

Study Protocol



Recruitment : EarlyBird incorporates a random sample of the 1995-6 Plymouth birth cohort. All Plymouth primary schools were identified and their head teachers asked for agreement to participate in the study. The 54 schools that consented were stratified into quartiles according to their proportion of free school meals as a socio-economic proxy, and a random selection from each made accordingly. Registration into the study as family units comprising the child and his/her parents was invited during parent induction meetings for school entry and parents expressing interest were given a full written explanation. The parents were included to make up trios for three reasons. They provide the closest approximation available to the child's metabolic status a generation hence, and they permit the separate analysis of father/offspring and mother/offspring analysis of inherited features. Phenotypic characteristics passing between father and offspring are more likely to prove genetic, whereas the same characteristic shared by mother and offspring might equally be a gestational (intergenerational) effect. The inclusion of parents is also critical to retention.

Exclusion criteria among the children included existing diabetes, pathological states likely to affect growth or body composition, moderate or severe physical disability, long term use of oral steroids, and in the parents, biochemical and anthropometric data were not used in cases of chronic illness requiring medication, long-term use of corticosteroids, pregnancy, lactation or use of the oral contraceptive. Following ethical approval and written parental consent, 307 children in total (137 girls, 170 boys, mean age 4.9 yr), starting school from Jan. 2000 to Jan. 2001, became the EarlyBird cohort.

Procedure : The children are reviewed fasting from 8am by a research nurse within a hospital department of child health. Anthropometric measures and blood pressure are repeated at six-monthly intervals and other tests every 12 months. A limited number of anthropometric measurements were made on the parents at base-line, and a single blood sample taken for DNA, insulin resistance and markers of the metabolic syndrome. All data are anonymised and electronically archived.

1. Candidate factors affecting insulin resistance

Anthropometry : Height is measured to the nearest 1mm (Leicester Height Measure), weight to the nearest 0.1kg, skinfold thickness at five sites (triceps, biceps, subscapular, suprailliac and para-umbilical) by Holtain skinfold calipers, mid-arm, waist, widest abdominal and hip circumferences by metal tape measure. A minimum of two 'blind' repeats are made of each anthropometric measure at each visit. Ultrasound was used at baseline to measure the intra-abdominal sagittal diameter between aorta and anterior abdominal wall as a proxy for visceral fat mass, but found to be insufficiently precise.

Body composition : this gives a more precise definition of true fat mass and, importantly, of its distribution in the body: Regional fat mass, %fat, fat-free mass, water and bone are deduced from total body measures by bioimpedance (Tanita Corporation, Japan) and by dual energy X-ray absorptiometry (DEXA; *Lunar Prodigy fan beam densitometer, GE Medical Systems, previously Lunar DPX-L pencil beam*).

- EnCore 2004 software calculated basic regions of interest (ROI):
Android ROI = Lower boundary at Pelvis cut. Upper boundary above Pelvis cut by 20% of the distance between Pelvis and Neck cuts. Lateral boundaries are the Arm cuts.
- Gynoid ROI = Upper boundary below the Pelvis cut line by 1.5 times the height of the Android ROI. Gynoid ROI height equal to 2 times the height of the Android ROI. Lateral boundaries were the outer Leg cuts.

Physical activity : Daytime physical activity at school, after school, and at weekends is measured continuously over seven-day periods by means of an MTI (formerly CSA) piezo-electric accelerometer (Computer Science and Applications, Fort Walton Beach FL). The monitor is fitted before the child leaves the department and retrieved from school one week later. The data are downloaded onto a PC for storage and analysis. Accelerometers provide extraordinarily precise information, and are re-writing the literature on the physical activity of children. They sample movement 600 times a minute, register the clock time, duration and intensity of each movement, and can generate a detailed 'day in the life of x' graph for any child.

Resting Energy Expenditure : Resting metabolic rate is measured in the lying position by indirect calorimetry using a GEM (Nutrem, Manchester) over a period of 15 minutes, during which time the child can choose to watch a video.

Food types : Standardised food frequency questionnaires, widely used by dieticians, are completed by parent and child.

Genetics and demography : Two ml aliquots from each serum sample are archived at -80°C after each visit, and blood cells retained from parents and children for DNA analysis of candidate 'obesity' genes. Parents complete a questionnaire detailing medical history and socio-economic circumstances (income band, educational achievement, occupation, post-code and eligibility for free school meals), which is updated every 12 months.

2. Behaviour of insulin resistance and insulin secretory capacity:

Insulin sensitivity (S) and insulin secretory capacity (beta cell function (B)) are derived from fasting measures of insulin and glucose in venous blood samples, using Homeostasis Model Assessment (HOMA) The earlier of two reports describing HOMA provides formulae for the calculation of insulin resistance and beta cell function, the later update supplies a software programme. We are using HOMA indices for S and B to trace the disposition of the glucose control loop as the cohort gains weight and moves

through puberty. The data are novel, and suggest that children who are insulin resistant when very young may already be losing the very beta cells needed to protect them from diabetes.

3. Metabolic Impact

Blood pressure (considered as a metabolic response to insulin resistance) is taken by semi-automated sphygmomanometer (Welch-Allyn, Beaverton, OR) and the mean of the second and third of three recordings used in the analysis. Cryogestic spray or topical analgesic cream (Emla, Astra-Zeneca) applied 1hr before venepuncture. **Insulin and sex hormone-binding globulin (SHBG)** are measured by immunometric assay on a DPC Immulite analyser, using kits manufactured by Diagnostic Products Corporation (Los Angeles). Insulin cross-reactivity with proinsulin is less than 1%. **Glucose, cholesterol, HDL cholesterol, triglycerides and uric acid** are measured on a Cobas Integra 700 analyser (Roche Diagnostics, Lewes, East Sussex, UK). A full **haematological profile** is recorded. **Glycated haemoglobin** is measured by automated high performance liquid chromatography using a Menarini Biomen HA 8140 analyser. **Follicle-stimulating hormone (FSH)** and **luteinising hormone (LH)** are measured by automated chemiluminescent sandwich immunoassay on an Advia Centaur analyser (Bayer Diagnostics, Newbury, Berkshire, UK). **Serum IGF1** (microELISA, Univ Glasgow), Tanner staging and peak height velocity are used to monitor the timing of puberty. Some new measures include: **adiponectin**, a recently discovered mediator of insulin action and regulator of fat mass, **leptin**, a satiety signal and indicator of fat mass produced by fat cells and a highly sensitive measure of **C-reactive protein** (hsCRP), a marker of inflammation (insulin resistance is fundamentally a state of low grade inflammation).

4. Cardiovascular Impact

Heart rate variation Beat-to-beat variation in heart rate is increasingly recognized as a measure of autonomic tone, itself a feature of insulin resistance. A two-lead rhythm-strip ECG is attached to the children while undergoing REE measurement, and the beat variation pattern analysed by specialist software. **Pulse wave velocity, waveform analysis, central blood pressure and augmentation index** are measured by tonometry (a pressure sensor placed over the arteries of the neck, wrist and ankle), and will detect the earliest signs of adverse cardiovascular response to insulin resistance. The heart is exposed to central blood pressure, which is different from the BP registered by an arm cuff, and the augmentation index is a critical measure of the extent to which the coronary vessels are subject to shearing forces.

Summary of Main Outcome Measures:

- *Children* :
 1. Candidate factors : Birth weight, height, weight, BMI, skinfolds at five sites, waist, mid-arm and hip circumference, body composition, blood pressure, resting energy expenditure, physical activity and diet.
 2. **Insulin resistance and secretory capacity** : HOMA-S and HOMA-B.
 3. **Metabolic impact** : Blood pressure, full blood count, haemoglobin and haematocrit, HbA1C, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, uric acid, IGF-1, gonadotrophins, IGF-1, leptin, adiponectin, hsCRP and SHBG. DNA is prepared and serum aliquots from each visit archived at -80°C.
 4. **Cardiovascular impact** : Heart rate variability and tonometry for pulse wave velocity, waveform analysis, central blood pressure and augmentation index
- *Parents* :
 1. **candidate factors**: Height, weight, BMI, waist circumference.
 2. **insulin resistance and insulin secretory capacity** : HOMA-IR and HOMA-ISC.
 3. **metabolic impact**: Full blood count, haemoglobin and haematocrit, HbA1C, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, uric acid, gonadotrophins, and SHBG. DNA is prepared and serum aliquots archived at -80°C.

Quality Control, Validation and Compliance:

We have formally assessed the precision of all anthropometric measures. The performance of the accelerometers has been rigorously investigated by ourselves and others. We have also established 12-month test-retest correlations on the full EarlyBird cohort for the accelerometers measuring physical activity ($r = 0.49$), GEM measuring REE ($r = 0.49$) and HOMA measuring insulin resistance ($r = 0.44$). HOMA is now well established in the estimation of insulin resistance in children. The euglycaemic clamp, gold standard for the measurement of insulin resistance, is both impractical and unethical in healthy young children. HOMA compares favourably, with correlation coefficients exceeding $r = 0.8$.

The special concerns for clinical investigation in small children are recognised. No investigation is considered unduly invasive, and none is performed without the child's assent. To date, the Study can claim over 95% compliance on all tests. Every effort is made to minimise attrition by close family support, group activities, newsletters, parties and so on. Parents are assured that all data will be coded and anonymised, and that any abnormal results will always be reported to their general practitioner.

Statistical Analysis:

In summary, all statistical analyses are performed using SPSS for windows version 16.0. Standard deviation scores (SDS) are calculated for birth weight, current weight, height and BMI in order to standardise for age and sex (based on 1990 UK reference curves) using the conversion programme issued by the Child Growth Foundation (London, W4 1PW, UK). T-tests or one-way analysis of variance (ANOVA) compare means, and correlations are established by Pearson's coefficient. Chi-Square is used for the analysis of categorical variables. A sample of size 300 will detect a correlation of 0.23 or more, assuming a two-tailed test at the 5% significance level. When considering males or females only, a sample size of 150 will detect a correlation coefficient of 0.27. EarlyBird has been accumulating data at annual time-points since January 2000, and increasing use is now made of trend analysis involving multi-level, mixed effects, modelling.