in the field setting by a non-specialist observer was comparable to an expert assessment. Intraclass correlation coefficient between field observer and expert was 0.964.
Validity of testicular self-assessment	Self-assessment of testicular volume with Prader's orchidometer is comparable to observer-assessed. 93% of measurements taken by the two methods were within one rank of each other. There was no evidence of systematic over or under-assessment on Bland-Altman plot.
Determinants of CVD risk (Chapter 6)	
Body size and composition	Increasing age and pubertal stage positively predicted all measures of body size and composition. Boys were taller but had similar BMI as girls (girls had more fat mass, boys had more fat free mass). Higher socio-economic position positively predicted height, fat mass and fat free mass.
Blood pressure	Increasing age, pubertal stage, height and fat free mass positively predicted blood pressure. BP was higher in boys. Stronger associations seen for systolic than diastolic BP.
Augmentation index	Increasing heart rate and height were inversely associated with augmentation index. Augmentation index was lower in boys.
Lipid concentrations	No effect of age or pubertal stage. Girls had higher lipid concentrations compared to boys. Socio-economic position and urbanisation weakly (positively) predicted cholesterol concentrations. Increasing fat mass was weakly predictive (positively) of total and HDL-cholesterol, and triglyceride concentrations.
Glucose homeostasis	No clear trends but fat free mass (not fat mass) weakly predictive of insulin and HOMA score

Table 8.2: *Summary of results from Chapter 7 (Supplemental nutrition and CVD risk)*

Outcome	Key results*
Pubertal stage	No effect of supplementation on timing of puberty onset
Body size and composition	Supplemented children were taller: 14 mm (4.1, 23.1); some evidence for greater BMI and fat free mass in control children
Blood pressure	Lower systolic blood pressure in the supplemented children, but little evidence against null hypothesis: 0.6 mmHg (-1.1, 2.3); p=0.4
Augmentation index	Lower augmentation index in the supplemented children, difference: 3.3 % (1.0, 5.7); p=0.008
Lipid concentrations	No difference in total, HDL, and LDL factions of cholesterol, or triglyceride concentrations
Glucose-insulin homeostasis	No difference in fasting glucose, but important differences in fasting insulin and HOMA scores. HOMA score 20% lower (3, 39; p=0.02) in the supplemented children
Sex-specific effects of intervention	None found
Central role of insulin resistance	The effect of supplemental nutrition on BP were roughly halved for a unit increase in HOMA score, suggesting that insulin resistance may mediate the effects of supplementation on BP
Insulin resistance syndrome	More prevalent in control area, odds ratio: 2.5 (1.2, 5.2); p=0.012

** Results presented are mean differences (control minus intervention) and 95% confidence intervals*

8.3. Interpretation of the findings in the context of existing literature

Descriptive data confirmed this as a low risk population for CVD, when compared to similar populations from more affluent settings. To illustrate this point, the mean values of risk factors are compared to a study in US adolescents, carried out to assess the relationship between birthweight and insulin resistance (Table 8.3).[297] This study is chosen as an example because of the closely matching age range, since values in puberty are often different than at other ages. Participants came from a population based sample of Minnesota school children, and the values are consistent with other studies from similar settings.

Table 8.3: *Characteristics of Hyderabad and Minnesota adolescents*

	Hyderabad (rural)		**Minnesota (urban)**	
	Boys (N=604)	*Girls (N=516)*	*Boys (N=164)*	*Girls (N=132)*
Age (yrs)	16.0 (0.9)	15.9 (0.9)	15.0 (1.3)	14.9 (1.2)
Height (cm)	159 (9)	152 (6)	174 (8)	165 (7)
BMI (kg/m^2)	16.7 (2.1)	17.8 (2.2)	23.3 (5.1)	23.8 (4.6)
Fat mass index (kg/m^2)	1.6 (2.1)	3.8 (1.1)	7.3 (5.1)	9.0 (5.8)
Systolic BP (mmHg)	111 (11)	107 (9)	111 (8)	105 (8)
Total cholesterol (mmol/L)	3.3 (0.6)	3.7 (0.7)	3.8 (0.8)	3.8 (0.7)
HDL-cholesterol (mmol/L)	1.0 (0.2)	1.0 (0.2)	1.2 (0.3)	1.1 (0.2)
Triglycerides (mmol/L)	0.8 (0.3)	1.0 (0.4)	1.0 (0.5)	0.9 (0.5)
Insulin (mU/L)	18.8 (11)	20.1 (11)	15.3 (9)	15.1 (9)

N=1,118 for systolic BP; N= 1,050 for lipids, and N=1,008 for insulin; Values presented are means (standard deviations)

Data show that the Hyderabad children were shorter and leaner than the Minnesota children. There were no notable differences in the systolic BP and lipid levels; however, fasting insulin levels in the Hyderabad children were clearly higher than those of the Minnesota children. This is surprising in view of

the far higher adiposity levels of the Minnesota children, with which insulin levels are generally associated.[269;316] If these differences are not due to study differences, they support the presence of a pro-atherogenic profile in undernourished children. Birthweights of these Minnesota children were 3.5 kg (boys) and 3.4 kg (girls), as compared to 2.7 kg (boys) and 2.6 kg (girls) in the Hyderabad study. Augmentation index was not measured in this study; however AIx in the youngest age band of the Anglo-Cardiff collaborative study (mean age: 19 years; N=305) was 2%,[275] as compared to 4.0% in Hyderabad (mean age: 15.9 years; N=870). As augmentation index increases with age, this again supports the possibility of a pro-atherogenic profile in undernourished children (bearing in mind the limitations of such cross-study comparisons).

8.3.1. Determinants of CVD risk

The determinants of CVD risk factors in this population were, in the main, consistent with what would be anticipated in this age group. However, there were important differences too.

Body size and the components of body composition were positively associated with age and pubertal stage, as expected. Boys and girls had similar BMI. Analyses of its components revealed that while girls had more fat mass, boys had had more fat free mass. This is consistent with what is known about the evolution of BMI and its components during this period.[6;62;239;244] BMI increases continuously in boys and girls during adolescence, as there is general growth of body mass throughout childhood. The relative contributions of fat and fat free mass to this growth, however, differ by sex. While females have a proportionate increase in fat mass, the rise in BMI in males is largely due to a disproportionate increase in fat free mass from the start of adolescence. The pattern for abdominal circumference generally resembles that of BMI (as was the case in this study), since it is a similar composite measure of fat and fat free mass.[62] Percentage of

body fat as measured by skinfolds or bioelectrical impedance is higher in females than males at all ages, and shows a sharp decrease in males after a peak at the age of 10 to 12 years.[62] The sex differences in the evolution of the components of body composition further supported the use of specific indices of body composition over composite markers such as BMI and waist circumference.

Higher socio-economic position was positively associated with height, fat mass and fat free mass. While the association between socio-economic position and height is consistently positive, the association between socio-economic position and fat mass tends to be in opposing directions in countries on either side of the epidemiological transition.[311] Unlike the trends in high-income countries in the recent decades, adiposity in low-income countries including India is positively associated with socio-economic position.[140;310;311] The association of socio-economic position with fat free mass may reflect better growth of all components of body mass generally, as a consequence of better nutrition in children from well-off households. Interestingly, data suggests that the established positive association between birthweight and later BMI reflects a positive association between birthweight and fat free mass (and not fat mass).[50;277-284] If true, these data would be consistent with the findings from the present study (i.e. a positive association between socio-economic position and fat free mass), since one would expect birthweight to be higher in those born in affluent households.

Urbanisation was not associated with body size and composition in this population. The absence of a relationship between urbanisation and body composition may reflect absence of sufficient variation in the exposure (the villages were quite similar in their development) or the young age of the population (social differences in adiposity generally manifest at later ages).

Increasing age, male sex and pubertal stage positively predicted blood pressure. Blood pressure is known to rise in adolescence and the relatively modest increase in girls during this age may explain the higher overall levels noted in boys.[74] They may also reflect differences in height, as blood pressure is associated with height.[306;307] Height, fat free mass and central-peripheral skinfolds ratio (but not fat mass) were positively associated with blood pressure, especially systolic BP.

The association between fat free mass and BP has been previously reported.[277;306;308;309] A large body of research supports the association of BMI with BP, which is assumed to reflect an association between adiposity and BP (generally in the absence of data on components of BMI). If both fat mass and fat free mass contributed to the association of BMI with BP (which may be a reasonable assumption, since greater pressure may be required to maintain perfusion over a greater mass, as is assumed for height-BP association),[306;307] then in a lean population the association between fat free mass and BP would dominate the association between fat mass and BP (lack of power to detect an association with fat mass due to insufficient variation in fat mass). Indeed, given the association between central-peripheral skinfolds ratio and fat free mass in this population, the association of central-peripheral skinfolds ratio with BP may also be reflective of the association between fat free mass and BP (rather than central adiposity, the presence of which had little face validity in this lean population).

Current socio-economic circumstances and village urbanisation showed no clear association with blood pressure. This may reflect a combination of relative homogeneity in the socio-economic circumstances of this population (both individual and village level), and the late manifestation of social differences in BP seen in most settings.[92]

Age and pubertal stage were not associated with augmentation index. Augmentation index increases with age and this association is more prominent at younger ages.[275] The narrow age range of the study population is the most likely reason why this association was not seen. Height and heart rate inversely predict augmentation index, and this was confirmed in the present study.[117;274-276] These associations are believed to be independent of the associations with other risk factors such as BP; hence, augmentation index is routinely adjusted for heart rate and height, as was done in this study. At all ages, females tend to have higher augmentation index as compared to males and this was confirmed in the present study.[117;274-276] The reason for this difference is not clear: differences in height and physical fitness have been proposed as explanations,[274;275] but the difference in augmentation index between the sexes persists despite adjustment for height and heart rate. Similar to BP, village urbanisation and measures of body composition bore no relationship to augmentation index, which again may be reflective of lack of sufficient variation in these exposures.

There was no clear trend for an association between lipid concentrations and age or pubertal stage. Lipid concentrations change only gently with age, and cross-sectional studies are often been unable to pick such weak trends.[6] There were modest, inconsistent but generally positive associations between lipid concentrations, fat mass and socio-economic position (or urbanisation). This reflects the pattern seen in other studies from similar settings. The positive direction of the associations presumably reflects the opportunities for better nutrition among children from well-off families. The inconsistency of these associations may reflect the age group: such associations have often been absent in children but present more clearly in adults.[92] Social differences in adiposity and its related measures are probably dependent on wide variations in diet and physical activity patterns to manifest, and these are generally absent in children.

There were no clear trends for associations between measures of glucose-insulin homeostasis with any of the determinants, except for a weakly positive association between fat free mass (not fat mass) and insulin (or HOMA score). This association is uncommonly reported and may reflect inadequate adjustment for fat mass.[277] Equally, it could be a chance finding. Similar to BP, it may also be speculated that the modest association of insulin with fat free mass simply reflects an increase in insulin requirement with greater body size.

8.3.2. Supplemental nutrition and CVD risk

Table 8.4 summarises the strength of evidence in support of early nutritional programming (for the main risk factors) from different sources (animal experiments, observational and other experiments) and relates it to results from the present study.

In the analyses on the effects of supplementation on CVD risk, no association of supplementation with the timing of the onset of puberty was seen. This was to some extent surprising given that improvements in nutritional status has been associated (although not consistently) with an earlier age of onset of puberty.[238;245;246] The most likely explanations include the relative modesty of the intervention (especially if one takes into account the incomplete uptake and possible sharing of the supplement), its uni-generational nature (many population studies showed secular tends over generations) and the inadequate precision of the tool used to assess puberty.

Supplemented children were clearly taller: the mean difference in height (control minus intervention) was 14 mm (4.1, 23.1); p=0.007. In the Guatemala trial, those given the high-energy supplement were on average 22 mm taller.[26] All the participants in the Guatemala trial received supplements, while probably only

about half of the participants received supplements in this study. Furthermore, the participants in Guatemala trial had completed their growth (mean age 24 years), while the children in the Hyderabad study are still growing. Nutrition supplementation trials from the UK have reported more modest height gains of 3.7 mm (food supplements for 12 months in childhood)[222] and 2.9 mm (milk supplements for 21 months in childhood).[221] It is unclear whether the modest gains in height in these studies reflect: (a) shorter duration of supplementation, (b) timing of supplement (childhood, not earlier), (c) less severity of undernutrition at the outset, or (d) all these factors combined. Shortness of stature is a long- and well-established risk factor for CVD in populations across the world.[49;317]

Table 8.4: *Summary of evidence: Early nutrition and CVD risk*

Outcome	Timing of exposure	Strength of evidence according to source			
		Animal experiments	Observational studies	Human experiments*	Hyderabad study
Increased BP	Antenatal	+	++	-	+/-
	Infancy	+	++	-	+/-
	Post-infancy	0	0	-	+/-
Dyslipidaemia	Antenatal	+/-	+/-	+/-	-
	Infancy	+	+/-	-	-
	Post-infancy	0	0	0	-
Adiposity	Antenatal	-	-	+/-	-
	Infancy	+	+/-	-	-
	Post-infancy	0	0	-	-
Abnormal glucose-	Antenatal	++	++	+/-	+
insulin homeostasis	Infancy	++	+	+/-	++
	Post-infancy	++	0	+/-	++

Strength of evidence: ++ = Strong evidence for an effect; + = Moderate evidence for an effect; +/- = Borderline/inconsistent evidence for an effect; - = Evidence against an effect; 0 = Lacks research

**Human experiments category includes the Dutch Winter and Leningrad famine studies, and the Guatemala trial*

There was some evidence for greater BMI and fat free mass in control children, but no difference in fat mass index and central-peripheral skinfold ratio. The mean difference in fat free mass index (control minus intervention) was 0.24 kg/m^2 (0.06, 0.43); p=0.01. There is no obvious explanation for greater lean mass in the participants from the control area, and appears to be a chance finding. One potential explanation could be that control children worked more in the fields, but the lack of attenuation of this association on adjustment for occupation of the child or the current socio-economic position (indeed current socio-economic position was positively associated with fat free mass index) argues against this, as children from more affluent households would be less likely to engage in fieldwork. In the Guatemala trial, the participants from the two supplement groups did not differ in their BMI or waist-hip ratio.[26]

Existing evidence on fetal programming of adiposity is weak and inconsistent, and to some extent favours overnutrition (not undernutrition) in early life. Birthweight is generally positively correlated with later BMI, although studies with more specific measures of adiposity (e.g. skinfolds, DXA scans) have been less consistent in their results.[50] Smaller sample sizes of many of these studies, along with inadequate control for confounding (especially socio-economic position) may account for this inconsistency. Some studies have found an association between rapid growth in infancy (and/or early childhood) and later adiposity.[51;80;173] Although this may be taken as evidence supportive of fetal programming (on the assumption that rapid growth in this period reflects catch-up following undernutrition in fetal life), the methodological limitations in analysing correlated growth data make it extremely difficult to discern if this is so, and more specifically if rapid growth is somehow causally involved in the evolution of obesity (rather than simply reflecting the process).

This study was also unable to confirm or refute the hypothesis that some of the effects of early nutrition on later risks of cardiovascular disease may be mediated

through poor development of muscle mass.[50;277-279;283] Poor muscle mass could potentially influence CVD risk through insulin resistance (blunted response of insulin receptors present in skeletal muscle) or altered vascular resistance.[11;12;278;279] Currently, there is limited evidence to support these suggestions. In this study, there was no evidence to support underdevelopment of muscle mass, as indexed by fat free mass index, which admittedly is a crude measure. Furthermore, there is now a growing number of studies showing a positive association between size at birth and fat free mass in later life. [50;277-284] It is possible to speculate that mass itself does not necessarily equate functionality: if underdeveloped muscle fibres were being compensated for by fat and fibrous tissue, then the fat free mass index would remain unchanged (or may even be more due to overcompensation).[318] However, there is no real evidence to support this. Further studies with more sophisticated measures of muscle composition and functionality may shed light on this.

Systolic blood pressure was 0.6 mmHg lower (-1.1, 2.3; p=0.4) in the supplemented children, although the difference was consistent with random variation. In the Guatemala trial, blood pressures were not different between the two supplementation groups.[26] Sample size calculations presented earlier suggest that both studies may have been underpowered to detect smaller differences. Systematic reviews of studies have suggested that the mean systolic BP decreases by 1-2 mmHg for each kilogram increase in birthweight.[21;160;161] Relating this figure to the present study (61g birthweight difference), one would expect to find ~0.1 mmHg difference in BP between the two arms. Assuming that the BP difference estimated in the study is accurate, this would be consistent with either of the two suggestions: birthweight provides only a crude summary measure of the effects of fetal undernutrition or birthweight is merely an index for nutritional (and other) conditions in early life (i.e. it includes pre- and post-natal periods). This is relevant here, of course, as the nutrition intervention was given until the age of six years.

Augmentation index was clearly lower in the supplemented group with a mean difference (control minus intervention) of 3.3 % (1.0, 5.7), p=0.008. There are no previous studies of nutrition intervention in humans or animals for comparison; however, few studies have examined the association of arterial stiffness with birth weight and arrived at differing conclusions.[126;128;129;230;319-321] A summary of these studies presented in Table 8.5 suggests an explanation for these differences. Arterial stiffness can be assessed either locally for a particular segment of the arterial tree (e.g. pulse wave velocity, distensibility, compliance) or more globally for the entire arterial system (augmentation index).[96;98] The arterial tree is made up of the large, elastic arteries (such as aorta and its main branches, including the common carotids) and the smaller, muscular arteries (such as the limb arteries, their branches and small arterioles).[95] Global measures of arterial stiffness such as augmentation index depend on the timing and magnitude of the reflected wave. The time of appearance of the reflection depends on the pulse wave velocity (related to the elastic properties of large arteries), while the magnitude of the reflected wave depends on the more peripheral muscular arteries and the peripheral resistance in the arterioles.[97] The results from these studies suggest that arterial stiffness in low birthweight individuals originates in the smaller, muscular arteries rather than the large, elastic ones. This is particularly relevant here as insulin's predominant action is to decrease peripheral vascular resistance by increasing blood flow in skeletal muscle.[322]

In the only study assessing the association between birthweight and augmentation index, AIx values were 3.9% in children with birthweight below 2.5kg and 0.7% in children with birthweight above 3.5kg.[128] The study was conducted in Spanish adolescents aged 7 to 18 years, but with a mean age of 11 years. The sample size was also small (n=219), with only 17 children in the below 2.5kg birthweight category. In the Hyderabad study, the AIx values were 5.6% (controls) and 2.5% (intervention). The multivariably adjusted estimate for change in augmentation index per kilogram of birthweight in Spanish adolescents was 1.1%. On this basis, the expected difference in augmentation

index between the two arms of Hyderabad study would be 0.07%, which is lower than the observed difference of 3.3%. This difference could be due to sample size and population differences, or may to some extent reflect the importance of postnatal nutrition.

Table 8.5: *Arterial stiffness and birthweight*

Study	N	Age (yrs)	Measure	Result by vessel type
Martyn[126]	208	55	L	LE = No SM = Yes
Montgomery[319]	528	25	L	LE = No LE + SM = No
Styczynski[321]	142	21	L	LE = No
Kumaran[230]	435	50	L	LE = No LE + SM = No
Oren[320]	422	28	L	LE = No*
te Velde[129]	281	36	L	LE = No** SM = No**
Lurbe[128]	219	11	S	Yes
Hyderabad	862	16	S	Yes

Measure (stiffness): L = local/regional (e.g. PWV, compliance); S = systemic (AIx)

*Result by vessel type: LE = large, elastic artery (aorta and its main branches); SM = small, muscular artery (limb arteries); Yes = supports fetal programming (i.e. inverse association between birthweight and arterial stiffness); No = does not support fetal programming; * Result goes in opposite direction, ** After height adjustment*

In a large cohort of 4,100 healthy, normotensive volunteers (Anglo-Cardiff collaborative trial) aged 18 to 90 years, AIx and PWV were measured to assess normal vascular ageing.[275] Age-related changes in the two measures followed different patterns: changes in AIx were more prominent in younger individuals (<50 years), whereas changes in aortic PWV were more marked in older individuals (>50 years), suggesting that AIx was a better marker of vascular

ageing in younger individuals. The mean augmentation index in the youngest age group (18 - 20 years) was 1%, as compared to 2.5% (intervention) and 5.6% (controls) in Hyderabad study. There is a suggestion here of the adverse effects of chronic undernutrition and its partial amelioration by supplemental nutrition; however, the differences in study design and populations do not allow for robust comparisons.

There were no differences in the lipid concentrations between the supplemented and control areas. This would be consistent with the lack of convincing evidence for programming of lipid metabolism from animal experiments and epidemiological studies.[18;166;167] A systematic review reported a 0.04 mmol/L fall in total cholesterol for each kilogram increase in birthweight.[167] Based on this estimate and the 61g birthweight difference in the Hyderabad study, the expected difference would be infinitesimally small and probably of limited public health relevance.

There was no difference in fasting glucose, but important differences in fasting insulin and HOMA scores. The HOMA score was 20% lower (3 to 39; p=0.02) in the supplemented children. In the Guatemala trial, modest effects on fasting glucose were seen, and in women only.[27] Capillary glucose was taken from finger prick (which is a less reliable method) and insulin was not measured. The programming of insulin resistance has strong basis in animal experiments and observational studies.[18;163] Consistent with the present study, epidemiological studies in children generally show an inverse association between birthweight and fasting insulin (but only inconsistently with glucose),[163] suggesting that insulin resistance may be one of the earlier steps in programming of cardiovascular diseases and diabetes.

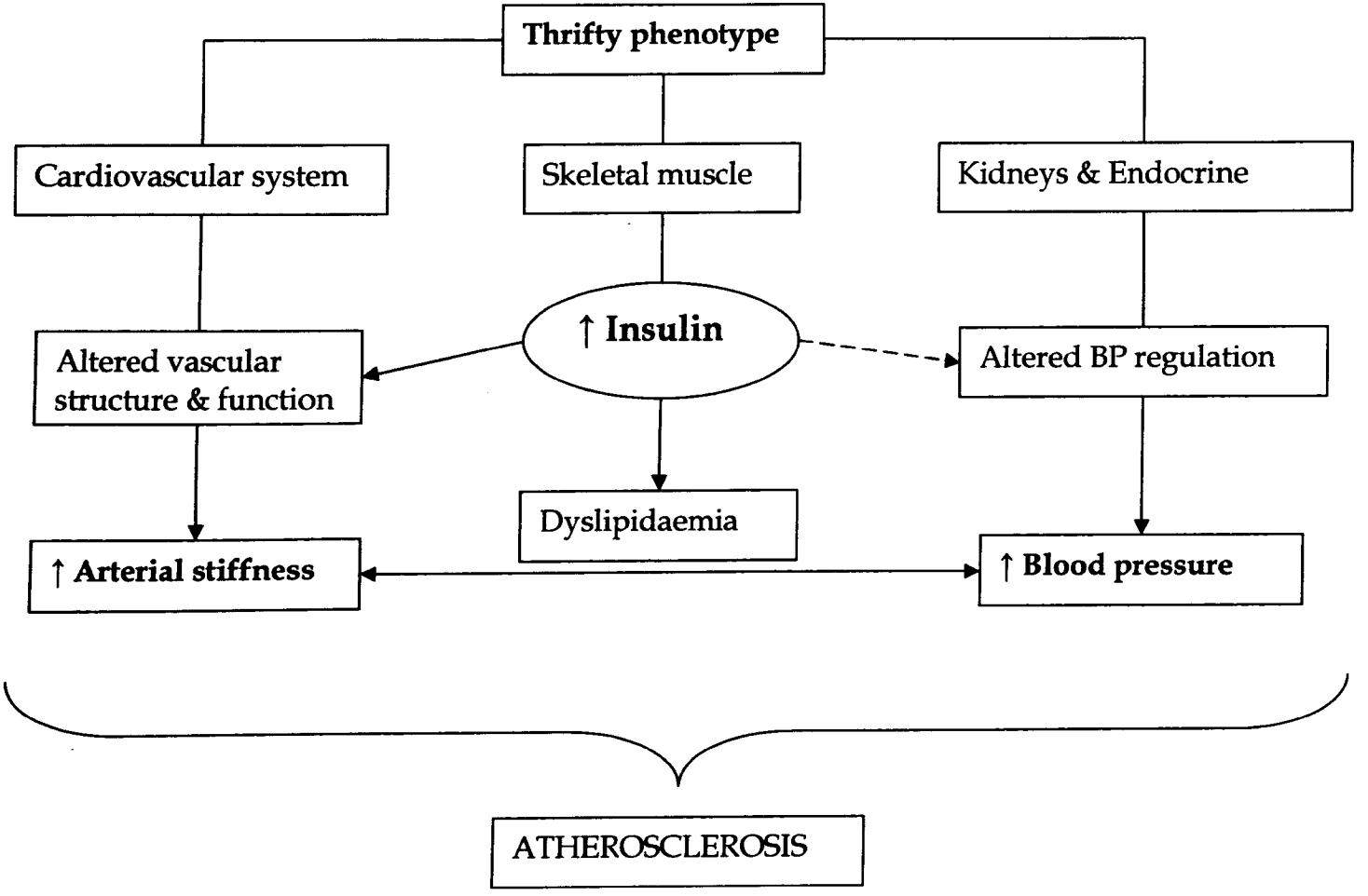
8.4. Potential mechanisms

The key findings in this study were the higher levels of insulin (resistance) and arterial stiffness in the unsupplemented children, accompanied by a modest rise in blood pressure. Figure 8.1 shows the potential pathways for these changes following early undernutrition.

8.4.1. Pathways to disease

Insulin resistance is believed to be central to many of the other changes attributed to the thrifty phenotype; however, the exact mechanisms by which undernutrition in early life results in insulin resistance are not known.[18;292] It has been suggested that insulin resistance is a consequence of persistent glucose-sparing metabolism.[323-325] Insulin is a major anabolic hormone in fetal life that promotes growth. In times of undernutrition, the fetus may develop insulin resistance in specific tissues such as skeletal muscle to conserve glucose and reduce growth, thereby improving its short term chances of survival.[323;325] However, persistence of insulin resistance in adult life is accompanied by a host of accompanying metabolic abnormalities, especially when food is available in excess.[12;316;323;326] Although the exact role of insulin resistance in hypertension is somewhat unclear, insulin resistance has been clearly linked with other risk factors of CVD (dyslipidaemia, adiposity, diabetes) and directly with CVD.[316;326-329] It is especially relevant to this study as Indians are known to have high levels of insulin resistance.[133-136] Whether this predisposition originates in genes, early environment or even later environment is not certain, although differences in insulin resistance are apparent even in childhood.[133;323;326;330-333]

Figure 8.1: Pathways to disease



For fetal programming of blood pressure, a range of mechanisms are postulated that involve the endocrine, renal, vascular and neural pathways.[11;325;334] Activation of renin-angiotensin system, increased sympathetic nervous activity and resetting of the hypothalamic-pituitary-adrenal axis can all potentially be induced as a result of fetal undernutrition and also lead to higher blood pressure.[11;335;336] Reduced number of renal nephrons (as a result of undernutrition) resulting in hyperperfusion and sclerosis in the remaining nephrons could also initiate a self-perpetuating cycle of progressive hypertension and glomerular injury.[337;338] However, potentially the most relevant mechanisms are those that relate to changes in the vascular system.

Arterial stiffness is a relatively novel risk factor for atherosclerosis.[105] There is ongoing debate as to whether it is merely a risk marker for atherosclerosis (i.e. reflects the process) or an independent risk factor in its own right (i.e. has an aetiological role).[105;118;121;339;340] While definitive answers must await results of ongoing longitudinal studies, current sum of evidence suggests that it may have some aetiological relevance, even if it is not the initiator.[118;120;121] Various other programming pathways are believed to culminate in a self-perpetuating cycle of increasing blood pressure and arterial stiffness, with altered shear stress in stiffer arteries further providing nuclei for atheroma formation.[105;339] The original hypothesis by Martyn and Greenwald proposed that the impaired synthesis of elastin in walls of large conduit arteries may be the initiating event in pathogenesis of hypertension following early undernutrition.[125] On the basis of the evidence so far (i.e. involvement of smaller muscular arteries primarily) this appears unlikely, although it cannot be ruled out entirely as even the smaller muscular arteries contain elastin; they may simply be manifesting earlier due to their narrower lumens. One of the several other mechanisms postulated for arterial stiffness may be responsible.

Redistribution of blood supply established at the time of fetal undernutrition to conserve nutrient supply to important areas such as brain could lead to persistent changes in structure and function of the arteries.[341;342] Changes can be the result of altered shear stress in the vessel lumen or reduced metabolism accompanying the reduction of blood supply in the peripheries. Proposed mechanisms include impaired angiogenesis (both the number of vessels and the pattern of the bifurcations), altered elastin-collagen ratio (reduced elastin production) or the tone of the smooth muscles in the vasculature (due to impaired development of muscle fibres).

However, the majority of evidence suggests a central role for impaired vascular endothelium. Vascular endothelium can be impaired as a result of altered shear stress[343] and reduced metabolism due to vascular redistribution (e.g. fetal undernutrition) or also by infections; impairment of vascular endothelium has been correlated both with low birthweight[127;344] and chronic inflammatory markers in humans[345] and undernutrition in animal experiments.[18] Vascular endothelium is involved in local regulation of blood pressure by release of vasoactive peptides such as Nitric Oxide (which act on smooth muscle tone),[346] but can also produce long-term changes by influencing elastin production.[347] Vascular endothelium is generally assessed as flow mediated dilatation in the brachial artery (using ultrasonography) and has been correlated with arterial stiffness and other CVD risk factors, especially insulin.[127;348;349] Indeed, keeping with its proposed central role, insulin can not only influence the functioning of the endothelium via stimulation of nitric oxide synthesis[350] but also result in structural changes by proliferation of smooth muscle cells[351] or enhancing the rate of elastin degradation over time, a normal process of vascular ageing.

In the present study, insulin levels were associated with BP but not with arterial stiffness. This may be because in the evolution of the cycle of increasing blood pressure-arterial stiffness, associations may go in the either direction in the short

term due to temporary compensations and decompensations.[339;352] Interestingly, insulin resistance (which can be associated with hypertension)[327] is accompanied by higher levels of insulin (which acts as a peripheral vasodilator through enhanced production of nitric oxide by the endothelium), suggesting a complex relationship between insulin sensitivity, hypertension and endothelial function.[353] Although one would expect the true relationships between these variables to become apparent over time in longitudinal studies, cross-sectional associations are likely to be uninformative and may even be misleading. It was for this reason that associations of blood pressure and arterial stiffness were examined with other explanatory variables but not with each other.

In terms of molecular-level changes underlying these overt changes at the level of organ systems, several mechanisms have been proposed, first by Lucas[354] and more recently updated and reviewed by Waterland and Garza who put them under five broad categories.[19] The five candidate mechanisms include: (a) induced variations in organ structure, (b) alterations in cell number, (c) clonal selection, (d) metabolic differentiation, and (e) hepatocyte polyploidization. The first one refers to gross morphological changes occurring during organogenesis (for example in organ vascularisation or innervation) to differentiate it from organ changes resulting from more microscopic changes in cell number (reduced proliferation of differentiated cells or even earlier selection of founder cells) and function (altered expression of genes resulting in changes in quantities of enzymes and hormones). In regard to the last, epigenetic mechanisms (heritable changes in gene function that cannot be explained by DNA sequence) show promise. Finally, polyploidization (more than the normal complement of chromosomes) of the liver cells at the developmental changes can lead to long-term changes in their metabolic activity. Each of these molecular mechanisms can apply to any of the above organ pathways and the evidence to date does not favour any of these over others. Further research is needed to identify which of these are relevant and the timing at which these may act.

8.4.2. Timing and nature of exposure

Since the supplementation was given during pregnancy, lactation and early childhood, it was not possible to investigate whether one period is more important than another i.e. if there was a critical window when such an intervention may be acting. It could be argued that the postnatal period was equally if not more important than the prenatal period because: (a) the observed differences in outcomes were greater than what could be explained by birthweight differences as a result of the intervention, and (b) the intervention to the child was more direct than the intervention given to the fetus (through maternal intake, which may not reflect fetal supply accurately). However, given that the birthweight is a crude indicator of fetal circumstances and most development takes place over the perinatal period including the first few years of life, the entire developmental period may be implicated.[11;23] Birthweight and infancy growth data would have been informative; however, although they were collected in the baseline study, they were too few (less than 50%) and potentially biased in the final study sample for any meaningful analyses.

Although this study shows that the causal exposure was operative in early life, once cannot be certain about its precise nature, as the intervention had multiple components including immunisation. Although immunisation is available free in all areas, previous evaluations have shown that ICDS programme areas tend to have relatively higher rates of immunisation.[40;45;46] Despite this, nutrition remains the primary candidate here: it is the centrepiece of the programme (and the main incentive for participants) and the differences in nutritional status of the participants were evident - birthweight, but also height which is consistently associated with risk for CVD.[11;23;49;317] Furthermore, nutritional programming is supported by a stronger body of research evidence than say chronic infections.[355] Even if immunisation and other measures are important, it is conceivable that

they act through improvements in nutrition: by helping to break the vicious cycle of infection-malnutrition in these settings; diarrhoea and other infections being one of the main risk factors for undernutrition.[356]

The nutrition supplementation given in this study was balanced protein-calorie. Research summarised in this thesis suggests that balanced protein-calorie malnutrition is more important than isolated protein deficiency. There is inconclusive evidence on the effect of some micronutrients on fetal growth, and on the risk of cardiovascular diseases (especially folic acid).[357-359] However, micronutrients were not added to the supplement in this trial, and previous evaluations have shown only modest improvements in the uptake of other nutritional programmes delivered through the ICDS scheme (available also in the non-ICDS areas).[45;46;360] This is particularly true of the anaemia control programme, which has regularly been shown to be ineffectual, in its impact on anaemia, as well as on the consumption of iron and folic acid tablets.[360] Therefore, on the basis of the intervention given, its effects on growth (i.e. birthweight and height of the children), and the strength of previous evidence, one would have to favour balanced protein-calorie supplementation as the causal exposure in this trial, although some facilitatory role for micronutrients cannot be ruled out.

8.5. Implications and further work

This study has shown that protein-calorie supplementation given to pregnant and lactating women, and the ensuing offspring in early childhood, can result in long-term changes in the physiological and metabolic profile of the offspring. These changes are discernable at adolescence and most apparent for vascular stiffness and glucose-insulin homeostasis, less so for blood pressure. Although these improvements in the pro-atherogenic profile of undernourished children appear to be modest, they are nevertheless important for several reasons.

Augmentation index was 3% less in the supplemented area, which suggests a less aged vascular phenotype. Using regression equations for the lowest age band (18 - 20 years) in the Anglo-Cardiff collaborative study detailed earlier, I estimated that the augmentation index changes by ~1% per year at this age.[275] Extrapolating this data to the Hyderabad study (3.3% difference in AIx between the intervention and control arms) suggests that the intervention improved (i.e. reduced) the vascular age of the recipients by around 3 years, a substantial gain given the mean age of 16 years.

A meta-analysis of existing studies found that 7 mU/L lower insulin level was associated with an 18% increase in occurrence of CVD. Assuming causality, extrapolating this association to the Hyderabad study suggests a 8% reduction in CVD risk among supplemented children (20% of 15 mU/L = 3 mU/L difference).[328] In a large study of Chicago men aged 18-39 years, 1 mmHg increase in systolic blood pressure was associated with a 1.6% increase in risk of death from CVD within 20 years.[72] This would translate to a 1% lower risk of death from CVD within 20 years in the supplemented children.

There are inherent assumptions and limitations of such extrapolations across studies (such as measurement protocols) and populations (such as differences in ageing profile). The apparent differences in CVD risk in this study are small. However, many of the CVD risk factors are known to track and also amplify with age.[6;7;361] Importantly, these children were just at the age when behavioural risk factors such as tobacco consumption and reduced physical activity start to develop. If, as has been previously proposed, the impact of these behavioural risk factors is disproportionally stronger in those predisposed by early undernutrition (i.e. effect modification), then one would expect a more prominent amplification in this risk difference.[12;325]

The intervention (i.e. nutrition supplementation) in this case was given within the framework of a public welfare programme.[45;46] The amount of supplement was small and often shared within family members, further reducing the amount. The programme often suffers from problems of food supply resulting in intermittent availability. Bearing these issues in mind, the study most likely underestimates the potential for benefit. On the other hand, the results do provide more realistic estimates of the expected benefit in the `real world'.

The ICDS programme is the largest national food supplementation programme in the world with over 25 million beneficiaries.[42;43] Similar programmes exist around much of the developing world.[362;363] These programmes were introduced to address undernutrition and infections, not for any perceived benefits in cardiovascular diseases prevention. Concerns have been raised recently about the advisability of such programmes in view of the obesity and cardiovascular diseases epidemic.[52;53] This study has confirmed that food supplementation in settings with high prevalence of undernutrition does not fuel obesity and may even reduce (not increase) the risk of CVD. Further programmes are not likely nor necessary on the basis of this evidence. However, this study provides further support for strengthening the existing programmes of supplemental nutrition in settings with undernutrition.

Several questions remain unanswered. This study was unable to identify the relative importance of different time periods (prenatal, infancy or post-infancy childhood) or the nature of supplementation (balanced protein-calorie, protein only or micronutrients) in influencing CVD risk. The relevance of birthweight and childhood growth could not be clarified. Nor could the importance of improvements in nutrition in settings where populations are generally well-fed. How these early maladaptations interact with later behavioural risk factors to influence the long-term morbidity and mortality due to cardiovascular and other

diseases can also only be speculated upon in this study. All these questions need to be answered to have a better understanding of the aetiopathogenesis of cardiovascular diseases and its links with early circumstances, especially nutrition. Suitable prospective studies are difficult to establish and take a long time to complete. Previous nutrition supplementation studies and programmes offer a useful setting to examine these issues.[362;363] Importantly, they provide a pragmatic study design that can answer important public health questions without necessarily allowing full understanding of underlying mechanisms that may be less relevant.

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APPENDICES

Appendix A: Ethics approval letter

Appendix B: Information sheet and consent forms

Appendix C: Abstracts of questionnaire & clinical datasheets

Appendix A: Ethics approval letter

NATIONAL INSTITUTE OF NUTRITION
Hyderabad – 500 007

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REPORT OF INSTITUTIONAL REVIEW BOARD

Sept. 1, 2005

This is in continuation of the Institutional Review Board meeting held on 2nd July, 2003 in connection with the ethical committee clearance of the project entitled "The impact of supplemental nutrition in pregnancy and infancy on cardiovascular disease risk profile in childhood" by Dr.K.V.R.Sarma, Dr.Ghafoorunissa and Dr.M.Vishnuvardhan Rao, wherein the committee had recommended that both PIs should submit revised proposal with the required information.

As directed by the Ethical Committee, the revised project has been submitted by the investigators. The committee, after being satisfied that different ethical issues have been addressed, hereby gives its clearance for the project.

(Dr. B. Sivakumar)
Director
NIN, Hyderabad

Appendix B: Information sheet and consent forms

NIN LOGO

Date and version number

NATIONAL INSTITUTE OF NUTRITION
INDIAN HEALTH RESEARCH CENTRE
HYDERABAD - 500 007.

Research on chances of getting heart disease in middle age:

effect of nutritious diet during pregnancy and birthweight

We invite you and your family to participate in a health research programme. This research is conducted by doctors and researchers at the National Institute of Nutrition, Hyderabad and University of Bristol, England.

Some doctors believe that there are chances of getting heart diseases during middle age due to poor nutrition during childhood, particularly from the time of being in the mother's womb. By this study, we are trying to know whether this is true or not. This important information will be useful in preventing these diseases by providing proper diet to pregnant women.

You (Girl/Boy) have been chosen for this study as your mother participated in an earlier study conducted by National Institute of Nutrition when she was pregnant with you. At that time, some but not all the participants were provided with extra food with the help of the Anganwadi. We are trying to know from this research whether the chances of getting heart diseases have been reduced in the children born to the women who got extra food.

As a part of this study, you will be asked some health related questions, undergo health check-up and blood test. You will not have any discomfort as these tests will be conducted by well trained research team along with an experienced doctor. As your health is related to your parent's health, we will ask them few health related questions, and if possible measure their height and weight.

The information obtained from this research will be stored in computers at Hyderabad and Bristol, and will be analysed along with information from the previous study to provide answers to research questions. The results will be published in research magazines and reports. The study participants will not be recognisable by others, either by their names or by any other details.

Any remaining blood sample will be preserved and will be used for genetic research. These results will be useful to know whether these diseases are hereditary. We may contact you in the future to know the details of your health.

You are free to decide whether to participate in the study or not. This research is being done to improve medical knowledge. You may not gain anything directly but you will be informed about any abnormality found in the tests and will be given medical advice.

We thank you for cooperating and participating. We hope you will enjoy participating in this study. If you have any doubts about this research feel free to contact Dr. Rameshwar Sarma at the National Institute of Nutrition.

NIN LOGO

Date and version number

NATIONAL INSTITUTE OF NUTRITION

INDIAN HEALTH RESEARCH CENTRE

HYDERABAD - 500 007.

CONSENT FORM

Research on chances of getting heart disease in middle age:

effect of nutritious diet during pregnancy and birthweight

Consent of the participant:

I have been informed about the nature of this research and also the role of my family. After taking sufficient time to know all the details of the study, I give my consent for the participation of my family in this study (by word/ signature/ recording).

Signature of participant..... Date.....

Signature of mother/father..... Date.....

Signature of the researcher..... .... Date.....

Signature of the witness .... Date.....

(in case of thumb impression)

NIN LOGO

Date and version number

NATIONAL INSTITUTE OF NUTRITION

INDIAN HEALTH RESEARCH CENTRE

HYDERABAD - 500 007.

VILLAGE HEAD CONSENT FORM

Research on chances of getting heart disease in middle age:

effect of nutritious diet during pregnancy and birthweight

The study participants from my village and I have been provided with sufficient information about the study and we were given adequate time to reflect and ask questions. I, hereby, consent to the participation of residents from my village in this study.

Name of the village.....

Signature of the village head

(or representative)..... Date.....

Signature of researcher..... Date.....

Appendix C: Abstracts of questionnaire and clinical datasheets

Questionnaire abstract (relevant questions)

No	Question	Response	Code
	DEMOGRAPHIC		
	Date of birth		[DD]
	Month of birth		[MM]
	Year of birth		[YYYY]
	Sex		[1=Male; 2=Female]
	What is your main activity?		[1=Student; 2=Employed; 3=Unemployed/seeking work; 4=At home doing housework; 5=Vocational training; 6=Other, specify]
	What is the educational level of your mother?		[1=Illiterate/no schooling; 2=Primary school/literate; 3=Middle school completion; 4=High school certificate; 5=High school + (HSC, ITI, Intermediate, Post high school diploma); 6=Other graduate (BSc, BCom, DME, DHMS, BPNA); 7=Professional degree (MA, MSc, MCom, BTech, MBBS, BE, MSW, PhD]
	LIFESTYLE		
	Have you ever used tobacco in any form (smoked, chewed, snuff) on a daily basis i.e. at least once a day?		[1=Never; 2=Yes, but don't any more (i.e. stopped over 6 months ago); 3=Yes, and still use it (up to last 6 months)
	Do you consume alcoholic beverages regularly (i.e. at least 10 days a month)?		[1=No, never; 2=Yes, but don't any more (i.e. stopped over 6 months ago); 3=Yes, and still do (up to last 6 months)]
	PUBERTAL STAGE: FEMALE PARTICIPANTS ONLY		
	Have you started having periods?		[1=Yes; 2=No]
	If yes, how old were you when your periods first started?		[YY:MM; age in completed years and months]
	If married, are you currently pregnant or breast feeding?		[1=Yes; 2=No]

No	Question	Response	Code
	STANDARD OF LIVING INDEX		
	What is the material used in the construction of your house (roofs/walls/floor)?		[1=Kutcha (made from mud, thatch or other low quality material); 2=Semi-pucca (part low quality and part high quality material); 3=Pucca (high quality material throughout including roof, walls, and floor)]
	Do you have a separate room that is used as a kitchen?		[1=Yes; 2=No]
	What is the main source of lighting for your household?		[1=Kerosene/gas/oil; 2=Electricity; 3=Other, specify]
	What is the main source of drinking water for your household?		[1=Pipe, hand pump or well (public); 2=Pipe, hand pump or well (residence/yard/plot); 3=Other, specify]
	What type of fuel does the household use for cooking mainly?		[1=Coal, charcoal or kerosene; 2=Electricity, liquid petroleum gas or biogas; 3=Other, specify]
	What type of toilet facility does the household have?		[1=Own flush toilet; 2=Shared/public flush toilet; 3=Own pit toilet/latrine; 4=Shared/public pit toilet/latrine; 5=No facility/bush/field; 6=Other, specify]
	Does the household own this house or any other house?		[1=Yes; 2=No]
	Does the household any agricultural land?		[1=Yes; 2=No]
	How much agricultural land does the household own?		[Land in acres; enter 0 for none]
	Out of this land, how much is irrigated?		[Land in acres; enter 0 for none]
	Does this household own any livestock?		[1=Yes; 2=No]

	Does the household any of the following goods:		
	A mattress		[1=Yes; 2=No]
	A pressure cooker		[1=Yes; 2=No]
	A chair		[1=Yes; 2=No]
	A cot or bed		[1=Yes; 2=No]
	A table		[1=Yes; 2=No]
	A clock or watch		[1=Yes; 2=No]
	An electric fan		[1=Yes; 2=No]
	A bicycle		[1=Yes; 2=No]
	A radio or transistor		[1=Yes; 2=No]
	A sewing machine		[1=Yes; 2=No]
	A telephone		[1=Yes; 2=No]
	A refrigerator		[1=Yes; 2=No]
	A black and white television		[1=Yes; 2=No]
	A colour television		[1=Yes; 2=No]
	A moped, scooter or motorcycle		[1=Yes; 2=No]
	A car		[1=Yes; 2=No]
	A water pump		[1=Yes; 2=No]
	A bullock cart		[1=Yes; 2=No]
	A thresher		[1=Yes; 2=No]
	A tractor		[1=Yes; 2=No]

Clinical examination data sheet abstracts (relevant data)

No	Measure	Value	Code
	BLOOD SAMPLING		
	Any minor illness within the last week?		[1=Yes; 2=No]
	Was this illness or some other reason responsible for reduction in food intake over the last week?		[1=No reduction; 2=Minor reduction; 3=Major reduction]
	Time of last meal		
	Day of last meal		
	Time blood taken		
	Success?		[1=No; 2=Partial; 3=All]
	If partial, tubes missing:		
	Heparin vacutainer		[1=Yes; 2=No]
	EDTA vacutainer		[1=Yes; 2=No]
	Gel plain vacutainer		[1=Yes; 2=No]
	Citrate vacutainer		[1=Yes; 2=No]

No	Measure	Value	Code
	STANDING/SITTING ASSESSMENTS		
	Time exam began		[HH:MM in 24-hr clock]
	Initial of researcher doing the exam		
	Weight		[Kg]
	Weight adequate		[1=Yes; 2=No]
	Standing height		[mm]
	Standing height adequate		[1=Yes; 2=No]
	Waist circumference 1		[Kg]
	Waist circumference 2		[Kg]
	Waist circumference adequate		[1=Yes; 2=No]
	Hip circumference 1		[mm]
	Hip circumference 2		[mm]
	Hip circumference adequate		[1=Yes; 2=No]

No	Measure	Value	Code
	ARM ASSESSMENTS & SKINFOLDS		
	Dominant arm (writing hand if literate; eating hand if illiterate)k		[1=Right; 2=Left; 3=No dominant arm]
	Side measurements taken (non-dominant side; left side if no dominant side)		[1=Right; 2=Left]
	Mid-arm circumference 1		[mm]
	Mid-arm circumference 2		[mm]
	Mid-arm circumference adequate		[1=Yes; 2=No]
	Triceps skinfold 1		[mm]
	Triceps skinfold 2		[mm]
	Triceps skinfold 3		[mm]
	Triceps skinfold adequate		[1=Yes; 2=No]
	Biceps skinfold 1		[mm]
	Biceps skinfold 2		[mm]
	Biceps skinfold 3		[mm]
	Biceps skinfold adequate		[1=Yes; 2=No]
	Subscapular skinfold 1		[mm]
	Subscapular skinfold 2		[mm]
	Subscapular skinfold 3		[mm]
	Subscapular skinfold adequate		[1=Yes; 2=No]
	Suprailiac skinfold 1		[mm]
	Suprailiac skinfold 2		[mm]
	Suprailiac skinfold 3		[mm]
	Suprailiac skinfold adequate		[1=Yes; 2=No]

No	Measure	Value	Code
	PUBERTAL ASSESSMENT IN BOYS		
	Self assessed testicular volume		[model rank]

No	Measure	Value	Code
	SUPINE ASSESSMENTS		
	Recent infection (last one week)		[1=Yes; 2=No]
	Known cardiovascular disease		[1=Yes; 2=No]
	If yes, specify		[Name of disease]
	Any other known medical ailment (past or present)		[1=Yes; 2=No]
	If yes, specify		[Name of disease]
	Current medications		[1=Yes; 2=No]
	If yes, give name and reason	(a) Name	(b) Reason
	BP (Right arm supine)		
	Initial of researcher doing exam		
	Time exam began		[HH:MM in 24-hr clock]
	Time of last meal		[HH:MM in 24-hr clock]
	Day of last meal		[1=Today; 2=Yesterday]
	Room temperature		[Degree Celsius]
	Cuff size		[1=Adult; 2=Small]
	Heart rate 1		[bpm]
	Systolic blood pressure 1		[mmHg]
	Diastolic blood pressure 1		[mmHg]
	Heart rate 2		[bpm]
	Systolic blood pressure 2		[mmHg]
	Diastolic blood pressure 2		[mmHg]
	Any problems taking readings		[1=No; 2=Left arm; 3=Anxious; 4=Other, specify]
	Other, specify		[Problem]
	Radial augmentation index 1		[%]
	Radial augmentation index 2		[%]
	Any problems taking readings		[1=Yes; 2=No]
	If yes, specify		[Problems]