Growth and Metabolic Outcomes in Children Born Preterm:

The Growmore study

Growth and Metabolic Outcomes in Children Born Preterm: The Growmore study

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Abstract

Background: The Growmore Study was an observational cohort study of adolescents born preterm, in Newcastle-upon-Tyne. The cohort were born between 1993 and 1998: mean gestation at birth was 31 weeks (range 24+5d to 36+2d) and birthweight 1392g (690-2200g). Individuals were originally recruited into one of two randomised, controlled trials ('growth', n=113; and 'protein', n=134). As separate groups they were followed up at intervals, undergoing assessment of growth and development. From 247 children originally recruited, 220 completed assessments to 24 months of age. At age 10 underwent cognitive assessment. Between 9 and 13 years old, the two cohorts were amalgamated to a single cohort and underwent further auxological and metabolic testing (n=153/247) including DEXA scan (n=109) and bloods (n=139). The current study revisited the cohort, aged between 12 and 18 years old.

Aims: The study aimed to explore relationships between: growth in early life; body fat deposition; mitochondrial oxidative capacity; and quantitatively assessed diet and activity in ex-preterms.

Methods: 60 of the 235 traceable members of the original cohort were recruited into this study. They underwent multimodal assessment, including: auxological measures; body composition measurement by air-displacement plethysmography and skinfold thickness; magnetic resonance spectroscopy (MRS), using a 3-Tesla scanner, custom-built coils and a tailored scanning routine to quantify skeletal muscle mitochondrial oxidative capacity, lipid content of the liver, and fat-containing tissue at the L2/3 vertebral level; a standard OGTT (bloods taken at 0 and 120minutes); and serum insulin, glucose, lipid profile, liver function and Vitamin D measurement. Dietary intake was assessed using a computer-based recall diary and physical activity by wearing of accelerometers. Data was analysed by using a variety of statistical methods including comparative, correlation and regression analysis.

Results: The 60 adolescents recruited for this study had a mean gestation at birth of 31 weeks (range 26+1d to 34+4d) with birthweight of 1370g (range 840-1870g). Their mean age at study was 15.5 years and M:F ratio was 1:1.4. Analysis showed they were not significantly different from their peers at the previous cohort assessment and both of the two original RCTs were almost equally represented ('growth': 'protein' = 1:1.07). The current study showed that amongst this cohort subgroup, vitamin D status

correlated with time of year (p=0.046) and current weight SDS (p= 0.039). Skeletal muscle oxidative function was significantly related to vitamin D status (p=0.021) and gestational age at birth (p=0.005); combined r^2:0.31; p=0.002). Earlier gestational age (GA) at birth and lower serum vitamin D was associated with reduced oxidative capacity. Physical activity was not associated with oxidative capacity. Visceral adipose tissue (VAT), circulating triglyceride (TG) and waist circumference were strongly associated with hepatic lipid content (all p<0.001); dietary intake was not. VAT and TG were highly significant when the model was adjusted for Tanner Stage (r^2: 0.4; p=0.0002). GA and birthweight were not related to hepatic lipid deposition. Insulin sensitivity by two different measures was predicted by triglyceride levels (p<0.001), light activity (P<0.05) and vitamin D levels (p<0.05).

Conclusion: Environment and early life both have an influence on adolescent physiology. The strength of association between vitamin D and muscle oxidative capacity has been observed in other conditions, but the contribution of gestation at birth in those born preterm is a novel finding. This may reflect either a variance in muscle fibre type or mitochondrial density directly related to developmental arrest or delay as a result of preterm birth. Vitamin D status also influences insulin sensitivity, as seen in other populations: Vitamin D status is an obvious target for dietary advice. Absence of an association between gestation and adiposity, and correlation between VAT and hepatic lipid deposition suggests that there are opportunities for children born preterm to improve their health in adolescence, and by implication, their future adult health.

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Abbreviations

2S	Two skinfold method
4S	Four skinfold method
ADP	Adenosine diphosphate
AGA	Appropriate (growth) for gestational age
AMARES	Advanced method for accurate robust efficient spectral fitting
ATP	Adenosine triphosphate
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BP	BODPOD™
DAG	Diacylglycerol
DEXA	Dual energy X-ray absorptiometry
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease (theory)
EMCL	Extra-myocellular lipid
FFMI	Fat-free mass index
FMI	Fat mass index
GA	Gestational age
GP	General (medical) Practitioner [family doctor]
HOMA	Homeostasis model assessment
HTA	Human Tissue Act
IHL	Intrahepatic Lipid
IS	Insulin sensitivity
IMCL	Intra-myocellular lipid
MAC	Mid-arm circumference
MRS	Magnetic resonance spectroscopy

MVC	Maximum voluntary contraction
MVPA	Moderate to vigorous physical activity
NAFLD	Non-alcoholic fatty liver disease
NHS	National Health Service
NMRC	Newcastle Magnetic Resonance Centre
NPTBGS	Newcastle Preterm Birth Growth Study (cohort)
OGTT	Oral glucose tolerance test
Pi	High energy inorganic phosphorus
PCr	Phosphocreatine
PKC	Protein kinase C
RNA	Ribonucleic acid
SAT(i)	Subcutaneous adipose tissue (index)
SCR	Summary care record
SD(S)	Standard deviation (score)
SGA	Small for gestational age (at birth)
SNR	Signal to noise ratio
τ½	Half-time
T2DM	Type 2 diabetes mellitus
TG	Triglyceride
VAT(i)	Visceral adipose tissue (index)
VLDL	Very low density lipoprotein
WC	Waist circumference

Chapter 1. Introduction

The notion that the adult phenotype is mainly determined by environmental influences encountered through life and the influence of inflexible, inherited genotype (in terms of health outcomes) is one that has been increasingly challenged by research evidence over the last 30 years.

In the late 1980's and 1990's, Barker et al used epidemiological methods to examine the relationship between birth weight, size in infancy and cardiovascular risk in adults (Barker, 1995). They found that low birth weight and weight at one year, small head circumference and ponderal index at birth was associated with increased risk of cardiovascular disease as an adult (Barker *et al.*, 1993). From this they concluded that intrauterine life and early childhood was important in laying down the foundations of adult health and predisposition to illness (now known as the 'Fetal Origins', or 'Barker', hypothesis). The mechanism was, however, unclear.

Further work led to the proposal of the 'thrifty phenotype' (Hales and Barker, 1992) which suggested that in-utero, the fetus samples the nutritional environment and alters the rate of organ growth to optimise growth of key organs (brain and liver). When in nutritionally poor times, this leads to asymmetrical growth by the time of birth, but enhances post-natal survival when poor quality or intermittent nutrition continues. By contrast, if energy dense food subsequently becomes available, the 'thrifty' mechanisms do not down-regulate and obesity and metabolic morbidity ensues. However, diet in the immediate post-natal period (after a normal pregnancy) can also have a profound effect on outcome (Desai *et al.*, 1996). Similarly, over-nutrition has been shown to have unwanted effects in later life in rodent models (Hahn, 1984). This suggests that the mechanisms are not simply the result of metabolic up-regulation secondary to a sub-optimal, in-utero environment, or 'thrift'.

This was taken further by Lucas (Lucas, 1994), who postulated that genetic make-up played an important role in the health outcomes by either:

"...induction, deletion or impaired development of a somatic structure during a *critical period*; or physiological 'setting' by an early stimulus or insult at a critical period, with long term consequences for function."

This is now known universally as 'programming'. Specifically, Lucas was referring to the role that nutrition in-utero *and* in early infancy might play in altering the adult phenotype. The notion that a 'programming' stimulus (nutrition, or otherwise) during any given critical phase of early development could have a predictable effect on somatic response to a stimulus or stress not yet encountered, led to an appreciation that modification of *adult* health outcomes might need to begin even before a disease process had started. The environments to which the gamete, fetus and infant are exposed are likely to exert significant influence on eventual adult health outcome by means of programming the way the body will react to future events (Gluckman and Hanson, 2004; Gluckman *et al.*, 2005).

Preterm delivery is a cataclysmic interruption of the 'normal' processes of human fetal development and modification of the environment in which the newborn finds itself. The interruption of normal gestation exposes the fetus to the physiological stress of trying to survive ex-utero with immature organ systems, as well as stimuli that it would not normally have been exposed to. In rats, the effect on offspring of even a slight difference in how well they were nursed as pups has been shown to greatly alter their behaviour in response to future stressors (Weaver *et al.*, 2004). It could be expected that being ex-utero at a preterm gestation would have a profound developmental effect. In addition, nutrition is provided to preterm infants using a mixture of tailored parenteral and enteral preparations which would not be otherwise encountered by the fetus in-utero. Therefore the potential to encounter one of Lucas' 'nutritional stimuli' at a 'critical' phase is greatly increased.

As an example: aggressive parenteral nutrition in extreme prematurity is extremely important to prevent catabolism and maintain growth in the immediate neonatal period (Embleton *et al.*, 2001; Ibrahim *et al.*, 2004). There is evidence, however, that growth restricted infants experience liver injury with this approach more often than their well grown counterparts (Baserga and Sola, 2004). It is also thought-provoking to note that very similar preparations of lipid are used in aggressive parenteral nutrition to the infusions which can be used to experimentally induce reduced insulin sensitivity in adults (Roden *et al.*, 1996). While the 'critical period' is not defined in this example, it is clear from evidence of damage and by virtue of the lipid infusion, that there is exposure to a stimulus which may lead to harm through programming. Body habitus

differences between infants born extremely preterm and their term born peers have been well known for 10 years (Uthaya *et al.*, 2005)

Nutrition is not the only possible stimulus to which fetuses and newborn infants may be exposed that might lead to altered adult health outcomes. Other events and interventions which might impact on the ex-utero preterm infant trying to survive are protein intake (both low and high), vitamin supplementation (especially vitamin A), glucocorticoid administration, exposure to inhaled nitric oxide and antibiotics interfering with normal bowel flora development (Kalhan and Wilson-Costello, 2013). All of these are regular occurrences in the Neonatal Intensive Care setting and could have profound impact on the future health of an individual if exposure did, or perhaps didn't, occur during a critical window.

It is important to note that this ever widening scope of potential interactions with delayed effect is reflected in the rapid 'academic evolution' of programming and the fetal origins hypothesis into the wider field of research under the label of Developmental Origins of Health and Disease (DOHaD) (Gluckman and Hanson, 2006). This field includes the science of epigenetic analysis (Groom *et al.*, 2010; Relton and Davey Smith, 2010). Inheritable changes made to DNA and DNA-associated proteins which influence and change gene expression *without* altering gene sequence may result from environmental exposure and therefore provide a significant mechanism by which programming, and much of what is encompassed within DOHaD, might plausibly be explained at cellular level.

1.1 Infant growth and health outcomes

There are four important groups of infant studied in the literature with respect to growth and outcome: Term infants who have growth appropriate for gestational age (AGA), preterm infants who are also AGA, term infants who are small for gestational age (SGA) and preterm SGA infants. The aim of neonatologists to date has been to provide adequate nutrition for all groups to ensure good growth. This, however, requires different amounts of nutrition for different infants. Embleton *et al* demonstrated that with accepted levels of nutrition (at the time), preterm infants inevitably accumulated a deficiency in nutrition in the first few weeks of life on the neonatal unit (Embleton *et al.*, 2001). This could be directly linked to postnatal growth

impairment and was unlikely to be recovered with 'normal' daily nutritional intake. In addition, there is good evidence that neurodevelopmental outcome is linked to early growth. Lucas et al found that in infants who have a higher early protein intake, neurodevelopmental deficits are reduced (Lucas *et al.*, 1998). In infants born prematurely and SGA, growth such that they 'catch up' to a more appropriate size was also linked to improved neurodevelopmental outcomes (comparable to AGA infants) (Latal-Hajnal *et al.*, 2003). The same study also demonstrated that AGA infants whose growth was poor (so that they had 'catch down' growth) had poorer neurodevelopment (comparable to SGA infants who had *not* demonstrated catch up growth). These outcomes are used to support the use of aggressive nutritional intervention in preterm infants, and would seem to justify trying to achieve and maintain 'catch up growth'.

To achieve an energy density suitable to promote catch-up growth in the growth-restricted and/or preterm infant, the nutrition provided often has very high lipid and glucose concentrations (Thureen, 2007). It is also difficult to achieve high levels of protein intake, since appropriate assimilation of protein for growth relies on provision of large amounts of non-protein calories to prevent the newborn using the protein as an energy source. The necessary high concentrations of glucose and lipid to achieve this (above that which the fetus in utero would experience) might be expected to have an impact on metabolic outcome, particularly on insulin-regulated processes. The current evidence specifically relating to preterm birth in humans and altered insulin sensitivity (IS) is summarised later.

Rat models suggest that in-utero hypoxia (as a method of inducing intra-uterine growth restriction and thus SGA offspring) is associated with changes in metabolism that lead to development of the metabolic syndrome when exposed to a high fat diet (Rueda-Clausen *et al.*, 2011). Indeed, catch-up growth in humans (usually defined as an increase of >0.67 standard deviation score [SDS] in a defined time period) has been associated with cardiovascular death, hypertension, T2DM and obesity (Hales and Ozanne, 2003). Conversely, in other mammals, 'beneficial under-nutrition' has been associated with improved life expectancy (Thureen, 2007). It is also very difficult to tease out the long term effects of perinatal exposure to other stimuli: for example, corticosteroids might have an influence on longer term glucose homeostasis, given

their in-vivo effects once administered and have been shown to affect insulin sensitivity later in life (Dalziel *et al.*, 2005). Recent work also shows that infants who are born at term and who are innately small (but would qualify as being SGA by population standards) are, in fact, metabolically healthy in later life(Milovanovic *et al.*, 2012). Aggressive nutrition in these infants might program for adverse health later in life. Infants in this category may also delivery prematurely, but it is not possible to accurately identify SGA preterms as innately SGA, or not, at delivery.

One of the measurable outcomes in neonates used to reflect the outcome of nutritional programming is body composition; especially the location and quantity of fat stores. DEXA, air-displacement plethysmography and MRI have both been used to quantify and describe body composition and lipid deposition in term and preterm infants. Uthaya and colleagues used MRS and anthropometric measures to characterise in-vivo compartmentalisation of adipose tissue in infants born aged <32 weeks gestation (at term corrected age) in comparison to full-term controls (Uthaya *et al.*, 2005). They found that the ex-preterm infants were lighter and shorter but with preserved head circumference, and reduced subcutaneous fat mass. However, they had increased intra-abdominal fat mass relative to their healthy controls. Early accelerated post-natal weight gain was associated with increased fat mass overall (subcutaneous and intra-abdominal), but illness severity was the major determinant of increased (inappropriate) intra-abdominal fat. The authors therefore concluded that illness in the preterm period altered the partitioning of lipid compartments and therefore begins to explain the association of prematurity with metabolic disease later in life. The same group also showed that specific intra-hepatocellular lipid deposition in preterms (at term corrected age) was greater than in healthy adults or term-matched controls (Thomas *et al.*, 2008). Gianni et al measured percentage fat-mass by plethysmography in premature and term SGA infants (Gianni *et al.*, 2009). They found that the more preterm the SGA infant was, the higher the percentage fat-mass it had by term. There is, therefore, evidence to support the abnormal deposition of fat in preterm and SGA infants, suggesting a potential pathway by which metabolic abnormalities might be developing *or* exerting influence. The immediate post-natal period was examined using DEXA by Cooke *et al* (Cooke *et al.*, 2010) who found that in the first year, from term correct age onwards, there was no increased adiposity by

giving infants nutrient-enriched formula *but* catch-up growth was seen. This suggests that *appropriate* extra nutrition (not just calories) can have a beneficial effect by increasing lean mass and that changes which may occur in the pre-term period are modifiable.

There is also evidence to suggest that as children get older, their contemporary body composition becomes more strongly associated with their health status and metabolic state, and that some associations which existed in the perinatal period become less pronounced (Jeffery *et al.*, 2006; Parkinson *et al.*, 2013; Tinnion *et al.*, 2014). Evidence from the ALSPAC cohort suggests that in the modern era, childhood and adolescence may be more directly influential times for excessive increases in body weight (and fat mass) than infancy and the perinatal period (Hughes *et al.*, 2011). Epidemiological evidence also supports the notion that postnatal, pre-pubertal rate of growth has a strong influence on health outcome, with those children underweight at birth who display greatest increase in body mass index by 11 years old being at greatest risk for hypertension and T2DM (Barker *et al.*, 2002). Therefore, there is a possibility that modifiable factors may be at work and provide a therapeutic/preventative option in at-risk individuals provided that the 'critical windows' can be identified.

It seems most likely that the immediate perinatal period has a significant role to play in 'setting' metabolism (as Lucas proposed) but as children grow older, the health outcomes at an individual level may well be dependent on multiple other contributory factors.

1.2 Metabolic syndrome and reduced insulin sensitivity

There has been a well-documented increase in obesity in the general adult population in westernised societies, especially over the last 20 years (Baskin *et al.*, 2005). Along with this obesity 'epidemic', there has been the emergence and characterisation of the associated 'metabolic syndrome'; a collection of clinical findings which, when present in an individual, are significant risk factors for developing cardiovascular disease and type 2 diabetes mellitus (T2DM), thus risking premature death. The diagnostic criteria required to make a diagnosis of metabolic syndrome (Alberti *et al.*, 2009) are:

- Increased waist circumference (specific cut-offs for differing ethnic groups)

- Hypertension: systolic blood pressure ≥ 135mmHg and /or diastolic blood pressure ≥85mmHg
- Elevated triglycerides (TG): ≥ 1.7mmol/L
- Reduced high-density lipoprotein cholesterol (HDL-C): ≤ 1.0mmol/L [male] or ≤ 1.3mmol/L [female]
- Elevated fasting glucose: ≥ 100mg/dL

These criteria cannot, however, predict the rate of development of subsequent illness. The proposed contributory factors to the obesity 'epidemic' are many (McAllister *et al.*, 2009). Various 'environmental' factors (such as infection and inflammation, calorie-dense, easily-available food, reduced exercise levels and sedentary lifestyle), 'genetic' factors (promoting the 'thrifty phenotype' (Hales and Barker, 1992)) combine to promote (in individuals) unhealthy deposition of fat. There is also compelling evidence that nutritional programming in early life has an important effect in determining adult health outcome (Lucas, 1994).

In the paediatric population, there is even less consensus(Ford and Li, 2008) about how to define and diagnose the 'metabolic syndrome'. It is clear that, while there is potentially greater morbidity and earlier mortality associated with acquisition of features consistent with the 'metabolic syndrome', three major problems exist. Firstly there is a tension between accurate diagnosis (with predictive power) and burden of investigation. The more detailed and multi-partite a definition is, the more tests need to be done. Correspondingly, the more room there is for error (for example in *where* to measure waist circumference or how to adjust for variation between ethnic groups). Secondly, children have periods of their lives where they are rapidly changing from both a hormonal and metabolic perspective. During these times, assumption of static, reliable, measurable parameters may be incorrect. At these times of growth and change, children also change at different rates within peer groups (especially during puberty) and even in a small population sitting within apparently tightly, age-limited definitions there will be child-to-child variations which could influence the effectiveness of the criteria to detect changes. Lastly, there are less data available as you look at younger children and it is harder to elucidate with certainty a causal association into middle and late adulthood (in terms of health outcomes).

The International Diabetes Foundation definitions(Zimmet *et al.*, 2007) try to strike a pragmatic approach to clear criteria for diagnosing metabolic syndrome in different ages of child (without relying on many reference value tables for different groups). This approach is clinically pragmatic, and therefore helpful in practice(Mancini, 2009; Poyrazoglu *et al.*, 2014), but has potential for under-detection through reduced specificity (fewer criteria) and sensitivity (fixed cut-offs).

While the underlying causal relationships between physiology and disease are still being elucidated in both animal and human models (Bremer *et al.*, 2012), it is clear that reduced sensitivity to insulin (either global or tissue-specific) is a significant common feature of the current, collective understanding of the metabolic syndrome. To understand the extent to which an infant born preterm might be at risk of adverse metabolic outcome, it is reasonable to consider, therefore, the evidence of association of decreased insulin sensitivity must be examined alongside the evidence for outcomes in preterm infants as they grow up.

1.3 Programmed Insulin Sensitivity and Prematurity

The studies to date which specifically address insulin sensitivity and prematurity are mostly observational cohort studies. I have published a literature review of these studies previously (Tinnion *et al.*, 2014). The studies reviewed were published between 2000 and 2013 and had participants whose ages ranged from a few days after preterm birth to middle adulthood. The studies were worldwide (New Zealand, Australia, UK, Turkey, Cyprus, Brazil, Chile, Netherlands, Italy, South Africa, Germany, Finland and Sweden), representing mostly 'first world' medical practice and described a total of 20 unique cohorts.

There were a number of different methods used to quantify or assess insulin sensitivity. Thirteen studies used a variation of a glucose tolerance test (GTT; intravenous short- or frequently-sampled-GTT(Leipala *et al.*, 2002; Bazaes *et al.*, 2004; Hofman *et al.*, 2004; Regan *et al.*, 2006; Willemsen *et al.*, 2009; de Kort *et al.*, 2010; Kerkhof *et al.*, 2012) or oral glucose load (Fewtrell *et al.*, 2000; Dalziel *et al.*, 2007; Hovi *et al.*, 2007; Pandolfi *et al.*, 2008; Chan *et al.*, 2010) or milk bolus(Gray *et al.*, 2002)) combined with insulin sampling and modelling (such as Bergman's Minimal Model or Homeostasis Model Assessment [HOMA]) to give a measure of IS. Three studies

(Rotteveel *et al.*, 2008a; Rotteveel *et al.*, 2008b; Rotteveel *et al.*, 2011) conducted hyperinsulinaemic-euglycaemic clamp studies to calculate glucose disposal, as a measure of sensitivity to insulin. The remaining studies used measures of glucose metabolism such as fasting insulin and glucose, 32-33 split pro-insulin and Insulin-like Growth Factor Binding Protein (IGFBP), combined with a modelled estimation of IS such as HOMA to quantify sensitivity to insulin. The study methodology reflected, in part, the age of the participants. Chronological age is a factor in normal variance of insulin sensitivity, for example during puberty (Kurtoglu *et al.*, 2010) and some of the cohorts were longitudinally followed-up through childhood. As the cohort in this study are adolescents, this is the age group that I will concentrate on here, though the full life-course can be seen in the systematic review. The logic model showing changing associations with insulin sensitivity over the life-course, based on the findings of the systematic review is shown in figure 1.

1.4 Effect of preterm birth on Insulin Sensitivity (IS)

1.4.1 Insulin Sensitivity in adolescence (10 to 18 years)

Three preterm cohort studies investigated IS in adolescence. Fewtrell and Singhal(Fewtrell *et al.*, 2000; Singhal *et al.*, 2003) followed-up a cohort originally enrolled into a nutritional intervention (<1850g, Median GA 31 weeks) (Lucas *et al.*, 1984). At 9-12years of age(Fewtrell *et al.*, 2000) fasting bloods (insulin, glucose and pro-insulin levels n=279) were strongly related to current weight SDS, 30-minute insulin levels and split pro-insulin: 1 SD increase in current weight SDS increased 30-minute insulin by 25.6% and split pro-insulin by 31.7%. Regression modelling showed that change in weight SDS from 18months to current age was related positively to insulin, proinsulin and split pro-insulin. Individuals with the greatest increase in weight SDS from 18months to follow-up had the lowest IS. Birthweight had a strong negative correlation with 30-minute glucose levels.

By 13-16 years of age (Singhal *et al.*, 2003) a decrease in IS for current weight SDS was seen (13.4% change in 32-33 split pro-insulin per BMI SDS score). Those adolescents randomised to higher nutrient intakes as neonates had greater split pro-insulin levels even after adjustment for potential confounders (coefficient 18.4%; p= 0.016). A step-wise increase in adjusted 32-33 split pro-insulin was found to mirror quartiles of

weight gain in the first two post-natal weeks (11.9% increase per 100g weight increase).

Chan et al(Chan *et al.*, 2010) investigated 11-15 year old children born preterm or term and measured fasting and 2 hour glucose and insulin (standard 75g OGTT). They showed lower glucose levels at 2 hours post-load in those born preterm (0.25mmol/L lower than term controls; p=0.03).

Reinehr et al (Reinehr *et al.*, 2010) studied SGA children 5-13 years age who were obese (BMI >97th percentile) at the time of study. The cohort consisted of 341 children of whom 24 were preterm. The authors assessed the effects of a multifactorial lifestyle intervention (aimed at promoting weight loss) on IS and other metabolic parameters. The authors found that reduced obesity was associated with improved IS (12mU/L drop in serum fasting insulin per BMI-SDS reduction). Birth weight had a small association with IS (4mU/L increase per additional kg of birthweight: p=0.001; 0.7 increase in HOMA index per additional kg birth weight: p=0.007). Weight loss only accounted for 10% of the variance in IS.

1.5 Comparison of IS between those born preterm and appropriate for gestational age vs. preterm and small for gestational age

1.5.1 Insulin Sensitivity in Adolescence (10 to 18years old)

Reinehr et al.(Reinehr *et al.*, 2010) demonstrated improved IS with programmed weight loss. Whilst SGA status predicted changes in HOMA status with weight loss (accounting for 14% of variance) both term and preterm infants were in the AGA/SGA groups. Chan et al(Chan *et al.*, 2010) found no difference between SGA/AGA groups in IS, however, preterm SGA children had higher insulin levels 2 hours after OGTT.

1.6 Summary of evidence

As described by the logic model, the impact of prematurity, and being born AGA vs. SGA preterm on subsequent insulin sensitivity is complex. The evidence suggests that for infants born prematurely, there is an association with an increased risk of reduced insulin sensitivity in childhood. By adulthood, the significance of preterm birth is surpassed by strong associations with body habitus and composition (Parkinson *et al.*, 2013) in line with findings of studies of insulin sensitivity in the general adult

population. The emergence of body composition as a key associate of sensitivity to insulin appears to coincide with puberty and adolescence. The evidence does not, as yet, provide any insight as to any mechanistic role of puberty in this transformation, though it is accepted that insulin sensitivity decreases during puberty in humans (Kurtoglu *et al.*, 2010; Sinaiko and Caprio, 2012). Unravelling the effects of puberty from the effects of prematurity in adolescents with respect to changing insulin sensitivity is something that is not addressed in any of the literature.

It is also not clear from the evidence whether reduced insulin sensitivity in childhood is causal in altering body composition or vice versa. The association of growth patterns and insulin sensitivity during childhood is similarly unresolved: as children move through childhood, the influences of disordered (excessive) catch up growth can be seen to adversely affect insulin sensitivity and there are suggestions of different critical periods of growth (in line with current programming theories) during which nutrition may have its greatest effect.

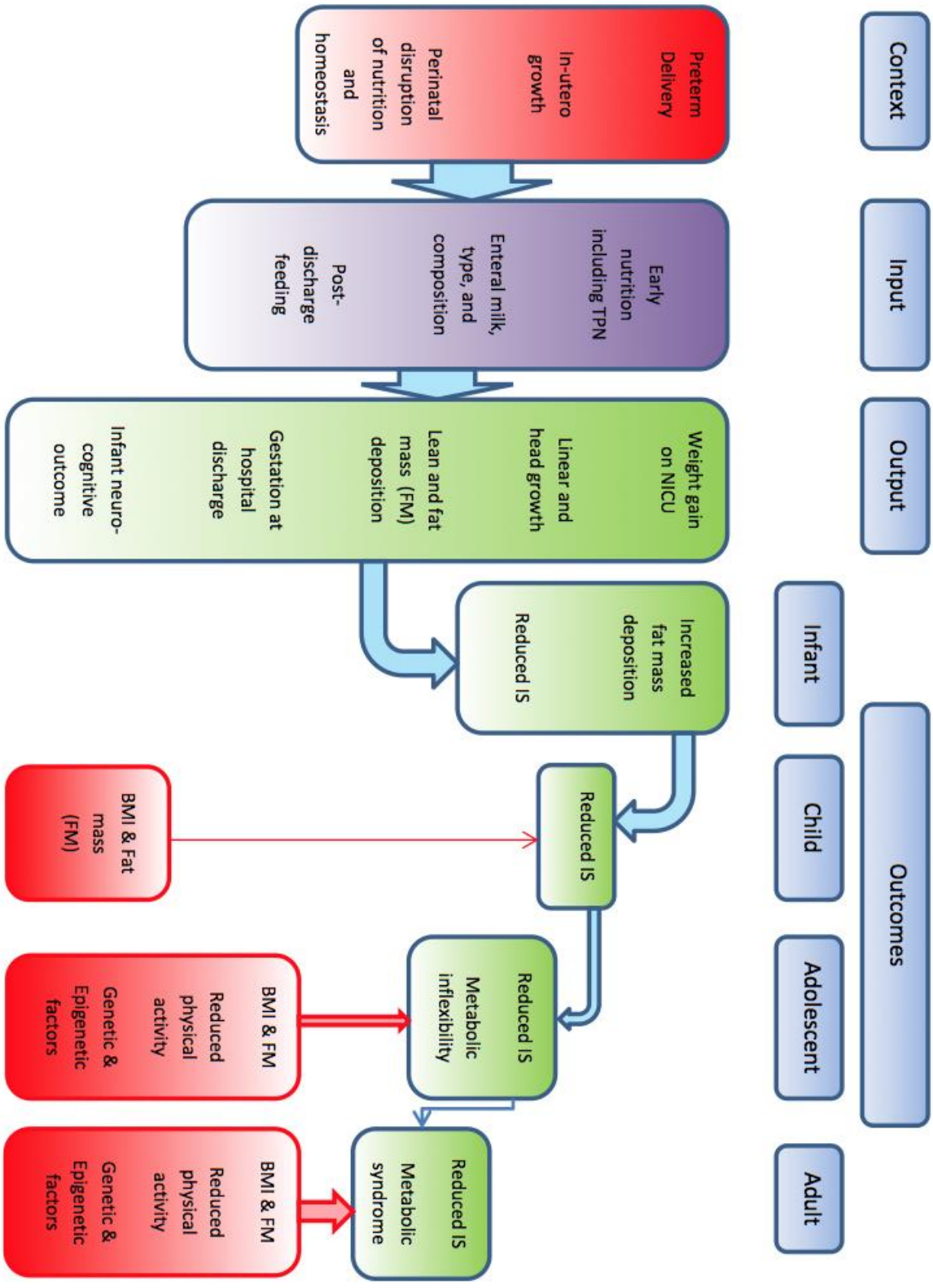

Figure 1: logic model representing influences on insulin sensitivity on individuals born preterm during the life-course (from Tinnion *et al*, 2014).

While early maintenance of normal protein and energy intake (preventing catabolism) is clearly important, subsequent enteral over-nutrition may well be less desirable than focussed supplementation and aiming for appropriate (only) catch up growth. Singhal et al (Singhal *et al.*, 2003)showed that a lower-nutrient diet during the neonatal period was associated with improved insulin sensitivity in childhood and Pittaluga et al (Pittaluga *et al.*, 2011) demonstrated that specific nutritional changes in the post-natal period had a measurable effect in infancy. Both studies support the potential for nutrition to program later metabolism and suggest that early *appropriate* nutrition and specific supplementation *without overnutrition* may be key in ensuring good long term metabolic outcome. Importantly, excessive lean growth (e.g. height beyond genetic potential) may be as harmful as excessive fat mass increase. However, there is still disagreement about how best to measure catch-up growth and use of BMI alone is misleading as a substitute for other measures such as truncal fat deposition or lean tissue mass (Wells *et al.*, 2010) which might have more metabolic significance.

The evidence appears to show that SGA and AGA preterms are born with similar levels of insulin sensitivity and that the immediate post-natal nutritional environment has potential to alter later normal growth, and thus alter measured insulin sensitivity. Through adolescence and adulthood, there is little evidence to support any difference between ex-AGA/SGA preterms and the strength of association with early growth pattern appears to wane. In three studies (Gray *et al.*, 2002; Leipala *et al.*, 2002; Pittaluga *et al.*, 2011) the measured glucose levels between AGA and SGA groups did not differ suggesting, perhaps, that the insulin sensitivity of a neonate/infant (using glucose levels as a proxy measure) is linked more strongly to GA at delivery (reflecting the in-utero environment) than with glucose metabolism. The potential interruption to ongoing production of growth factors such as Insulin-Like Growth Factor (IGF) 1 occurring because of preterm delivery(Yumani *et al.*, 2015) may also affect circulating glucose levels and thus postnatal interpretation of insulin sensitivity in these babies. Modern neonatal medicine, and the ability to support babies born at very early gestations to term equivalent age and beyond, is young enough that it is unlikely that there is any evolutionary survival advantage to having a reduced baseline insulin sensitivity as a neonate, when born preterm. It is possible, however, that surviving this

early state becomes a disadvantage after puberty leaving, unmasked, metabolic problems with the residual levels of reduced insulin sensitivity.

It is important to mention, specifically for children born preterm, there is still no clear answer to the nutrition conundrum facing clinicians: should early care be focussed on catch-up growth to promote neurodevelopment at the risk of long term metabolic morbidity, or should more focussed protein nutrition be implemented and maintained without focus on optimised overall growth? (Harding *et al.*, 2013)

1.7 Potential Aetiologies of Reduction in Insulin Sensitivity

Reduced sensitivity to insulin is closely associated with a metabolically inflexible, obese phenotype in the general adult population (Kelley, 2005). However, it has also been possible to study insulin sensitivity in the offspring of those who have developed T2DM and who are not obese and do not have T2DM themselves. Reduced insulin sensitivity can be induced in healthy, insulin sensitive individuals(Roden *et al.*, 1996) by administering a lipid infusion intravenously. This mechanism is particularly pertinent in view of the association of reduced insulin sensitivity with a high-fat, western diet and the metabolic syndrome and also because one of the commonest interventions in the nutritional management of preterm infants is administration of parenteral lipids while enteral milk feeds are being introduced slowly.

The lipid-induction theory offers an explanation for the development of insensitivity to insulin (Samuel *et al.*, 2010). It may also provide a mechanism by which to explain the association of prematurity and young children with reduced insulin sensitivity via hepatic lipid(Sandboge *et al.*, 2013), as well as lifestyle and body-habitus-associated reduction in insulin sensitivity in teenagers and adults. The lipid induction theory is particularly relevant to the Growmore study because of the possibility, ultimately, that lipid-induced insulin insensitivity may be modifiable from very early on in the life of infants born preterm. This in turn could preventing significant disease burden in later life. For discussion, lipid-induced insulin insensitivity has been divided into hepatic and peripheral.

1.7.1 Hepatic Lipid Induction of reduced Insulin Sensitivity

Non-alcoholic fatty liver disease (NAFLD; sometimes referred to as non-alcoholic steato-hepatitis) is a well-established clinical entity (Angulo, 2002). It is closely

correlated with reduced insulin sensitivity and obesity (Petersen *et al.*, 2005a), and is seen in adults and children (McCullough, 2004). Recent research has also suggested that nutritional programming may have a role to play in the eventual phenotypic expression of NAFLD: being born SGA or having excessive catch up growth and becoming obese in adulthood after SGA status at birth has been shown to increase risk of adult hepatic steatosis (Sandboge *et al.*, 2013).

The action of insulin, at the cellular level in the liver, is mediated by tyrosine phosphorylation of insulin receptor substrates 1 and 2 at the cell membrane. These then interact with 1-phospatidyl-inositol 3-kinase (PI3K). This has two main effects: inhibition of gluconeogenesis and promotion of glycogen synthesis (by inhibition of Glycogen Synthase Kinase; GSK) (Previs *et al.*, 2000). Thus, in a post-prandial, high-insulin state, glucose is taken up into the liver and converted to glycogen whilst serum glucose levels would remain normal.

In lean adult subjects with reduced insulin sensitivity who had comparable visceral fat-mass to lean, insulin-sensitive controls, a glucose load was been shown to cause an elevation in serum insulin levels but a blunting of muscle glycogen synthesis as measured by 13C-MRS. Alongside this, there was a concurrent increase in hepatic triglyceride content and increased fasting plasma triglyceride levels (Petersen *et al.*, 2007). Accumulation of hepatic lipid has been shown to be associated with activation of PKCε in rodent models (Savage *et al.*, 2006). This novel PKC directly inhibits the signalling pathway (described above) in the insulin-sensitive liver, thus inhibiting the normal changes in the cell metabolic processes usually stimulated by insulin. This therefore reduced metabolic sensitivity to insulin. It has also been shown that in individuals with reduced hepatic insulin sensitivity that in parallel to a deceased ability to inhibit gluconeogenesis in the face of elevated serum glucose and insulin levels there is a parallel and paradoxical enhancement of hepatic lipid deposition stimulated by insulin (via the sterol regulatory binding protein (SREBP)-1c. This increases intrahepatic triglyceride deposition as well as formation of very low density lipoproteins (VLDL) for export around the body (associated with increased cardio-metabolic risk). The increase in these lipids directly stimulates activation of PKCε, thus reinforcing the reduction in sensitivity to insulin and propagating metabolic inflexibility (Kelley, 2005; Brown and Goldstein, 2008)

Hepatic triglyceride levels have also been shown to be higher in lean Asian-Indian men who, though lean, are genetically at a higher risk of being insulin-insensitive compared to weight-matched white male controls (Petersen *et al.*, 2006). When transgenic mice which have an over-expression of *ApoC3* are given an high-fat diet there is increase in hepatic DAG and insulin compared to wild-type mice (Samuel *et al.*, 2010). Polymorphisms in the *ApoC3* gene have been described, in man, in individuals at higher risk of NAFLD and reduced insulin sensitivity (Petersen *et al.*, 2010). In the ApoC3 transgenic mice, there also appears to be hepatic activation of PKCε. When PKCε is blocked using antisense compounds, hepatic insulin sensitivity is preserved (Savage *et al.*, 2006).

It is likely, therefore, that during the development of reduced global insulin sensitivity, hepatic insulin insensitivity may occur in tandem, or precede, peripheral insensitivity (see below) due to high levels of circulating lipid overwhelming the hepatocyte and adipocyte capacity for processing, leading to secondary ectopic deposition of lipids(Weiss *et al.*, 2013). Importantly, NAFLD (in adults with T2DM) has been shown to be reversible with exercise (Petersen *et al.*, 2005a; Trenell *et al.*, 2008) and therefore may be modifiable by diet and exercise at a younger age before clinically significant reduced insulin sensitivity is reached.

1.7.2 Adipose tissue and reduced insulin sensitivity

Adipose tissue is not an inert 'store' in the metabolic milieu of the body as perhaps once thought. In addition, not all fat is 'equal': differential distribution and deposition of fat in adipose tissue had different effects on the baseline homeostasis. Visceral fat is less sensitive to the effects of insulin but is more sensitive to catecholamines (Wajchenberg, 2000). If lipolysis occurs in visceral fat, the released free fatty acids will (physiologically) drain via the hepatic portal system(Bjorntorp, 1990) which might contribute to hepatic steatosis via overload, as a mechanism by which visceral fat directly leads to reduced hepatic insulin sensitivity. Subcutaneous fat increase is associated with reduced insulin sensitivity in obese individuals but a causative link has not yet been demonstrated. Work by Taksali and colleagues suggests further that it may be the ratio of visceral to subcutaneous adipose tissue that is important in the overall final effect, independent of total volume of either in any given individual (Taksali *et al.*, 2008).

1.7.3 'Peripheral' reduction in Insulin Sensitivity

The term 'peripheral' is used to describe the changes in sensitivity to the action of insulin that occurs, in some conditions, in skeletal muscle. The literature regarding this is focussed mainly around Type 2 Diabetes Mellitus (T2DM) as this chronic condition provides a population of individuals who have varying degrees of reduced sensitivity to insulin, phenotypes and who are well enough to participate in research. There is obvious overlap between this group (where genetic inheritance will have a key role) and those who become insulin resistant because of other causes, but caution must be exercised before any assumption that the processes ongoing are the same, despite the similarity of outcome.

Lipid deposition in muscle and potential effects

In obese patients with T2DM, there is a clearly documented association between reduced insulin sensitivity and 'inappropriate' or 'ectopic', intracellular lipid deposition within skeletal muscle (Samuel *et al.*, 2010). Adult children of parents with T2DM (who have measureable reduction in insulin sensitivity without clinical T2DM and who are not clinically obese) have also been shown to have significantly higher levels of intramyocellular lipid (IMCL) than closely-matched, insulin-sensitive, control subjects (Petersen *et al.*, 2004). Muscle biopsies from non-diabetic, male, Pima Indians (who are more likely than the general population to go on to develop diabetes) have also shown increased levels of IMCL (Pan *et al.*, 1997). Petersen *et al* also showed that with normal ageing, IMCL content increased and in tandem with reducing peripheral insulin sensitivity with no specific increase in the triglyceride content of the liver (Petersen *et al.*, 2003).

The lipid induction theory describes a possible association between decreased insulin sensitivity, impaired cellular glucose uptake and increased IMCL, by impairment of cell uptake of glucose (similar to the process in the liver). IMCL may impair mobilisation of inducible glucose transporters (Shepherd and Kahn, 1999)) which usually provide an additional route into the cell for glucose when serum insulin is in high concentration. Translocation of these proteins to the cell wall is impaired in in-vitro cell cultures from individuals with T2DM (Ciaraldi *et al.*, 1995; Garvey *et al.*, 1998) thus suggesting a mechanism for reduced response to the action of insulin. This process is also reduced

in individuals receiving lipid infusions who demonstrate marked reduction in insulin sensitivity (Dresner *et al.*, 1999) suggesting it does not get activated in lipid-induced reduced insulin sensitivity.

Lipid in muscle and oxidative function: cause or effect?

In unhealthy, obese individuals, the accumulation of intramyocellular lipid by virtue of persistent delivery of excessive dietary free fatty acids to the myocyte is one explanation of the source of lipids associated with reduced insulin sensitivity. The other possibility, as outlined above) is that as other sources of lipid production overwhelm storage capacity in the liver and adipose tissue, excess VLDL is deposited as 'ectopic' deposits in muscle. This occurrence may lead to precipitation of increased morbidity in individuals, or may reflect a metabolic tipping point beyond which increased morbidity follows quickly, but not actually be a primary cause in the development of metabolic syndrome(Weiss *et al.*, 2013).

Skeletal muscle metabolism in healthy individuals displays a robust preference for fat oxidation, but can flexibly switch to glucose metabolism under the control of insulin (whilst fat oxidation is correspondingly inhibited). The degree to which this oxidative flexibility exists in vivo has been studied using MRS. In individuals with reduced insulin sensitivity, it has been demonstrated (using 31-P MRS) that the rates of ATP synthesis and rates of flux of the citric acid cycle (using 13C-MRS) are decreased by ≈30% (under hyperinsulinaemic/euglycaemic clamp conditions when compared to healthy controls)(Petersen *et al.*, 2004; Petersen *et al.*, 2005b; Befroy *et al.*, 2007). It has also been demonstrated that there is a reduction in the intramyocellular transport of phosphate in these individuals (Petersen *et al.*, 2005b).

The observations described above have been made in individuals who are already overweight and/or insulin insensitive, or in healthy subjects under experimental 'supra-physiological' (hyperinsulinaemic or hyperlipidaemic) conditions. In addition to this, the selection of subjects for examination took the extreme (Petersen *et al.*, 2004) phenotypes (most and least insulin sensitive) in their study groups and so the differences in effects at mitochondrial level would potentially be exaggerated in both directions from a 'normal' phenotype.

There is strong evidence from MRS that exercise can improve lipid oxidation in T2DM without changes in mitochondrial function (Trenell *et al.*, 2008) and that the time course of turnover of ATP in skeletal muscle under hyperinsulinaemic conditions *does not* support it as a rate limiting step in initiation of insulin-stimulated glycogen synthesis (Lim *et al.*, 2010). Indeed, in insulin-sensitive subjects, an increase in ATP was not seen during the first 45 minutes of a euglycaemic/ hyperinsulinaemic clamp, suggesting the acute metabolic effects of insulin are not directly related to oxidative capacity (Lim *et al.*, 2011c).

There is compelling evidence that raised IMCL in the setting of T2DM has an effect on insulin sensitivity within the cell (by reduction of glucose transport into the cell) and that reduced insulin sensitivity is associated with the presence of raised IMCL in the metabolically inflexible phenotype. Measured reduced oxidative capacity does not, however, suggest a true primary underlying defect in mitochondrial function leading to increased IMCL: reduced insulin sensitivity and impaired oxidative capacity have not been described in the absence of raised IMCL. It is as plausible that the reported reduction of oxidative capacity simply reflects a primary reduction in mitochondrial number.

Therefore it seems likely that the primary focus for lipid induction of reduced insulin sensitivity in this study should be in the liver and visceral adipose tissue: the evidence is not conclusive enough at present to look at skeletal muscle as a primary focus. It seems likely that skeletal muscle changes are 'downstream' of insulin insensitivity in the liver and visceral adipose tissue (Bremer *et al.*, 2012) and in a population of children, searching for early changes in lipid should be focussed where it is likely to be found. However, concurrent assessment of oxidative capacity of the muscles with 31P-MRS to ensure that this is as expected in the study group is sensible in view of the reported changes that can occur, as a proxy for early muscle metabolic alteration.

Other influences on skeletal muscle insulin sensitivity

Though the lipid theory of induction of reduced insulin sensitivity is prominently cited in the literature reviewed for this thesis, there are other mechanisms which have been proposed for influencing 'peripheral' insulin sensitivity. As skeletal muscle is one of the largest metabolic organs in the body when considered as a single entity, any process

that alters the skeletal muscle insulin sensitivity has potential to alter the metabolic flexibility of the muscle to change from fat oxidation in muscle to glucose oxidation when under insulin stimulated conditions and thus have huge effect on the body(Kelley, 2005). In addition, the presence of high levels of lipid in myocytes has to be put into context: elite athletes can have high levels of myocellular lipid as a store of energy to use during exercise but are usually very sensitive to the actions of insulin: the threshold for when they convert from fat oxidation is altered and, most likely, there is compartmentalisation of lipids in a different way to metabolically inflexible individuals(Goodpaster *et al.*, 2001). This is also seen in some knockout mouse models(Liu *et al.*, 2007).

As outlined above, a primary mitochondrial defect does not seem to be the underlying problem in T2DM (as an insulin resistant state) as apparent basal reduction in mitochondrial function can be overcome with imposed hyperglycaemic conditions. Alongside the proposed mechanism by which intramyocellular lipid reduces insulin sensitivity, other processes interrupting expression of glucose transporters (GLUT) on the cell surface in response to insulin signalling, or ones which decondition and reduce the volume of skeletal muscle, have potential to alter skeletal muscle insulin sensitivity.

Myostatin is a negative regulator of skeletal muscle growth. It has been noted in individuals with T2DM that they often have a raised level of myostatin(Brandt *et al.*, 2012). In animal models, inhibition or absence of myostatin has been associated with increased expression of glucose transporters, specifically GLUT-4(Takahashi *et al.*, 2014). Myostatin, therefore, may have an important role to play in insulin resistant state.

Adipokines (including adiponectin and leptin) are a group of hormones which are intimately involved in appetite, metabolism and glucose homeostasis, and exert effects on skeletal muscle. Leptin, in animal models where diabetic states have been induced by streptozotocin, has been shown to restore some glucose uptake and enhance the lipid oxidation in skeletal muscle(Hidaka *et al.*, 2002). Adiponectin is another adipokine which is present in lower amounts in the serum in those with T2DM(Arita *et al.*, 1999) and which is known to be insulin-sensitising(increasing glucose uptake into skeletal muscle and increasing lipid oxidation). In animal models of T2DM, it has been shown

that the induction of adiponectin production improves insulin sensitivity(Liu *et al.*, 2009b). While the adiponectin and leptin levels measured in the study cohort when they were reviewed between age 9 and 13 did not show any correlation with either body composition or insulin sensitivity (Korada, M; personal communication) the potential for adipokines modifying insulin sensitivity over a longer time, in a population of ex-preterm infants, is huge through programming or other mechanisms.

Inflammation and the associated generation of reactive oxygen species has also been linked with reduced insulin sensitivity through transcriptional and post-translational modification which ultimately inhibit insulin action, specifically in the pathogenesis and maintenance of T2DM, as well as in the metabolic syndrome(Hotamisligil, 2005; Shoelson *et al.*, 2006). Preterm infants are exposed to metabolic stress, inflammation and infection as part of intensive care. They are also often given gaseous oxygen as part of respiratory care which can lead to serum hyperoxia, in turn leading reactive oxygen species damage. The extent to which this neonatal exposure may lead to longer terms effects in skeletal muscle is not known, but this mode of damage and influence on insulin sensitivity cannot be discounted as at least part of the overall picture.

1.8 Body composition assessment

Assessment of body composition in clinical practice and research can be done using numerous methods but may be limited by both access to technology and the cooperation of the patient/subject. In the literature looking at fat deposition, lipid partition and sensitivity to insulin, several different strategies are used by authors to obtain the information needed.

1.8.1 Body fat assessment

Indirect measures

Measurement of skinfold thickness is a quickly and easily performed procedure which, when the investigator is trained (and their technique appraised regularly) can produce consistent measurements of the depth of subcutaneous fat deposits. Use of an averaging technique to ensure smoothing of the data from each site, followed by a summation of the totals, generates a number which can then be entered into standard equations to produce a percentage of total body fat (Durnin and Rahaman, 1967;

Durnin and Womersley, 1974; Slaughter *et al.*, 1988). This technique is superior to simple calculation of body mass index (BMI) in measurement of 'fatness' as it involves direct measurement of the subcutaneous fat compartment. However, it is subject to some of the same assumptions about consistency of distribution of fat between compartments across individuals (i.e. it does not necessarily take account of body 'shape'), and it does not allow assessment of individual fat compartments (visceral vs. subcutaneous). As, the confidence intervals around any single measurement will be wide (between 3 and 11% of the estimate(Wang *et al.*, 2000)). It is also subject to limitations around the normative data which produced the original conversion equations, much of which was generated in North America and is therefore not necessarily reliably generalizable to a UK population. It is also quite old data with normative z-scores last generated in 1993; (Davies *et al.*, 1993). In recent years the changes in childhood body composition in the population mean that, again, generalisability is not guaranteed. Some work using skinfold thickness in addition to BMI showed that it improved accuracy of estimation of body fat, but only in non-obese children(Freedman *et al.*, 2007). It remains an important method to consider, however, as it can be readily applied in a clinical setting.

Air displacement plethysmography (using the BODPOD™) is used to provide a variety of data about body composition and metabolic rate. The cost of the BODPOD™ means that it remains, for the most part, a research tool. Air-displacement plethysmography utilises comparison of measurement of air displacement against a standard volume in conjunction with mass measurement to generate a body density. The technique uses the mean of two readings within 150ml measured volume or takes a third if the two originals are more discrepant than that. In order to measure total body volume, the BODPOD can calculate an estimated lung volume based on height and weight parameters or the test subject can perform a series of manoeuvres via a closed breathing circuit which allow a direct measurement. The literature suggests that with the latter, the BODPOD is accurate to within 2% of other gold-standard measures for generating a body volume (such as hydrodensitometry) but it has the obvious advantages of being a dry method, quick, and less complex for younger subjects(Ball, 2005). BODPOD has been validated for use in children (Fields *et al.*, 2002; Fields *et al.*, 2004b) Once a body density is calculated, the BODPOD uses a 2 compartment model

based on either the equations of Siri or Lohman (Siri, 1961; Lohman, 1989). It is, therefore, subject to some of the same imposed methodological limitations as outlined for skinfold measurements.

The two other commonly used methods for estimating fat mass in humans are bioelectrical impedance analysis (BIA) and Dual-Energy X-ray Absorptiometry (DEXA). DEXA is, like air-displacement and skinfold measurement, an indirect technique with the added disadvantage of involving exposure to X-rays. In teenagers, this is an exposure to radiation at a time when the human body is rapidly growing and therefore as a technique it is hard to justify when non-radiation alternatives are available. BIA is also subject to predictive equations but has an even wider margin of error than skinfold measurement (+/-6 to 8kg in one individual adult) in estimating total fat mass(Piers *et al.*, 2000). For these reasons, we did not choose to use either of these methods in assessing body fat in our study.

Direct Measurement of body fat: MRI assessment of compartmental body fat

As described above, the literature suggests that potential mechanisms of reduced insulin sensitivity are correlated to the compartmentalisation of lipids and, specifically, the presence of intrahepatic lipid. The only tool we consider to be currently appropriate for use in a cohort of healthy adolescents, both ethically and practically, to assess intra-hepatic lipid (also called Intrahepatocellular lipid; IHL or IHCL) is magnetic resonance imaging. This is because it is non-invasive, involves not exposure to radiation and still gives quantifiable measurements of adipose tissue in both visceral and non-visceral compartments.

Magnetic resonance imaging scanners detect the signal released by magnetic nuclei in chemical compounds after application and removal of a magnetic field to a specific area of tissue. The signal generated depends on the type of magnetic nuclei (e.g. ^{1}H, ^{31}P, ^{23}Na), and the compounds in which they sit. This determines the deflection that occurs when the magnetic field is applied and thus the energy release when the field is removed and the nucleus returns to its original position. When used for 'plain' imaging the average signal returned from any given area is Fourier transformed to give a grey-scale image for clinical interpretation. This does not differentiate between individual compounds in the tissue. It is possible, however, to use a subtractive technique to

leave the residual image showing only compounds of interest. In identifying fat compartments, the main other molecule contributing to the picture generated will be water. As there is not much water in adipose tissue, it is possible to take a sequence of images generated using application of a magnetic field at a frequency which will target water molecules, applied at 0° and 180° to the subject in sequential applications. At release of the magnetic field, the different applications produce equal but opposite signals from any tissue containing large amounts of water. When the images generated are subsequently combined, the water signal effectively cancels itself out so anything left has a low water content (i.e. is mainly fat). This is the Dixon method (Dixon, 1984). Processing subsequently can generate a quantifiable cross-sectional area of adipose tissue in which it is easy to delineate compartments as the water rich tissue between compartments is absent.

A similar approach can be used to interrogate a much smaller voxel (sampling window) and by looking at the frequency of the received MR signal in relation to a reference (known) frequency, the chemical compounds within the voxel can be identified. The signal intensity can be measured at any given frequency and so quantification in comparison to the reference can also be made. In essence, for any voxel sampling tissue, a chemical spectrum can be generated which identifies compounds within the voxel and how much is there. This is magnetic resonance spectroscopy (MRS)(Qayyum, 2009). MRS can be used to measure chemical composition of any tissue in vivo at any given time and so repeated application of the magnetic field and capture of the resulting signal can measure chemical flux over time. Thus MRS has superseded many techniques reliant on biopsy or in-vitro cell culture to follow metabolic processes at a cellular level.

MRS is not without problems, however. It requires more intense signal concentration and is much more susceptible to heterogeneity across the voxel disrupting the signal. Similarly, the signal to noise ratio (SNR) needs to be as wide as possible to accurately identify small concentrations of compounds. For example, the predominance of water in cells (which contains magnetic H nuclei) can mask trace compounds unless the SNR is optimised. In 1H-MRS (which is used to identify IHL) this optimisation is done by selecting a voxel within a major lobe of the liver avoiding blood vessels if possible, using a higher rated scanner (3T instead of 1.5T improves frequency separation of the

compounds on the spectrum generated) minimising respiratory movement during signal acquisition (by employing breath-holds by the subject) and using a PRESS (point resolved spectroscopy) sequence which trades voxel definition for greatly improved SNR. Water is the reference peak generated in 1H-MRS and by comparison of the area under the peaks, a percentage of lipid can be calculated for that subject's liver. The literature shows that in cooperative subjects, the technique can be used at most ages. It has been successfully used in preterm infants at term(Uthaya *et al.*, 2005), term infants (Thomas *et al.*, 2008) and older children/adults(Thomas *et al.*, 2005; Brambilla *et al.*, 2006; Petersen and Shulman, 2006).

1.9 Measurement of insulin sensitivity

Measurement of glucose homeostasis and specifically sensitivity to insulin requires serum sampling and so in looking through the literature when designing a paediatric study, a method which is accurate but minimally invasive is desirable. Historically the 'gold standard' euglycaemic hyperinsulinaemic clamp study was used to obtain the relevant measurements: insulin sensitivity was measured by quantifying how much intravenous glucose was required to maintain serum glucose levels when administering an insulin infusion: the greater the glucose amount needing to be infused to maintain serum levels, the more insulin sensitive the subject(Sinaiko and Caprio, 2012). This method is, however, quite intensive and requires repeated sampling with the concomitant risks of administering insulin intravenously. Other methods have been validated against this 'gold standard' and provide a better alternative. Some studies have used a fasting insulin level and serum glucose to estimate baseline sensitivity to insulin, but this gives no indication of response to a glucose challenge. Normal indices at baseline may mask a blunted response to glucose loading in the early stages of decreasing sensitivity to insulin(Weiss *et al.*, 2013). However, the Homeostasis Model Assessment (HOMA2-IR) method is widely used to do this in both adults and children, and correlates reasonably well with the clamp method (Yeckel *et al.*, 2004).

To provide a glucose challenge in a structured way, a glucose tolerance test is most common in paediatric studies (both clinical and research), and the *oral* glucose tolerance test (OGTT) is most common due to ease of administration. This technique requires only one serum glucose/insulin measurement before giving the glucose load,

then one measurement afterwards (usually between 30 minutes and 2 hours)(Colley and Larner, 1990). From these measurements, an assessment of glucose disposal can be made and a higher than expected glucose level (≥7.9mmol/L at 2 hours) indicates reduced insulin sensitivity. It is also possible to calculate the 'Matsuda Index' from these values which has also been validated alongside the 'clamp' glucose disposal rate (though it does make assumptions about the area under the curve during calculation and is optimised with an intermediary measurement at 30 or 60 minutes)(Matsuda and DeFronzo, 1999; Yeckel *et al.*, 2004). From a paediatric study perspective, the OGTT with a fasting and 2 hour sample is the best compromise in measuring insulin sensitivity.

1.10 Effect of exercise and diet on insulin sensitivity

As has been outlined above, the liver appears to be central in reduction of insulin sensitivity and development of metabolic syndrome. While some of the biochemical processes are undoubtedly contributed to by genetic or early life programming, the literature review conducted suggests that with age, the influence of the environment and lifestyle becomes more important in insulin sensitivity. In simple terms, the chance of hepatic steatosis increases with an excess of energy. The surfeit of energy comes from one of only two alternative origins: too much intake or not enough energy used(Wiskin *et al.*, 2011)

1.10.1 Exercise in modifying health outcomes

One half of the energy balance equation is exercise (i.e. energy use). Kajantie et al (Kajantie *et al.*, 2010) researched the activity levels of adults who had been born preterm. They showed that even amongst those who had not got any sort of impediment from their preterm background (either neurological or respiratory conditions) those born preterm were less likely to take regular or prolonged exercise than their term-born peers. When leisure time spent in physical activity is examined, those born preterm and low birth-weight take less exercise than their term-born peers (Kaseva *et al.*, 2012). It is clear from direct measurements of their capacity for aerobic exercise that those born preterm are not at a disadvantage or significantly less capable of exercise than their peers(Clemm *et al.*, 2012).

Reinehr (Reinehr *et al.*, 2010) offered an intriguing glimpse into the link between programming and environment with an exercise intervention in a child population when they showed that the ability to achieve reduction in adolescent obesity was not linked to prematurity or SGA/AGA status *but* that being previously SGA was more significant in improving insulin sensitivity within the group during weight loss than the degree of weight loss itself. Work with the Helsinki Birth Cohort showed that young adults born preterm have a higher directly measured resting energy expenditure than those born at term (Sipola-Leppanen *et al.*, 2011). It is one possibility that until early adulthood, this is part of the process that contributes to maintenance of energy balance in the face of intake of dietary energy in ex-preterms (protective against obesity). However, if this means that less energy expenditure is required by exercise and a habit is established, it may be that at some point the balance tips towards accrual of energy: at that point apparently reduced exercise becomes a potential causal mechanism for becoming obese.

It is not possible to say what proportion of metabolically disadvantageous body compositions are due purely to environmental exposure and what proportion are a combination of programming during early growth priming a metabolic 'time bomb' unmasked by the changes of puberty and beyond. However, there is clearly a residual link and opportunity to beneficially modify body composition, the metabolic effects of which may still be strongly linked to early life.

In the wider paediatric population there are studies which have explored structured intervention in school-age children (Kriemler *et al.*, 2010; Puder *et al.*, 2011) which on the face of the results show improvements in aerobic exercise capacity and reduction in adiposity. However, the changes are measured immediately after the intervention and therefore the longevity of the change is not clear. The changes in fat mass are also very small and not of statistical significance. In addition there is some evidence to suggest that increased activity done at school leads to a concurrent reduction in exercise at home so that the overall effect of a structured intervention is minimised unintentionally (Wilkin *et al.*, 2005). It is also well known that a simple increase in exercise to reduce obesity is usually ineffective as the body upregulates appetite to take in more calories in compensation.

In adults with proven NAFLD (IHL >5%), resistance exercise has been shown to reduce IHL and improve glycaemic control independent of measured weight loss, with possible better sustainability than programmed aerobic exercise (Hallsworth *et al.*, 2011). This has not been examined in a paediatric population.

Measuring activity in children can be achieved in two ways: by reporting (either parent or child) or by accelerometric assessment. Evidence clearly shows that reported exercise overestimates the true amount of exercise done(Basterfield *et al.*, 2008) when measured directly. For this reason, the accepted standard is now direct measurement using accelerometers worn by study subjects. These devices measure acceleration in anything from one to three axes depending on the sophistication of the device. They monitor a number of counts per minute (one count representing a detected movement in a monitored direction/axis) which then allows stratification of the level of activity into (commonly) sedentary, mild, or moderate-to-vigorous activity (MVPA)(Metcalf *et al.*, 2002) using cut-off points. Once defined, a 24 hour period can be interrogated and the time at any given level of activity defined. Accelerometery has some limitations. Compliance (wear-time) is potentially an issue but use of an activity diary and clear instructions on use can maximise wear time. In addition, use of cut-offs allows definition of a minimum wear time above which the validity of the data is acceptable and representative of the level of activity done(Basterfield *et al.*, 2011b). Certain activities preclude the wearing of the accelerometer (e.g. swimming) and so on rare occasions, the measured activity may underestimate the actual physical activity done. Similarly, some types of activity measured on uniaxial devices may not trigger the accelerometer reliably (e.g. cycling whilst wearing a vertical-axis displacement accelerometer). Again, a well completed diary can aid interpretation of the data recorded.

Using accelerometers to assess activity in the Gateshead Millennium Cohort (another cohort of children from North Eastern England, born in 2000a.d.) it has been shown that a reduction in MVPA is associated with increased BMI(Basterfield *et al.*, 2012; Pearce *et al.*, 2012) and importantly for our cohort, the amount of time in MVPA decreases as children enter adolescence(Basterfield *et al.*, 2011a). Therefore, to help address influencing factors on energy balance in our cohort, accelerometery provides a method of reliably and accurately measuring direct activity.

1.10.2 Assessment of skeletal muscle oxidative capacity

When considering activity, energy expenditure and metabolic flexibility, it immediately draws attention to the skeletal muscle and, more particularly, mitochondrial oxidative capacity. As noted evidence from adult populations shows that a primary mitochondrial defect is unlikely to be a primary cause for reduced muscle insulin sensitivity (Lim *et al.*, 2010; Lim *et al.*, 2011c) and the presence of IMCL is most likely to result from overload of hepatic capacity, at the tipping point into illness. However, the ability of the muscle to respond to glucose and the oxidative capacity under exercise is something that influences the global metabolic flexibility in an individual (Kelley, 2005) which may be influenced by many factors such as number of mitochondria and the ability to transport glucose into cells. In adolescents born preterm these are factors that could relate to gestation, nutrition and growth or later environmental influences. As the evidence shows, reduced time in MVPA and increased BMI appear to be related in the wider paediatric population and so when considering insulin sensitivity in adolescents born preterms it is sensible to have some measurement of skeletal muscle oxidative capacity as an objective measure of exercise capacity in the study group. In addition, part of the rationale for this study (see section 1.8.4) was to look at potential assessments that could be used in a serial fashion to assess an intervention (either lifestyle of diet) that might measure improved metabolic outcomes. Measurement of oxidative capacity would allow an assessment of improvement of metabolic flexibility in this situation.

Non-invasive measurement of mitochondrial oxidative capacity is well described using the same MRS principles as outlined for IHL measurement. The technique is modified to detect the 31P nucleus which is present in the compounds which flux during exercise (ADP, ATP, Pi and PCr) to maintain ATP levels for the muscle to keep working. If the spectra are collected during exercise and afterwards, the level of phosphocreatine (which is the intrinsic muscle store for high-energy phosphate) can be measured as it depletes releasing high energy phosphate (measurable as an increasing Pi peak), then replenishing afterwards by mitochondrial oxidation. The half-time of replenishment is then a quantitative measure that is indicative of the oxidative capacity of the muscle which is being sampled in the MRS voxel (Chance *et al.*, 2006; Boesch, 2007; Kemp *et al.*, 2007). The method has been shown to be acceptable to

subjects and very reproducible making it a robust comparison across, and within, groups (Bendahan *et al.*, 2003; Befroy *et al.*, 2007; Layec *et al.*, 2009; Lim *et al.*, 2010). It has all of the advantages outlined for 1H-MRS (non-invasive etc.) and therefore in measuring skeletal muscle oxidative function in children, is the most acceptable method available.

There are some recognised limitations of the technique which could be relevant to a paediatric population. Firstly the exercise protocol needs to be standardised across the cohort and younger participants might find it more difficult to comply with this. Secondly, the technical placement of a voxel aims to sample a single muscle group. If more than one group is sampled, the PCr in different groups may deplete and recover at different rates and the loss of a unified recovery (known as inorganic phosphate splitting) precludes accurate interpretation of the results. Lastly, the minimum pH of during exercise has been shown to affect the rate of recovery of PCr and therefore in analysis consideration of standardised post-hoc corrections has to be made. This has potential, therefore, to affect comparisons across groups and generalisability of the results to other patient groups(Iotti *et al.*, 1993).

1.10.3 Vitamin D

Vitamin D is a steroid hormone produced in the skin from 7-dehydrocholesterol. UV light passing through the skin creates pre-vitamin D_3 which then undergoes a temperature sensitive rearrangement to produce vitamin D_3. Vitamin D_3 then undergoes hydroxylation twice over (once in the liver and then in the kidney) to produce dihydroxylated vitamin D. 25-hydroxyvitamin D_3 is the most abundant form of vitamin D in the circulation and is mostly found bound to vitamin D binding protein. The enzyme in the kidney responsible for producing 1,25-dihydroxyvitamin D_3 (the active form of vitamin D) is a mitochondrial enzyme (CYP27B1). Alternative 24-hydroxylation of the 25-hydroxyvitamin D_3 can also occur in the kidney to produce the inactive 24,25-dihyroxyvitamin D_3. Production of this alternative form of dihydroxyvitamin D is induced by the presence of 1,25 dihydroxyvitamin D and part of the mechanism for regulating itself. Some vitamin D can be acquired through dietary sources but the vast majority is generated in the skin.

The effects of vitamin D are mediated throughout the body by vitamin D receptors. The vitamin D receptor is a nuclear steroid receptor and highly conserved across species of vertebrate (mammal, fish, bird). The human vitamin D receptor joins with the retinoid X receptor (RXR) ligand in order to form a working complex which can then bind with co-regulatory complexes known as vitamin D response elements(Haussler *et al.*, 2013). The vitamin D response elements are specific genomic DNA sequences which bind the activated vitamin D receptor and promote transcription. Once bound, the effectiveness of transcription appears to be dependent on recruitment of co-activators, and the diversity in this process may partly explain the diversity of effects seen with vitamin D/vitamin D receptor interactions(Christakos *et al.*, 2016).

Vitamin D is described as having 'classical' actions through involvement with skeletal homeostasis(Shaw and Mughal, 2013a), but in recent years it has been appreciated that there are vitamin D receptors in many non-skeletal tissues throughout the human body(Shaw and Mughal, 2013b; Wacker and Holick, 2013). As this knowledge has progressed, given the high prevalence of vitamin D deficiency in the western hemisphere, vitamin D replacement and supplementation has become the focus of therapeutic investigation in many different clinical disciplines, from treatment of psoriasis, hypertension and myalgia, to reduced muscle strength and glucose control in diabetes(Girgis *et al.*, 2013).

The association with high BMI and reduced serum vitamin D levels is well known(Samuel and Borrell, 2013): in those who are vitamin D deficient and obese, standard supplementation is less effective in raising serum vitamin D levels than in non-obese subjects(Forsythe *et al.*, 2012; Saliba *et al.*, 2013). It is also reported that young adults born preterm are less likely to have adequate intake of dietary vitamin D or calcium when compared to their term-born peers(Kaseva *et al.*, 2013).

Evidence in the literature also demonstrates that vitamin D deficiency in adults has a measurable and reversible impact on mitochondrial oxidative function when measured using the 31P MRS technique (Sinha *et al.*, 2013). The mechanism behind this observed phenomenon is unknown, but it is methodologically essential now when using the 31P MRS technique to control for, or at least measure, vitamin D status in volunteers.

Serum Vitamin D levels have been investigated in relation to insulin sensitivity in adults, adolescents and children, though the results are mixed (Girgis *et al.*, 2013). In recent studies there appeared to be no difference in insulin sensitivity in obese children and adolescents who were vitamin D deficient and there was no difference in insulin sensitivity between obese and non-obese subjects (Torun *et al.*, 2013). In healthy young adults who had vitamin D deficiency, supplementation into the normal serum range did not improve insulin sensitivity when measure using a euglycaemic hyperinsulinaemic clamp study (Grimnes *et al.*, 2011). Some studies of supplementation of vitamin D, however, have reported improvements in glycaemic control and insulin sensitivity in type 2 diabetes (Mitri *et al.*, 2011) and gestational diabetes (Asemi *et al.*, 2013) where a pre-existing condition exists and improvement is desired. In adolescents who were obese and vitamin D deficient, high-dose supplementation of vitamin D resulted in a return to normal serum vitamin D levels and in an improvement of insulin sensitivity measured using fasting insulin and HOMA-IR modelling(Belenchia *et al.*, 2013). It should be noted, however, that BMI did not change in the study period and no response to glucose loading by way of assessing metabolic flexibility was made. Kelishadi et al supplemented children who met their study definition of having metabolic syndrome with very high dose vitamin D supplements (300000iU) over 12 weeks and looked at whether this improved indices of glycaemic control(Kelishadi *et al.*, 2014) such as the HOMA index. In the supplemented group there was measured improvement in these indices, but as with Belenchia's group there was no measure of response to dynamic loading with glucose.

The breadth of possible interactions of vitamin D with the areas of metabolic interest in this study suggest that investigation of vitamin D levels in this cohort would be both novel and important in order to interpret any metabolic findings.

1.10.4 Diet

Balancing against energy expenditure in examining whether there is a net energy accrual or loss, is dietary intake. The literature is clear about how important diet is as a contributory factor in development of NAFLD both in terms of direct excess of calories and exposure to substances which trigger deposition of IHL such as fructose and alcohol(Bremer *et al.*, 2012; Lustig, 2013; Weiss *et al.*, 2013) and, as outlined in 1.7.3, in some instances of dietary deficiency. In adults with type-2 diabetes, dietary intake is

central to the dual cycle hypothesis of development and persistence of the condition(Taylor, 2008). In newly diagnosed type 2 diabetes, the beta cell dysfunction (previously regarded as irreversible) can be interrupted and reversed by adherence to a highly calorie restricted diet. During this process, the first measurable changes that occur are pancreatic and IHL clearance (well before other adipose compartments reduce in size) (Lim *et al.*, 2011a).

Diet, however, is hard to robustly assess due to recall bias, intentional intake bias (when the subject knows they are commencing a period of monitoring) and non-expert variation in interpretation/estimation of components (e.g. fat, carbohydrate, protein) of the food during analysis of what has been reportedly consumed. The standard options for assessment include prospective food diaries, weighed-food records and recall questionnaires. In designing a paediatric study where access to a dietician would be limited, and not available until data analyses, it was important that the technique used was robust, reproducible and, ideally, removed some of the analysis work by having pre-set values for reported intake.

The SCRAN24 program chosen for this study is a piece of laptop-based software which engages children to input types of food and portion size from provided photographs for the previous 24 hours food intake. It has been validated for use against conventional methods of dietary assessment and, if anything, tends to slightly underestimate intake for total energy(Foster *et al.*, 2013a). It uses division of the day into epochs and prompts throughout to improve recall, and is simple enough for children in primary school to use it successfully. The database attached to SCRAN24 has 98% of the most common foodstuffs consumed by children in the North East of England accessible in such a way that, by selecting a food type and portion size, it generates automatic output files with a nutritional breakdown for that item. This means that only a limited number of foods will ever have to be assessed by a dietetic expert and therefore the time and cost of producing valid results is greatly diminished. In these respects it has great advantage over the other methods which could have been used.

1.11 The study cohort

The Growmore Study is the latest review of the Newcastle Preterm Birth Growth Study cohort (children who were born preterm in Newcastle in the 1990's) (Wood *et al.*, 2013). The children were recruited soon after birth into two separate studies looking at nutrition in preterm infants. The Growth Study (Cooke *et al.*, 1998; Cooke *et al.*, 1999) recruited infants who were ≤1750g birth weight and ≤34 weeks gestation. They were randomised to be fed specific preterm- or term- formula from discharge to 6 months of age, or the specific preterm formula until term corrected age, then term formula until 6 months old. The Protein Study (Embleton *et al.*, 2001; Embleton and Cooke, 2005) used similar methods and randomised recruited infants (≤1750g birth weight and ≤34 weeks' gestation) into 3 groups. The groups were then given one of three milk formulas which had different protein concentrations (2.7g, 3.0g and 3.3g/100ml formula) but the same calorie density. They were fed the selected formula from time that they began enteral feeding until 12 weeks after term (corrected gestational age). There was a small time period of overlap between recruiting for the two studies and thought the recruitment criteria above were clear, one set of low birthweight (but 36 weeks GA) triplets were include as breastfed controls, as were siblings of eligible infants whose birthweight was >1750g.

Both studies looked at outcome measures including body composition (by Dual Energy X-Ray Absorptiometry [DEXA] and skinfold thickness), anthropometry (height, weight, length, head circumference) and involved taking serum samples to assess body biochemistry (including lipids). Extended follow-up visits, when other measures of development were assessed (mental and psychomotor developmental indices), were undertaken until 24 months of age. In total, more than 200 infants took part in the initial studies.

At the age of 10-12 years, the cohort was revisited in a further study looking at growth and insulin sensitivity. In this study, the anthropometric measures were repeated and further blood samples taken to examine insulin sensitivity with respect to contemporary body composition, growth in the intervening years and early (infant) growth patterns. Of the original cohort, 153 children consented to assessment, 139 had assessment including DEXA and of these, 109 had blood sampling done.

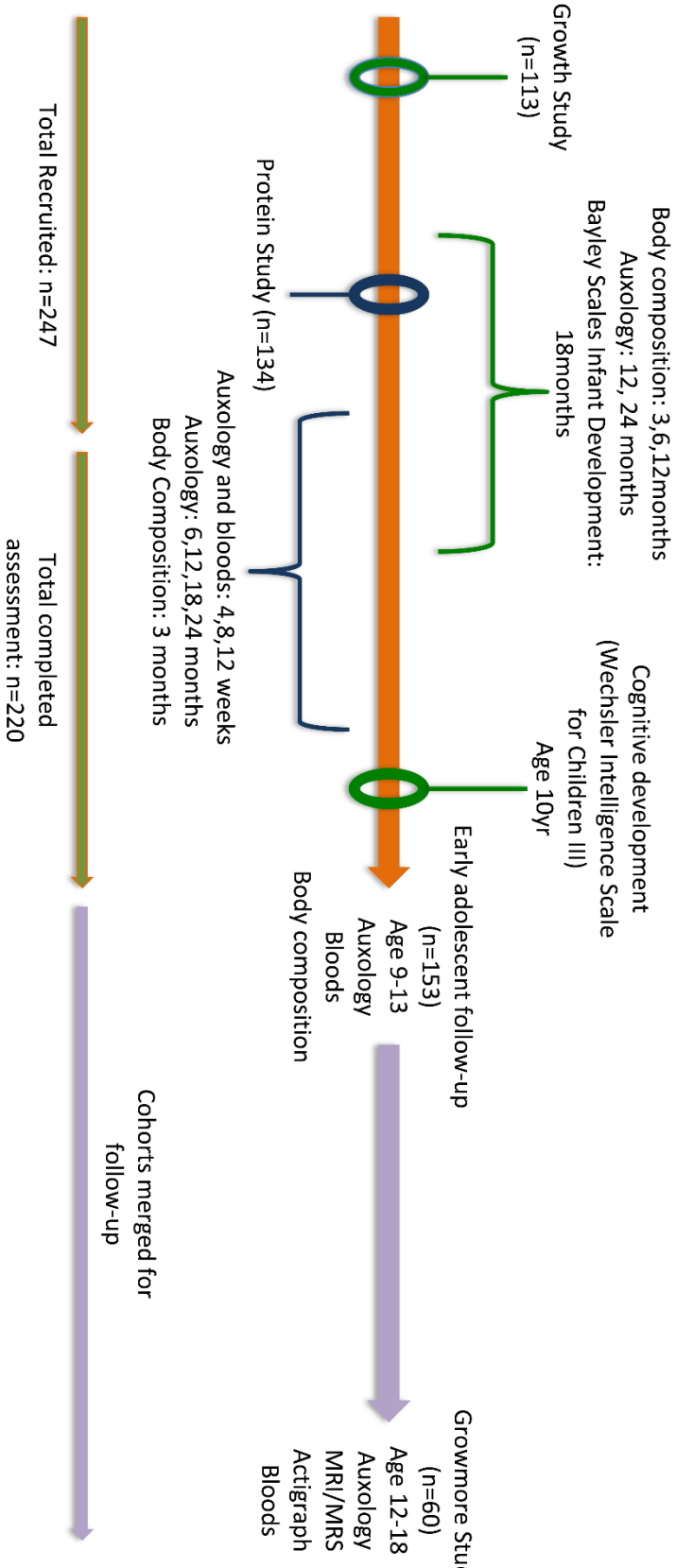

Figure 2: NPTBG Study Cohort: recruitment and studies chronology

1.11.1 Protein Study

The protein study looked at the differences that occur when preterm infants are fed iso-caloric formulations with different protein concentrations. It demonstrated that both weight gain and nitrogen accrual were improved in children who were fed the high-protein formula once enteral feeding had been established (Cooke *et al.*, 2006). This was without any of the feared side-effects of a high protein formulation expressed in contemporary literature such as acidosis and excess nitrogen compounds in the blood. The study also showed that on the high-protein formulation, the rate of growth was greater than that which would have been expected in utero: this demonstrated that the protein intake (not calorie density) of the formulation was responsible for good catch-up growth. It also showed that weight gain was best in boys fed the high protein formula, suggesting that even high protein formula may not be meeting the growth needs of girls in this critical period. The study also demonstrated that a high protein formulation would promote better growth *prior* to hospital discharge (Embleton and Cooke, 2005).

1.11.2 Growth Study

The growth study looked at the differences that occur in preterm infants when fed different 'whole' formulas (rather than altering a single component in the protein study). Specifically, there was inclusion of a preterm formula group (higher calorie, protein and mineral contents). This demonstrated that differences in very early growth persisted to 6 months of age and that, dependent on the calorie density of the milk, children would up- or down-regulate their intake (volume) to get the calories they required for growth (Cooke *et al.*, 1998). By 12 months, those fed on the preterm formula consistently had increased lean and fat mass (therefore no increase in % fat mass)(Cooke *et al.*, 1999; Cooke *et al.*, 2010) which is at odds with the suggestions of some authors (Ong, 2007) who have suggested preterm infants are more likely to deposit 'central' fat mass disproportionately to lean tissue mass. In the 'preterm' formula group the magnitude of catch-up growth shown was greatest, and boys grew more than girls (Cooke *et al.*, 1998; Cooke *et al.*, 1999; Cooke *et al.*, 2010). This disparity between the sexes was not previously reported in preterm infants.

Developmental outcomes were studied in the growth cohort at 18 months of age (Cooke *et al.*, 2001). It was found that girls have better psychomotor and mental

developmental indices than boys but there did not seem to be any difference in the cohort ascribable to the formula given to the children. Differences in the growth patterns observed previously were also maintained.

1.11.3 Most recent assessment

The cohort was seen last at the ages of between 10 and 13 years of age. Children from both the protein and growth study were reviewed. Data taken at the time (as yet unpublished) showed that the development of reduced insulin sensitivity was not associated with rapid growth and weight gain in infancy. However, the change in BMI since infancy was correlated with contemporary fat mass and level of insulin sensitivity (Korada, M. *et al*. Presented at PAS, Baltimore, 2009). A small number of the cohort also had epigenetic analysis which showed that 'patterns of weight gain or body composition are related to gene expression, and differences in DNA methylation of specific genes' (Groom, A. *et al.* Presented at DoHAD, Chile, 2009). These data suggested strongly that in the cohort, there had been strong influences on health which were not directly attributable to infancy despite the marked difference in growth that had been demonstrated by nutritional manipulation started prior to discharge home.

1.12 Study design

It is important to acknowledge that this study sits within a larger piece of research following up the Newcastle Preterm Birth Growth Study (NPTBGS) cohort (Wood *et al.*, 2013) longitudinally since birth. While there is a degree of opportunism in the study design (i.e. there is a 'bigger picture' imperative to try to optimise the amount of data collected to enrich the longitudinal dataset) all of the assessments used were planned in order to minimise the inconvenience to the families and children who volunteered. To this end, serious consideration was given to the merits of spending some of the limited funds for the study on age-matched controls to perhaps place the data in a more generalizable context. While this would have provided an interesting comparison for some of the MR studies and serum data, the control group would not have been big enough to match the spread of ages within the study cohort nor provide enough power for the control group data to be robust (due to lack of numbers). The study, therefore, remained an observational cohort study. This model offers most

opportunity for investigation of the cohort group at this time, while preserving the longitudinal data collection ethos underpinning the research as a whole.

When planning this study, it was also acknowledged that it might be the last time that a significant number of the cohort could be recruited into a study episode. The NPTBGS study group felt, therefore, that it was an important opportunity to look at the use of different and novel modalities of investigation (e.g. MRS, BODPOD, SCRAN24, accelerometers) from the perspective of how they could be used, closely integrated within single study visits, to provide data. This would then perhaps go on to inform future study planning for longitudinal (serial visits), multimodal assessment in interventional studies with future cohorts of children born preterm. This is reflected in some of the data presented in the results chapter.

1.13 Study Aims and Hypotheses

1.13.1 Growmore Study Hypotheses

From the data collected at previous visits from the study cohort and the evidence in the literature, there are four hypotheses underpinning the current study. These are presented below as null hypotheses regarding a cohort of adolescents born preterm:

1) In adolescence, muscle mitochondrial oxidative capacity quantified by 31P MRS will show no association with physical activity levels, adipose tissue distribution, sensitivity to insulin, or vitamin D status.
2) In adolescence, IHL accumulation will show no association with nor be determined by insulin sensitivity, serum lipid profile and compartmentalisation of body fat deposits.
3) In adolescence, there will be no measurable association between body metabolic outcomes (specifically insulin sensitivity) and body composition, nutritional intake or directly measured physical activity.
4) Auxological methods (specifically skinfold thickness measurement techniques) of assessing body composition will not correlate well with gold standard

'research methods' for quantifying body composition (BODPOD), nor will they correlate with directly measured lipid deposition in the cohort (1H-MRS).

1.13.2 Growmore Study Aims

The study had the following aims which guided the study design, the investigative methods chosen and the data analysis:

- To investigate whether contemporary fat mass, lipid distribution and compartmentalisation in adolescents, who were born preterm, is related to directly measured metabolic outcomes.
- To investigate associations between reduced insulin sensitivity, exercise, prematurity and mitochondrial function in the cohort.
- To determine the potential interactions between adipose tissue compartmentalisation, lipid deposition, reduced insulin sensitivity and other specific factors (such as diet and measured physical activity).
- To determine the feasibility of using the study visit protocol as repeated assessments in a future, controlled interventional trial.
- To collect serum samples for future epigenetic/RNA and metabolomic analyses.

Chapter 2. Methods

2.1 Cohort selection and recruitment

As described, this observational cohort study recruited from a cohort of individuals who have previously been involved in research studies. The study participants recruited attended a single visit to the Newcastle Magnetic Resonance Imaging Centre (NMRC), on the Campus for Ageing and Vitality at Newcastle University, during which all but one of the investigations were completed within a four-hour timeframe. At the end of the study visit, the participants were issued with a pre-programmed accelerometer (Actigraph) and instructions for using it in the three days immediately after the study. Due to the cost of the multiple tests the study was limited to 60 participants. The protocol for the study has been peer reviewed and published (Wood *et al.*, 2013) as part of a broader-spectrum article discussing the research in which the study cohort have participated since birth.

2.1.1 Recruitment

153 of the original cohort participated in the most recent study. We had previous contact details and permission to contact 247 of the children from the original NPTBGS cohort. It was anticipated, therefore, that recruiting 60 children from the same cohort would be feasible in the timescale allowed for the study. No active selection process was used (i.e. no discriminators based on previous study results were applied to the selecting participants from the cohort to avoid bias in the process of recruitment).

In line with the previous studies involving the cohort, we used a standard, paper-based recruitment with three phases:

1) The NHS Summary Care Record (SCR) was accessed in a strictly limited fashion by me (as per ethical approval) to obtain current General Practitioner (GP) details for the cohort using the NHS number for each child, which we held already from participation in previous research. For those with a registered GP, who were listed as alive on the SCR, we contacted the children's GPs to ensure the contact details we had were correct and that the children were alive and well. The GP was required to reply with a standard form stating that either the details we held were correct, or providing any updated details as appropriate.

Of the 247 previously-contacted children, we were able to find current complete details for 235. The relevant GPs universally returned the information slips within the time frame of recruitment and we contacted everyone for whom information was confirmed by the GP as correct or updated during the recruitment period.

2) Once we had a response from the GP (final n=235), we contacted the parents by mail (enclosing the project information sheets) to ascertain interest in the study. We required a response by mail using a standard reply sheet and stamped, addressed envelope. A maximum of two invitations were sent to each traceable cohort member.

3) Once the family confirmed interest in the study, a follow-up contact to provide any extra required information and to arrange a visit date was made.

The study visits were then assigned on a first-come first served basis according to the parental replies.

Written consent for participation was taken at the study visit, after a review of the information sheet and verbal briefing about the process. Both parent- and child-specific forms were used and the both had the option to accept or decline any part of the testing, at any time during the study visit.

2.2 Investigative methods

Participants attended the visits following an overnight fast with no food after midnight prior to the morning of the visit. All visits were carried out in the morning to facilitate this. Participants were allowed a small drink of water up to two hours before the study visit start time.

The following methods for data collection were used during the study visit. The visit flow chart can be seen in figure 3.

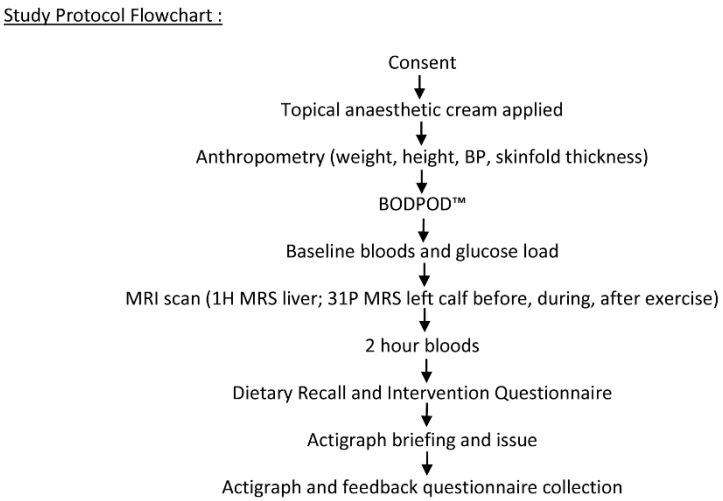

Figure 3: Study protocol flowchart

2.2.1 Anthropometry

Each participant had measurement of: height (to the nearest 0.1cm) using a telescopic stadiometer (SECA, Birmingham, UK) with the head positioned so that the Frankfort plane was correctly attained and the stadiometer arm was resting on the crown of the head (to ensure standardisation between participants); weight (to nearest 0.1kg) taken from the BOD POD™ (COSMED, USA) during plethysmography; waist circumference (to the nearest 0.1cm) using a self-retracting, soft, plastic measuring tape (BMI measuring tape, model no. CWSG26852, CTO group, China) with the waist taken as the transverse plain midpoint between the lower margin of the ribs and the upper edge of the superior iliac crest; and blood pressure (mmHg; sitting, right arm) using an automated oscillometric sphygmomanometer (Carescape vital signs monitor, GE Healthcare systems, UK) with appropriately sized cuff (to cover the upper arm as much as possible, but not including the elbow nor impinging on the axilla. Each measurement was taken up to three times (if two concordant readings, this was recorded; otherwise three were averaged) with the exception of the BODPOD which took two concordant readings of body weight during its use. From these measurements, body mass index was also calculated. Height readings were also used in analysis to generate fat-mass and fat-free mass indexes, as well as indexing the intra-abdominal lipid measurements. Standard deviation (z) scores (SDS) were calculated for height, weight and BMI using

standard UK reference data (Cole, 1997; Cole *et al.*, 1998) (http://www.cdc.gov/growthcharts/percentile_data_files.htm) and an online calculation tool to generate the score (Quesgen Systems: http://www.quesgen.com/tools/BMIPedsCalc). The accuracy of this tool was checked during analysis and cross-referenced to another online tool which generated the same values as the chosen tool (http://www.stokes.chop.edu/web/zscore).

2.2.2 Body composition

Holtain (Crymych, UK) calipers were used to measure skin-fold thickness in four areas following the techniques published by Durnin, Womersley and Rahman (Durnin and Rahaman, 1967; Durnin and Womersley, 1974): biceps, triceps, subscapular and suprailiac. Each measurement was repeated up to three times: if two concordant readings were obtained, this was recorded; otherwise three were taken and the average recorded. The investigator was trained in the technique of skinfold measurement by members of the Diet and Nutrition Survey of Infants and Young Children prior to the study commencing.

From these measurements, a measure of body composition was calculated using the logarithm of the sum of four skinfolds to generate a body density estimate. The body density formulae used (Durnin and Rahaman, 1967) are sex-specific and age dependent (<18 or ≥18 years old). The density estimate was then used in age-specific formulae to calculate percentage body fat (see below). In addition, the equations for estimate of body fat used by Slaughter (Slaughter *et al.*, 1988) from two skinfold measurements (triceps and subscapular) were calculated. These measures are commonly used in North America. Six different equations were required as per Slaughter's research, specifically one for each group within the cohort defined as: pre-pubescent (Tanner Stage 1+2), pubescent (Tanner Stage 3) and post-pubescent (Tanner Stage 4+5) white males with sum of two skinfolds <35mm; all females with sum of two skinfolds <35mm; any males with sum of two skinfolds >35mm and any females with sum of two skinfolds>35mm.

Air-Displacement Plethysmography was used to provide a second measure of body composition using the BOD POD™ system (COSMED, USA). This non-invasive method measures the density of the test subject and has been validated in children (Fields and

Goran, 2000; Fields *et al.*, 2002; Fields *et al.*, 2004a; Fields *et al.*, 2004b; Ball, 2005). The BODPOD used has also been validated 'in-house', in adults, in comparison to the electric bioimpedance technique also used at the NMRC.

All volunteers who underwent BODPOD measurement were in a fasted state to try to standardise hydration status and density measurements. The measurement process involved calibration of the measurement chamber (empty, then with a standard cylinder of known volume) then the participant sits in the measurement chamber. The difference in volume by comparison with the calibration volumes allows calculation of the participant volume and thus, in conjunction with the measured mass of the participant, the body density can be calculated. The software programme that controls the BODPOD measurement requires two consistent readings. In the event of disparate readings, it takes a third to produce a usable measurement. Our participants wore a tight-fitting swimsuit or equivalent (to minimise the loose clothing which might interfere with the reading) and a tight-fitting hat to prevent hair-volume isothermic interference with the measurement. The biggest difficulty that was faced was ensuring the children sat still during the measurement, but all who agreed to be measured in the BODPOD (n=58/60) managed to achieve a measured density.

Unlike the method used by Fields (Fields and Goran, 2000; Fields *et al.*, 2004a; Fields *et al.*, 2004b) we did not directly measure the children's thoracic gas volume. The BODPOD software gives an estimated thoracic gas volume based on the height of the participant. Data from a preliminary study by Holmes et al. suggests that, compared to hydrodensitometry and air-displacement plethysmography using measured thoracic gas volume in those aged 8-12 years old, use of the estimated thoracic gas volume is acceptable(Holmes *et al.*, 2011). Using this estimate meant that we minimised the length of time in the BODPOD for the participants in order to minimise any discomfort associated with being exposed in a swimsuit. In a study with adolescents where there were many different measurements being done, we decided that the evidence supported this pragmatic approach to minimise the length of time spent in the BODPOD. It should also be noted that direct measurement of thoracic gas volume as described is difficult to achieve in paediatric subjects (Fields *et al.*, 2004b). As such, while using the predicted thoracic volume might introduce error in any individual

measurement, it should be a consistent error across the cohort which therefore minimises impact on the correlation analyses.

Converting body density into percentage body fat

For both the skinfold thickness (sum of four) and BODPOD techniques, a body density measurement is generated. In order to convert this to a percentage body fat measurement the density measurement is entered into an equation of body composition: either that by Siri(Siri, 1956; Siri, 1961), applicable to those aged 18 or over; or Lohman (Lohman, 1989) which is a modified version of the Siri equation to take account of the age of the child (by using age-stratified constants in place of the fixed values in Siri's equation). This was done manually for the skinfolds results, but was calculated automatically by the BODPOD software and a percent fat reading supplied (in addition to the fat mass and fat-free mass readings in kilograms). For the skinfold and BODPOD techniques, a fat mass was calculated using the percentage result and known bodyweight, then indexed to produce an FMI and FFMI comparable to that generated by the BODPOD technique. This ensured that there was some correction for body habitus and allowed better within-group comparison (VanItallie *et al.*, 1990).

2.2.3 Blood sampling and oral glucose tolerance testing

The study participants fasted for around 8 hours overnight prior to attending the study visit. This allowed a standardised fasting-state oral glucose tolerance test (OGTT) to be performed (Colley and Larner, 1990)

For those participants who consented to this investigation a venous cannula was inserted (22G Jelco, Smiths Medical, Kent, UK) prior to the administration of the standard oral glucose load (2.6ml/kg of Polycal™ [Nutrica Clinical Care, Wiltshire, UK] up to a maximum of 113mls, equivalent to a maximum of 75g anhydrous glucose). A series of baseline blood tests (for lipid profile, liver function, glucose, insulin, sex hormones, RNA, cellular DNA and serum to store) were taken at insertion of the cannula. Those participants in whom it was possible to site a cannula had a repeat sampling (for serum glucose and insulin) two hours after the oral load was finished.

The whole-blood samples taken for glucose were immediately tested using the glucose oxidase method glucose analyser (Yellow Springs Instrument [YSI] 3000, Yellow

Springs, OH). They were then centrifuged (at 3000 rpm for 10 minutes) to isolate serum which was then tested using the same machine. Prior to use and at regular intervals throughout the visits, the YSI3000 was calibrated using a 10mmol/L quality control solution and variance of ±0.2mmol/L was considered acceptable. Attempts were made to spin the samples for glucose within minutes of collection but this was not always possible because of demand for use of the centrifuge. Therefore, due to the extra processing step and the sometimes unavoidable delay in centrifuging samples, the whole blood sample glucose readings were used in the final analyses in preference to the serum glucose.

The samples for insulin analysis were collected in a preservative free vacutainers and allowed to coagulate before centrifugation within 30 minutes of collection. The serum was then pipetted and stored separately at -70°C. Once the study cohort visits were finished the samples were transported from NMRC to the laboratories, frozen, and analysed immediately once thawed in the laboratories. The insulin samples were analysed as a batch, together, (courtesy of Annette Lane, Institute of Cellular Medicine, Newcastle University) using the Dako Insulin ELISA Kit (Dako Denmark, Glostrup, Denmark) with the final plates read photometrically. Repeat measures were carried out every seventh sample to ensure quality control and all were within acceptable tolerances (accepted coefficient of variation for this ELISA is 7.5%). For analysis, the fasting insulin and glucose measurements were used to estimate insulin sensitivity and resistance using the Homeostasis Model Assessment (HOMA) method (Matthews *et al.*, 1985; Levy *et al.*, 1998; Wallace *et al.*, 2004). In addition, the index pioneered by Matsuda et al (Matsuda and DeFronzo, 1999; Yeckel *et al.*, 2004) was also used to examine the disposal of glucose after OGTT in analysis, as it offers the advantage of quantifying the physiological response to a glucose load (not offered by the HOMA method).

The blood for lipid profile and, liver function was analysed off-site at the Royal Victoria Infirmary (RVI) Biochemistry laboratories immediately after the study visit finished. In between sampling and delivery to the laboratories, the samples were stored in a 4°C fridge, on site. Samples were transferred from NMRC to the RVI on ice in a freezer bag by the investigator.

Spare serum was separated from using the same methods as for the glucose samples and stored frozen -70C freezer on site at the NMRC) for potential future metabolomic analysis, at the Newcastle Biomedicine Biobank facility. During the final stages of our study data collection, Sinha et al (Sinha *et al.*, 2013) demonstrated a clear association between skeletal muscle mitochondrial oxidative function and serum vitamin D levels in patients with Growth Hormone deficiency using the same 31-P MRS methodology as we had in our participants. Preterm infants in infancy and later in life may be nutritionally disadvantaged compared to their term peers and we therefore decided to use some of the saved serum to assess fasting vitamin D levels in our cohort. We felt that this would be important in correct interpretation of our MR data. The frozen serum was transported to Manchester (courtesy of Dr. Jacqueline Berry, Manchester University) where it was assayed using the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method to obtain a 25-OH Vitamin D quantitation. This was then included in the analysis of the 31-P MR data (see later methods).

2.2.4 Epigenetic sampling

Participants who consented to blood sampling were asked to provide two samples for later epigenetic analysis: RNA collected in a PAXgene™ Vacuum sample tube (PreAnalytiX GmbH, Switzerland), and an EDTA vacutainer sample from which a cellular 'pellet' was extracted. All participants were also asked to provide 2ml of saliva into an Oragene™ Pot (DNA Genotek, Canada) from which DNA analysis could be done. Once collected, these samples were stored in a 4°C fridge until the end of the study visit and then transferred to the International Centre for Life (ICFL) in Newcastle, in a freezer bag, by the investigator.

As all three samples types contained cells, they were subject to the Human Tissue Act (HTA), 2004(*Human Tissue Act* 2004). In compliance with this and the local HTA licence (no. 12534, held by Newcastle Biomedicine Biobank) the samples were processed at the International Centre for Life (ICFL, Newcastle, UK) to extract the genetic material using standard methods(Groom *et al.*, 2012; Relton *et al.*, 2012; Turcot *et al.*, 2012) within 7 days of the sample being taken. Once extracted, the genetic material was no longer subject to the HTA and was prepared for storage on site at the ICFL for future epigenetic analysis. Study participants were made expressly aware that they were providing genetic samples for *future* analysis and given the opportunity to opt out of

this process. All genetic samples were labelled and stored anonymously using study numbers to ensure that there was no potential for misuse of any genetic information found in the future analysis.

2.2.5 Pubertal staging

All study participants were asked to self-assess their pubertal status using a booklet outlining the standard Tanner staging (Tanner *et al.*, 1966b; Tanner *et al.*, 1966a) of pubertal development. Female participants were also asked whether they had undergone menarche and, if they have an established menstrual cycle, to estimate what day of their cycle it is at the study visit. This information was used to control for pubertal status when assessing insulin sensitivity.

2.2.6 Magnetic Resonance Imaging studies

All of the Magnetic Resonance Imaging (MRI) studies were carried out by the Research Radiographers at NMRC, who ensured the safety and comfort of the participants in the MR suite. A separate, NMRC-specific MRI consent and safety questionnaire was administered by the Radiographers (in parallel with the Study Consent Forms) for all of the children prior to starting the MR sequences. The children were allowed to be accompanied by a parent into the scanner room if they wanted, but none took up this opportunity. Of the 60 participants, one was deemed to be unsuitable for scanning as they had permanent metallic cosmetic implants in their anterior abdominal wall that would have posed a thermal injury risk and also have degraded the study images. The 59 children who underwent the scan protocol all provided images of suitable quality for analysis.

The MR study protocol was trialled with the investigator as a test subject prior to any participants being scanned to ensure that the scan protocol could be completed within one hour (as stipulated by the Study Protocol submitted to, and approved by, the Ethics Committee). The 3-Tesla Achieva MRI scanner (Philips, Andover, Massachusetts) was used in conjunction with in-house modified coils to generate the images and data for analysis (Trenell *et al.*, 2008; Lim *et al.*, 2010; Lim *et al.*, 2011c). Analysis of the results was undertaken by the investigator after tuition in the methodology by Professor M. Trenell and Dr K. Hollingsworth (NMRC).

The scan protocol for each participant started with abdominal scanning (head into the MR scanner). After approximately twenty minutes, the participants changed position under the guidance of the radiographers and went feet-first into the scanner for the exercise scans.

Measurement of intrahepatic lipid

Two methods for measurement of intrahepatic lipid (IHL) were used to collect data in the study: the Dixon method (Dixon, 1984; Glover and Schneider, 1991; Hardy *et al.*, 1995) and ^{1}H-Magnetic Resonance Spectroscopy (MRS)(Hallsworth *et al.*, 2011; Lim *et al.*, 2011a).

For both techniques, the participant was laid supine, head first into the scanner, and a Philips Sense Cardiac 6 Channel coil (Philips, Andover, Massachusetts) placed over them, centred a the xiphisternum. Standard survey and reference scans were then carried out to ensure correct positioning of the subject prior to data collection.

Dixon Sequencing

A 3-point Dixon sequence was then taken from mid- to superior-liver in 6 cross-sectional slices, 10mm thick with single-slice gaps) to provide the images required. The Dixon method uses in- and out-of-phase images to produce a 'pure water' image of the scanned structure, with the difference compared to the original image (non-water area) being pure fat. Thus an amount of intrahepatic lipid can be estimated. In our participants, however, the levels of intrahepatic lipid were found to be so low that we were unable to obtain accurate results from analysis of the scan data. There was insufficient fat content in the liver to allow reproducible estimation of fat content using the available software. This method was, therefore, discontinued after interim review of the data obtained.

^{1}H-MRS

Following the Dixon sequence, the participants underwent point-resolved spectroscopy (PRESS) imaging to determine the levels of fat in the liver by generating ^{1}H-liver spectra(Qayyum, 2009). The participants were required to perform several breath-holds in order to accurately place the sampling voxel (30x30x30mm) in the superior portion of the right lobe of the liver, avoiding any large liver vessels. They were then required to perform a further 16.8 second breath-hold during which T2

PRESS scanning was performed to measure the relaxation times of the spectroscopy peak signals over time. This was done in all subjects who underwent MR scanning. Individuals required coaching and encouragement during the scan to maintain a suitable breath-hold as failure to do so gave distorted results during the PRESS T2 scan.

Initial analysis of the ^{1}H spectra (for an example see figure 4) was done using the Advanced Method for Accurate, Robust, and efficient Spectral fitting (AMARES) algorithm in the jMRUI v3.0 software package(Vanhamme *et al.*, 1997). Once this was complete, further processing was done to calculate the %IHL. This is, by convention, the % of CH_2 lipid peak signal amplitude relative to the water peak signal amplitude after correction for T1 and T2 relaxation times(Naressi *et al.*, 2001; Thomas *et al.*, 2005; Gardner *et al.*, 2012). This method, however, was found to be inaccurate for a number of children at the interim review because their IHL levels were so low that the AMARES program could not be used to accurately plot the fat peak during relaxation in the T2 sequence. In order to overcome this, the area under the curve at the beneath the water peak (at 4.26ppm) and lipid peak (between 0.9 and 2.2ppm) was integrated(Qayyum, 2009) using a Matlab (The Mathworks Inc, MA, USA) algorithm designed by Dr Kieren Hollingsworth, after AMARES processing of the PRESS T1 scan. By using the assumption of a standard relaxation time for the water peak (26ms) and fat (71ms) this then allowed, as outlined above, expression of the lipid peak as a % relative to the water peak. While this method did not use directly derived water or fat relaxation times, it did allow reproducible estimates of IHL content for all of the participants who had a scan. It is this data that is included in the subsequent analysis.

Measurement of intra-abdominal fat

While in position for abdominal imaging, the participants also had a further Dixon sequence taken to determine intra-abdominal fat content. The 3 point Dixon sequence was centred anatomically around the L2/3 junction in order to allow reproducibility between participants and give some measure of centrally located, intra-abdominal fat. This level was also chosen as it allowed the scan to be commenced without the need or repositioning of the body coil, which contributed to the scan protocol being achievable within the specified time frame of 1 hour.

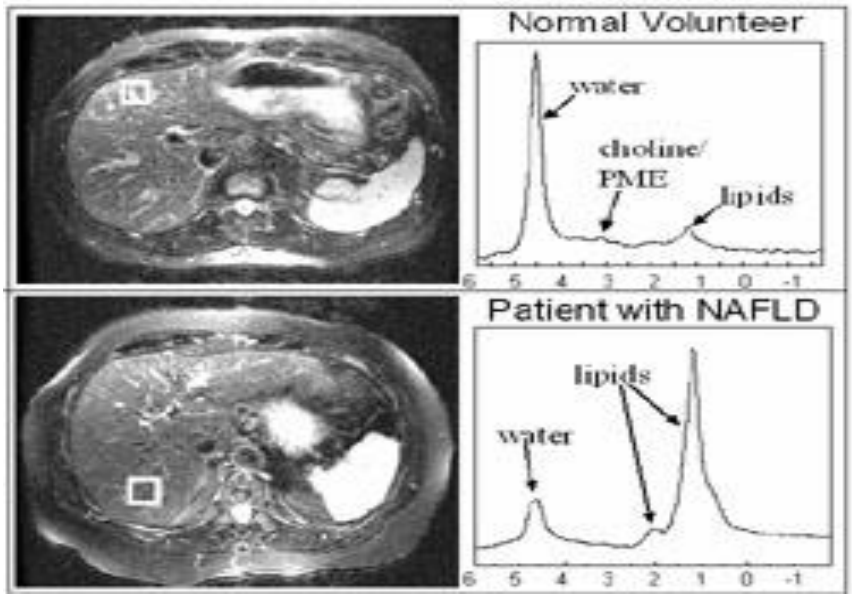

Figure 4: An example of 1H-MRS spectra in a normal and fat-loaded liver (courtesy of NMRC)

The abdominal Dixon sequence took 6 cross-sectional slices (as for that outlined for the intra-abdominal lipid content) with the third slice corresponding to the L2/3 landmark. Once taken, the fat percentage data was converted using Matlab from .PAR to .hdr format which could be read by imageJ software (opensource image reading program, National Institutes of Health). Using imageJ the fat percentage image was converted to a binary representation (pixels containing fat or not) and then the total area of fat was calculated (cm^2). This was then further classified as either visceral adipose tissue (VAT; i.e. that which was found inside the abdominal cavity) or subcutaneous adipose tissue (SAT; adipose tissue outside the abdominal cavity). These values were then converted into a VAT/SAT ratio (Brambilla *et al.*, 2006) and also indexed against height to provide some reference to body habitus given that the area was of a single cross-section only. These data were then used in analysis.

Measurement of mitochondrial oxidative function

Once the abdominal sequences were finished, the children were repositioned, feet first into the scanner and with their left foot placed in a custom-made exercise rig as described previously in the literature (Jones *et al.*, 2010; Sinha *et al.*, 2013) to allow a standardised exercise protocol during which ^{31}P-MRS was carried out to assess mitochondrial oxidative function.

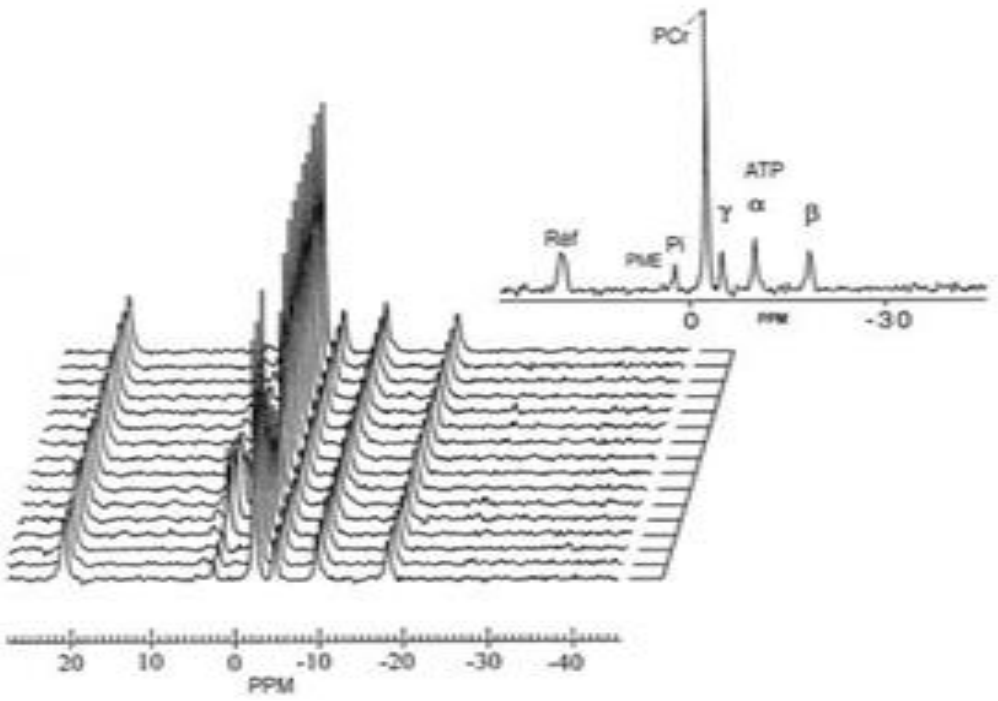

Figure 5a: A typical spectrum (top right) generated using 31P-MRS techniques, and stacked spectra demonstrating the change in PCr levels over time during exercise (depletion) and recovery.

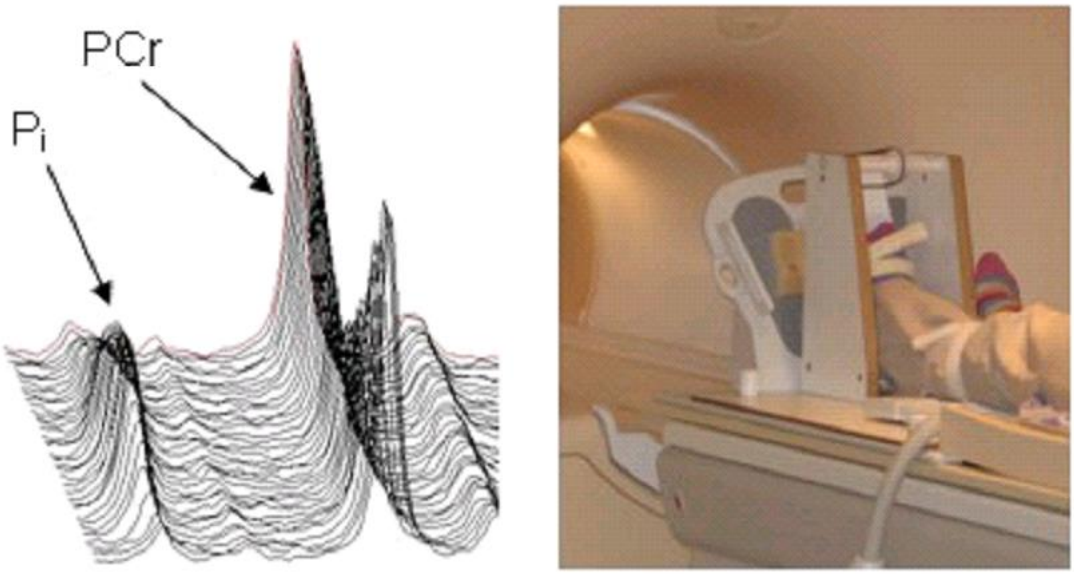

Figure 5b: an example of 31P-MRS spectra generated using the custom-built rig (courtesy of NMRC, Newcastle University)

The custom-built exercise rig allows plantar flexion movement between 0° to 30° with the foot starting in the anatomical position for the left leg only. This effectively isolates the calf muscles during the plantar flexion without other muscle groups being involved. Resistance to plantar flexion can then be programmed into the exercise rig at any level the operator sets. The participants were supine (as with the abdominal scans) and leg restraints were used to prevent deliberate use of thigh muscles in assisting work against the resistance rig during exercise (particularly quadriceps). The exercise rig was used prior to the exercise testing to quantify the calf muscle group maximum voluntary contraction (MVC) for each participant.

In order to generate the ^{31}P spectra (figure 5a), a 10cm diameter ^{31}P surface coil was placed over the calf muscles, centred on the widest part of the gastrocnemius/soleus complex, for transmission and reception of the signal. Once the MVC was recorded and the participants had had time to recover, the exercise rig (figure 5b) was set to provide resistance of 35% of MVC, and ^{31}P spectra were recorded over a three minute time window to assess resting mitochondrial function. This was immediately followed by a three-minute exercise period during which the participants were required to perform plantar flexion against the set resistance at a frequency of 0.5Hz. An audible cue at this frequency was provided to ensure regular rhythmic flexion. It was reinforced by tapping on the shoulder if the children were unable to match the frequency with rhythmic plantar flexion in time to the audible cue alone. With these measures, all of the children who attempted the exercise were able to accomplish the task. After the three-minute exercise, there was a rest period. MR phosphorous spectra were gathered every 10 seconds during the rest-exercise-rest protocol using adiabatic One-Dimensional Image-Selected In-vivo Spectroscopy (1D-ISIS). This protocol has been shown to robustly measure mitochondrial oxidative function during recovery from exercise (Jones *et al.*, 2010; Sinha *et al.*, 2013). Using a 35% MVC protocol alone (rather than a 25% then 35% in sequence) also allows collection of pH change data by spectroscopy without the need for a longer time in the scanner. This allowed us to ensure that PCr readings are representative with respect to pH influence on the Pi-PCr flux and also calculate τ ½ ADP from the measured data without unnecessarily prolonging the scan time for the children.

The phosphorus spectra were analysed in batches using the AMARES algorithm in jMRUI using a prior knowledge base, and from this quantifications of PCr, inorganic phosphate (Pi) and pH throughout the protocol were obtained. The data were then processed to calculate the various components of oxidative metabolism as described by Kemp and Radda(Kemp and Radda, 1994). Change in pH was calculated by measurement of the changing horizontal displacement of the Pi peak from the PCr peak on the ^{31}P MR spectrum(Kemp and Radda, 1994). A single exponential fit was then used to estimate the half-time to recovery within the muscle represented by recovery of equilibrium of PCr (τ½PCR) and ADP (τ½ADP) as described by Jones et al (Jones *et al.*, 2010). Occasionally, this technique did not yield properly fitted ADP curves due to inorganic phosphate splitting. This phenomenon occurs when the sampling voxel crosses two or more muscle fibre groups such that the flux being measured is effectively two or more independent groups, hence no clear single peak is registered: instead there are several smaller, asynchronous peaks which cannot be analysed in the standard fashion. On the few occasions when this was observed during data analysis, the results had to be excluded from correlation analysis.

In the post data-collection processing it became clear that the end exercise pH reached by our volunteers varied considerably. It is recognised that the pH can have an effect on the τ½PCr values and there is a well-established post-hoc correction that can be made(Iotti *et al.*, 1993) which references the results to the mean minimum pH achieved across the cohort. This changes the absolute half-time results but allows referenced comparison within the group for comparison and correlation to other measured variables.

2.2.7 Dietary recall: SCRAN24

To assess the participants' diets, we used the SCRAN-24 programme developed by the team at the Human Nutrition Research Centre (HNRC) at Newcastle University(Foster *et al.*, 2013a; Foster *et al.*, 2013b). It is a computer-based, guided software program which took around 30 minutes to complete, on a laptop computer, per participant. It is a recall diary tool.

SCRAN-24 uses a series of steps to determine the times of food/drink intake over the 24 hours prior to using the program. It asks the user to list chronologically the items of

food or drink consumed in the 24 hour period under a series of headings (breakfast, mid-morning snack, lunch etc.). It then breaks down each intake into their component parts. For the majority of foodstuffs (98% of those) regularly consumed by children in the North-East of England, the program contains a listed item. Non-listed items have to be entered in free-text. Once the items are selected for the full 24 hour period the program then revisits each episode of food intake and prompts the participant to assess and log portion size based on a series of photo-based choices. A final step prompts the user to try to link food and drink intake with activity through the day and highlights any times of the day during which nothing was ingested as particular areas to focus on. This is done to try to improve recall and thus improve the sensitivity of the diary. Once the participant has completed the portion sizes for all chosen items, the program saves this information in a database file.

The database file contains pre-programmed nutritional information for all of the listed items chosen and based on the quantities selected (portion size) for each item. As these have been seen and selected by the participant, it removes some of the interpretive steps that might have occurred in a traditional written recall diary. It also avoids the bias of a prospective diary which occurs when dietary modification occurs due to observation. For the free text items entered in the program, significant dietetic experience is still required to accurately assess and enter the required data into the database. SCRAN-24 has been validated for use in children in the North-East of England aged 11 years of age or over.

The SCRAN data was only collected on one occasion (during the study visit). Data from the recall would be more robust if repeated on 2-3 separate occasions per child, but due to the extensive nature of the tests at the study visit and the fact that the logistical framework to conduct follow-up home assessments was not available, it was decided to restrict this to a single assessment. This was approved by the REC who felt that minimising interruption to the participants' lives was important. It was accepted that natural variation in dietary intake due to extrinsic factors (e.g. weekend vs. school day; after-school activities etc.) may therefore have had an impact on the reliability of the results based on one recall session alone. However, it is not unreasonable to assume that those with high-fat, high-carbohydrate diets are unlikely to have changed to a

healthy diet the day before the study visit. Other sources of bias such as failed recall are equally applicable to SCRAN-24 as a written recall diary.

2.2.8 Direct Measurement of Physical Activity

The children were given the opportunity to take home an accelerometer at the end of the study visit in order to directly measure their physical activity for a period of three days. The process was explained and wearing of the accelerometer demonstrated prior to the candidate agreeing to take it home. All of the children agreed to try the process. A pool of six, calibrated accelerometers were used to collect the data. Accelerometers have been used for many years to measure directly physical activity in children(Metcalf *et al.*, 2002) and they are well validated for use being practical to use, reliable, calibrated to 'gold standard' techniques (such as indirect calorimetry) and more accurate than self-reporting of exercise(Basterfield *et al.*, 2008; Evenson *et al.*, 2008; Basterfield *et al.*, 2011a; Trost *et al.*, 2011).

The accelerometers used were ActiGraphs™ (GT1M model, MTI, Pensacola, USA). These are small and light-weight, uniaxial accelerometers. They were pre-programmed to begin and stop recording activity prior to issues to participants. They measured acceleration of different intensities in one (vertical) plane during sequential, pre-defined, short epochs (10 seconds) which were then downloaded onto a laptop computer and analysed after completion of the data collection. They were worn by convention on the right hip, during waking hours, and the participants were instructed as to how to wear them correctly to ensure accurate data collection. During wearing, any displacement of the hip (i.e. pelvis tilt or changing body position) during activity causes vertical displacement of the accelerometer which is recorded, and thus quantitative measure of activity ('counts per minute', cpm) can be calculated. It is recognised (and was explained to the participants) that the ActiGraphs were not waterproof and therefore were not wearable during activities such as swimming. They are also not reliable at recording exercise intensity during cycling (Evenson *et al.*, 2008). The accelerometers also measured the number of steps taken. The accelerometers were worn for three consecutive days including at least one weekend day and one weekday as described in the literature as this provides a reliable assessment of activity as long as there is a minimum of 6 hours weartime per day recorded (Basterfield *et al.*, 2011b; Pearce *et al.*, 2012). We decided, given the age

group (12 years and upwards), the requirement from the REC to ensure disruption to the participants lives was minimised and the fact that we only had a few accelerometers to use on a rolling basis, that a 72 hour data collection period starting at 7am the morning after the study visit would be in keeping with the aims of the study. We assumed that most of the participants would be able to adhere to this.

The participants kept a written diary to keep track of the time the accelerometers were put on and taken off through the day in order to aid analysis and identification of non-wear periods (Basterfield *et al.*, 2008; Basterfield *et al.*, 2012). The accelerometers were returned after the period of wear by either drop-off to the researcher or by post, in pre-paid envelopes.

Analysis of the activity data was done using the Actilife program (version 5, MTI, Pensacola, USA) under the supervision of Dr Laura Basterfield (Human Nutrition Research Centre, Newcastle University). Using pre-defined, calorimetrically-referenced cut-offs (Evenson *et al.*, 2008) the data recorded over three consecutive days was classified into sedentary, light, moderate to vigorous physical activity (MVPA). The cut-points used were: Sedentary ≤100cpm; Light Activity 101-2295cpm; Moderate Activity 2296-4011; Vigorous activity ≥4012cpm. MVPA therefore was any activity ≥2296cpm. These cut points are slightly lower than those used by other studies (Puyau *et al.*, 2002; Basterfield *et al.*, 2008) but they are calorimetrically referenced and because Evenson used ROC curves in her analysis, when compared to other cut-off points by Trost (Trost *et al.*, 2011) these cut-points performed best across a wide range of ages of children. It is also notable that adult cut-offs for moderate to vigorous activity are generally lower than those of children and so in an adolescent cohort, using these cut-points is appropriate.

From this, it was possible to calculate the time spent (minutes) and percentage time in sedentary or MVPA. MVPA was chosen rather than looking individually at different activity levels as it has been shown in non-preterm children in the North East that MVPA seems to be most closely associated with body habitus change when followed longitudinally(Basterfield *et al.*, 2012) Non-wear periods were defined manually during analysis cross-referenced to the weartime diary kept by the children and any long zero-count periods (no activity recorded for >1 hour) removed from the analysis. Any complete record which had wear on less than 3 days, or less than 6 hours of weartime

per day, was rejected. Of the 60 children who agreed to participate in this part of the study, 2 participants failed to wear the device (one not returned), 5 did not wear it for three consecutive days and one failed to achieve 6 hours weartime per day. This equated to a satisfactory completion rate of 84%.

2.2.9 Questionnaires

The study participants completed a paper questionnaire during the study visit regarding their view on what children would prefer for structured activity within a programme designed to encourage a healthier lifestyle in children and their families. A total of ten choices were presented as statements and the participants were asked to rate each one on a ten point Likert scale. The median and interquartile ranges were calculated for each outcome. A free text box was included to allow suggestions for other interventions which the children thought might be used. This data was collected in order to inform potential future interventional studies about which interventions might be most accepted by a similar teenage cohort and is presented in section3.5.

As part of good research practice, each family who came to a study visit was given a feedback questionnaire asking them to tell us what we did well, what they didn't enjoy, and what we could do to improve the study. This was filled in anonymously and they were given the opportunity to return them on the day or at a later date. The information from this allowed us to make minor changes in approach to improve the experience during the study visit (for example ensuring accompanying parents knew that they would have a period of 'downtime' while their children were being scanned or that the glucose drink for the GTT was best drunk in one go rather than sipped due to its viscous consistency).

2.3 Ethical Approval

A favourable ethical opinion for the Growmore study was gained (after amendments) on 9th February 2011 from Sunderland Regional Ethics Committee (study number 10/H0904/67). Following this, the Newcastle Hospitals NHS trust Research and Development department confirmed approval on 23rd March 2011 (study number 5530). One substantial amendment was approved during the course of the study (2nd September 2012) for a 'young adult' consent form (where a participant >18 years old living with parents might attend alone). This was not, in the event, needed.

2.4 Statistical analysis

As this is an observational study with many different measured variables, calculating a required number of study volunteers needed for the results to have standard statistical power to achieve significance is difficult. To this end, the following sample size estimates were made after discussion amongst the NPTBGS study group members:

1. Based on effect size data from the cohort, at the last visit, relating fat mass index to insulin sensitivity (R^2=0.12), an 80% power at 5% significance level would require approximately 60 study subjects to detect any significant within-cohort difference.
2. The work using 31P-MRS by Sinha et al showed that to demonstrate a reduction in τ½PCr of 10% with supplementation of vitamin D to the same power level as required in this study (of 80% at 5% significance) required only 10 subjects (Sinha *et al.*, 2013).

Based on these data, it was decided that a study size of 60 individuals would be appropriate as a target for recruitment in order to detect significant within-cohort variation within the variables measured.

It was decided that the data generated would be analysed in various ways suitable to the comparisons being made (Wood *et al.*, 2013). Comparison between the volunteers making up the study cohort and the cohort as a whole at the last visit was made using student t-tests or Mann-Whitney U tests to examine whether the study cohort (fewer in number) were representative of the overall cohort for key variables such as gestation and birth weight. Bland-Altman plots were used to compare and assess agreement between different methods examining body composition (Bland and Altman, 1986; Bland and Altman, 1995; Krouwer, 2008).

Correlation analysis (either Spearman's Rho or single variable linear regression) was used to examine relationships between pairs of variables such as IHL and insulin sensitivity, and oxidative function and vitamin D status. More complex linear regression were then developed using insulin sensitivity, fat deposition and tau½PCr (as a measure of oxidative function) as dependent variables. When constructing these models, adjustment was be made for confounding factors such as sex, gestation, pubertal stage and birth weight z-score.

Primarily the data was analysed using the Stata data analysis program (v11/13, StatCorp LP, Texas USA) available through Newcastle University Computing Services. Specific analysis of MRS, dietary and exercise data was made using some open source programs and some bespoke software as outlined above, prior to statistical analysis.

Chapter 3. Results

As outlined previously, the cohort who volunteered for the current study were part of the NPTBGS cohort, formed by amalgamation of two original study cohorts. The recruitment for this study was non-selective to try to minimise bias at entry to the study (i.e. the traceable members of the original cohort (n=235) were all sent two invitations which contained a response form and a patient information letter). The first 60 to respond and volunteer were recruited, so it was necessary to consider whether these individuals were representative of the original NPTBGS cohort. If the current study cohort was broadly representative of the NPTBGS cohort, the study results would be more translatable to a wider, ex-preterm cohort outwith the study group.

3.1 The study cohort: representativeness of overall preterm cohort

When comparing the current cohort to the original cohort, data from the previous time that the cohort was assessed was used (Wood *et al.*, 2013). Gestational age and birthweight SDS were used as variables representative of the growth status at birth of the individuals being compared. The dates of birth of the NPTBGS were used to confirm that the current study cohort was representative of the original cohort in age (and that the current cohort was not an older or younger sub-group).

In order to make some comparison of the metabolic status of the current study cohort with the other members of the NPTBGS cohort, at the time of the last study, the recorded data for body mass index (BMI), fat mass index (FMI) and percentage body fat (the latter two measured by DEXA and BIA) were compared. There was no way to confirm that the NPTBGS individuals who were compared to the current study cohort would have taken the same life course trajectory in terms of metabolic outcomes, so it was only possible to make an assessment of whether the current cohort was 'metabolically' similar to the overall group at the last assessment. The comparisons were made using either t-tests (for normally distributed data) or Mann-Whitney U tests (for skewed data). Chi-squared testing was used for categorical data analysis.

3.1.1 Comparison of birth weights and gestation

The characteristics of the current study cohort at the time of this study are shown in table 1.

Characteristic	Mean (range)
Gestation at birth (weeks)	31 (26+1 to 34+4)
Birth weight (kg)	1.37 (0.84 to 1.87)
Age at study (years)	15.5 (12.1 to 18.8)
Male:Female (n)	25 : 35

Table 1: cohort characteristics

The inclusion of a maximum weight cut-off in the original study enrolments leads to an intrinsic bias which cannot be overlooked when considering the generalisability of the results, such that the older the gestational age (GA) of the individual at birth, the lighter they had to be in comparison with their peers (as measured by birthweight SDS) in order to be recruited into the original studies (figure 6).

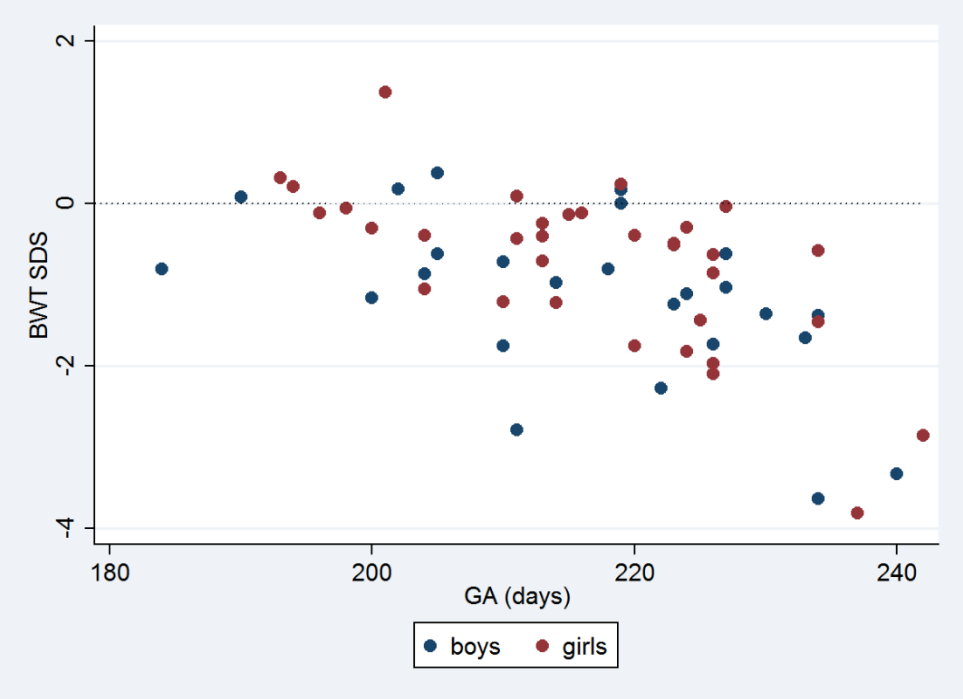

Figure 6: birth weight SDS plotted against gestational age at birth in the current study cohort.

	Gestational Age at birth (days)		
	Mean	**SD**	**95%CI**
Complete NPTBGS cohort (1)	216	14.87	±1.85
NPTBGS cohort without current cohort included (2)	216	15.40	±2.20
Current Study (Growmore) cohort (3)	217	13.14	±3.39

Table 2: Comparison of gestational age at birth between NPTBGS cohort and the Growmore Cohort. (2) vs. (3): p = 0.89 (NS); (1) vs. (3): p = 0.92 (NS) using two-tailed Student t-test

	Birth weight SDS score	
	Median	**Interquartile range**
NPTBGS cohort without current cohort included (1)	-0.75	-1.58 to -0.15
Current Study (Growmore) cohort (2)	-0.715	-1.41 to -0.195

Table 3: Comparison of birth weight SDS between NPTBGS cohort and the Growmore Cohort; (1) vs. (2): p = 0.95 (NS) using Mann-Whitney U test

	Age at time of current study (years)			
	Median	**Q1**	**Q3**	**Range**
Complete NPTBGS cohort (1)	14	12	15	11 to 17
NPTBGS cohort without current cohort included (2)	14	12	15.75	11 to 17
Current Study (Growmore) cohort (3)	14	12	15	11 to 17

Table 4: comparison of cohort members' age at start of the Growmore Study recruitment period; p = 1.0 (NS) using Chi Squared test

Tables 2 to 4 show that when comparing the current study cohort with the remainder of the NPTBGS cohort, there is no significant difference for gestational age, birth weight SDS or age at the start of the current study recruitment period

3.1.2 Comparison of BMI, FMI and percentage total body fat

Table 5 shows the comparison of BMI, FMI and percentage (total) body fat (measured at the last study visit), between those tested who subsequently volunteered for this study and those who did not. Of note, not all of the NPTBGS cohort who were *not* recruited for the current study had all of the above indices measured at the last visit. In addition, not all of the 60 who took part in the current study had all of the indices measured at the previous study.

These data show that there was no significant difference between those who subsequently volunteered for this study and those who did not, in the group who had their BMI, FMI and percent body fat measured at the last study visit.

		BMI	**FMI**	**% body fat**
NPTBGS cohort without current cohort included (1)	***n****	100	89	89
	Median	18.1	5.57	30.6
	IQR	16.1 to 20.8	4.07 to 7.96	24.3 to 38.9
Current Study (Growmore) cohort (2)	***n****	52	46	46
	Median	17.5	5.24	28.3
	IQR	15.9 to 19.7	3.67 to 6.84	24.3 to 35.4
Mann-Whitney U	***p* (1)vs.(2)**	0.23	0.22	0.16

Table 5: comparison of BMI, FMI and % body fat. (*the number of volunteers who had these measurements made at the previous study i.e. not the whole NPTBGS cohort)

Investigation undertaken	***n* (%)**
MRI scan	59 (98)
MR results suitable for post-hoc analysis (Aτ½PCr)	50 (83)
Activity Monitor (completed and returned)	44 (73)
BODPOD	58 (97)
Auxology	60 (100)
Serum sampling	46 (77)

Table 6: number of volunteers who underwent the aspects of multimodal assessment during this study

3.1.3 Participation in multimodal assessment in the current study

As was observed in the previous studies, not every volunteer in this study consented to taking part in every aspect of the study. Table 6 outlines the number of subjects out of the total volunteers (n = 60) that consented to, and took part in, the different aspects of the current study. Of these, not all the data collected was of sufficient quality to analyse.

3.1.4 Comparison between 'original' study groups.

Within the current study cohort there was a mix of children recruited to the original protein (n=29) and growth (n=31) studies. This could possibly lead to confounding. Analysis was done on those variables reflecting status at birth and those that were thought to be important with respect to the study aims. Mann-Whitney U tests were used to assess the difference within variables, categorised by original study (table 7). There were no statistically significant (P≤0.05) differences detected, but IHL measured by MRS was approaching significance between those enrolled into the different studies. The significant difference for age at study visit reflects the fact that the

children enrolled into the protein study, which recruited after the growth study, were younger as a subgroup within the cohort.

Variable	**Z value (Growth vs. Protein Study)**	***p* value**
Birth Weight SDS	0.488	0.63
Gestational Age at Birth	-0.23	0.82
Age at Study Visit	6.18	<0.001**
Intrahepatic Lipid (IHL)	-1.78	0.07
Fat Mass index (from BODPOD data)	0.15	0.88
BMI SDS	0.38	0.70
VAT/SAT ratio	-0.75	0.45
HOMA (% insulin sensitivity)	0.40	0.69
Matsuda Index	-0.19	0.85
Vitamin D (total)	-0.44	0.65
Moderate to Vigorous Physical Activity	-1.02	0.31

Table 7: Mann Whitney U test comparison between growth and protein studies within the GROWMORE cohort

3.1.5 Comparisons between Boys and Girls

Within the study cohort there was an increased number (n=35; boys n=25), and increased proportion of girls, compared to the original NPTBGS cohort. Comparison was therefore made in the current study cohort to assess whether there was a demographic difference between the boys and girls at time of study (table 8). The only significant difference in the comparison variables describing the two groups was age at

study: the girls were on average 1 year older than the boys (boys mean: 14.8yrs; girls mean: 15.8; p=0.036).

Variable	**Z value (M vs. F)**	***p* value**
Birth Weight SDS	0.488	0.63
Gestational Age at Birth	-0.23	0.82
Age at Study Visit	-2.09	0.04
Tanner Stage	-0.87	0.38
BMI SDS	-0.43	0.67

Table 8: Mann Whitney U test comparison between boys and girls within the GROWMORE cohort

3.1.6 Discussion

The analysis of these data shows that the cohort which volunteered for this study is representative, in a broad sense, of the NPTBGS cohort using metrics which reflect uterine and neonatal status as well as limited representation of metabolic status in late childhood/early adolescence. There were no significant differences arising from children being in different studies originally, and there was minimal difference in baseline measures between the male and female cohort members. The original cohort were all selected for being in good health in the context of preterm birth, in order to minimise the effects of 'acquired' morbidity having been born preterm. These individuals, from a population perspective, are those most likely to be those who subsequently lead lives in society which are outwardly least distinguishable from their term born peers. Under such circumstances, delayed or programmed adult metabolic health problems may have a profound effect on their lifetime need for use of healthcare resources, when compared to their term born peers. The results of this study specifically arise from a small group of individuals who are representative of the wider NPTBGS cohort: this, in turn, means that the results will have some generalisability with reference to preterm infants who pass through neonatal care

apparently unscathed. Further analysis of measured variables by male/female or original study enrolment is discussed in the relevant sections of the results (section 3.2 to 3.4).

3.2 Body composition and lipid distribution in body compartments

As described earlier, one of the intentions of this study was to look at some of the different methods that might be used in future studies to monitor efficacy of any lifestyle or research intervention over an extended period. When looking at body composition, it was of particular interest to compare skinfold measurements with air displacement plethysmography (ADP) as methods for estimating total body fat. A reduction in total body fat would be a simple measurement useful in monitoring progress of an intervention, as well as quantify any relapse after an intervention was stopped. ADP is measured using the BODPOD™ as described. While it is more convenient than using other research methods such as immersion, it is still expensive and remains the domain of research studies. For a future study with larger numbers use of skinfold measurement would potentially allow more rapid data collection, but only if the data generated were accurate.

The other outcome of interest was comparing the general measures of body composition with the directly measured MRS and Dixon method outcomes for IHL and visceral/subcutaneous adipose tissue distribution, to see if they held any associations.

3.2.1 Comparison of methods for measuring body composition

When using the four-skinfold method as outlined in the methods, and the BODPOD™, a body density is generated which can then be converted to a percentage body fat using the equations of Siri and Lohman, depending on age. The calculations for the two-skinfold technique produces a 'percentage body fat' without using these equations directly. The total body fat-mass and fat-free mass was then calculated (kg) from the volunteer's weight, and then indexed against height (VanItallie *et al.*, 1990) as a means of adjustment for body habitus and to improve comparability across the group. Table 9 shows the descriptive summary for the data generated using these techniques. Table 10 shows the correlation between these body composition techniques and each other, and also the correlation between these measures and other auxological measures commonly used to track changes in body composition. Table 10 also compares all of these measures to the directly measured lipid deposits from 1H-MRS and Dixon imaging. While it appears that all of the auxological and body composition measures have a good correlation with the directly measured visceral and subcutaneous adipose tissue, there is not a good correlation with the lipid content as

measured in the liver using 1H-MRS: only fat mass index measured using the BODPOD or 2-skinfold technique appears to be correlated with the measurement of intrahepatic lipid.

Technique	FMI BP	FMI 2S	FMI 4S	FFMI BP	FFMI 2S	FFMI 4S
5%	1.37	1.62	1.82	13.39	13.19	13.02
50%	3.43	3.60	4.47	15.89	16.42	15.70
95%	10.82	10.02	9.01	19.73	19.08	19.38
Mean	4.75	4.54	4.83	16.12	16.32	16.02
Standard Deviation.	3.29	2.79	2.33	1.84	1.78	1.97

Table 9: Descriptive data for the three techniques from which Fat Mass Index can be generated. All data in kg/m^2. (FMI: Fat Mass Index; FFMI: Fat Free Mass Index; BP: BODPOD™; 2S: two skinfold technique; 4S: 4 skinfold technique)

While the correlation statistic would suggest that the measures obtained by BODPOD and the 2-skinfold technique are potentially correlating well enough to be used interchangeably, this must be formally tested. Figures 6 to 11 show Bland-Altman plots generated to compare the results from the methods for Fat-Mass Index (FMI, kg/m^2) and figures 12 to 17 show the same comparisons for Fat-Free Mass Index (FFMI). For each comparison, there are two plots: one showing the differences plotted against the mean of the two techniques (as recommended by Bland and Altman(Bland and Altman, 1995) and one showing the differences plotted against the 'reference' method (Krouwer, 2008) which was assumed to be air displacement plethysmography (or the two-skinfold measurement when directly comparing the skinfold techniques, based on the correlation analysis).

	FMI BP	FMI 2S	FMI 4S	BMI	WC	MAC	BMI SDS
FMI BP							
FMI 2S	0.92 <0.001						
FMI 4S	0.90 <0.001	0.96 <0.001					
BMI	0.81 <0.001	0.82 <0.001	0.87 <0.001				
WC	0.77 <0.001	0.76 <0.001	0.79 <0.001	0.91 <0.001			
MAC	0.53 <0.001	0.58 <0.001	0.68 <0.001	0.87 <0.001	0.78 <0.001		
BMI SDS	0.79 <0.001	0.79 <0.001	0.79 <0.001	0.94 <0.001	0.88 <0.001	0.77 <0.001	
% IHL	0.29 0.03	0.28 0.03	0.18 **0.17†**	0.19 **0.15†**	0.25 **0.06***	0.07 **0.59†**	0.24 **0.07***
VAT	0.41 0.001	0.43 <0.001	0.38 0.002	0.49 <0.001	0.59 <0.001	0.33 0.01	0.56 <0.001
SAT	0.91 <0.001	0.92 <0.001	0.93 <0.001	0.86 <0.001	0.84 <0.001	0.62 <0.001	0.82 <0.001
VATi	0.44 <0.001	0.45 <0.001	0.38 0.003	0.45 <0.001	0.53 <0.001	0.24 **0.06***	0.55 <0.001
SATi	0.9295 <0.001	0.9329 <0.001	0.9178 <0.001	0.8253 <0.001	0.7962 <0.001	0.5448 <0.001	0.8154 <0.001

Table 10: Spearman's correlation analysis for the three techniques from which Fat Mass Index can be generated, and other auxological techniques used to track body composition. Values shown are: Rho; *p*-value. **†**Not significant; *****p<0.1 (FMI: Fat Mass Index; BP: BODPOD™; 2S: two skinfold technique; 4S: 4 skinfold technique; %IHL: % Intrahepatic lipid content; VAT: visceral adipose tissue; SAT: subcutaneous adipose tissue; i: indexed against height-squared)

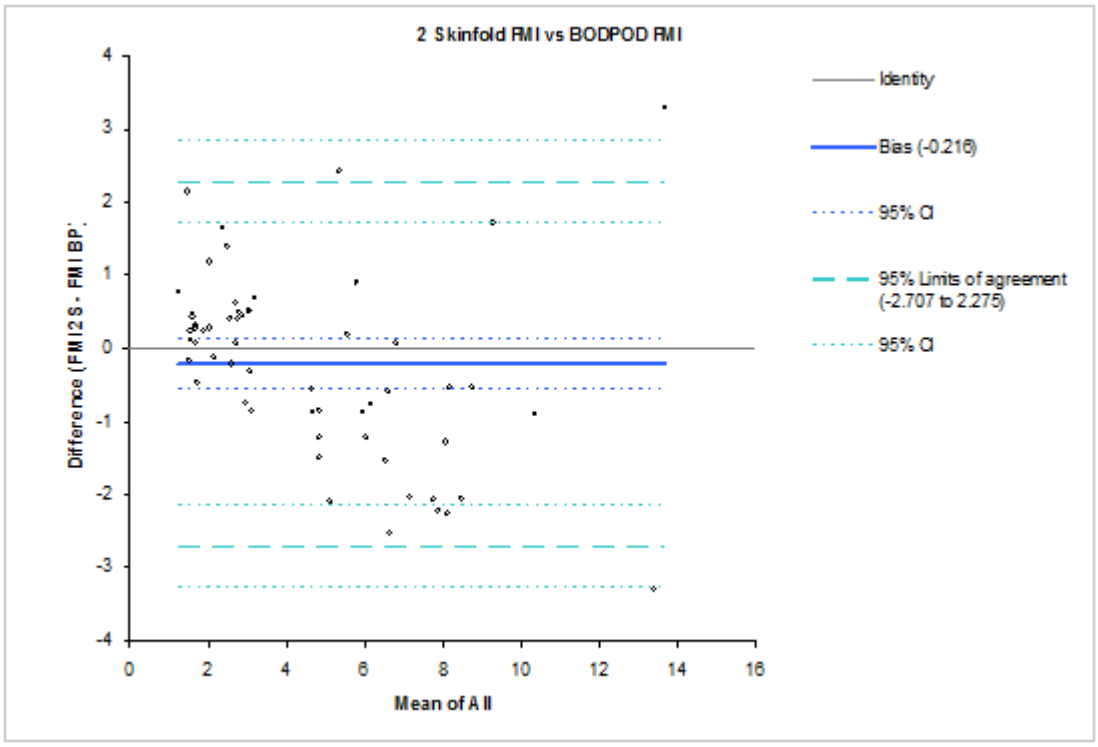

Figure 6: Comparison of Fat Mass Index (BODPOD [BP] vs. Two Skinfold Technique [2S]); Differences vs. Mean

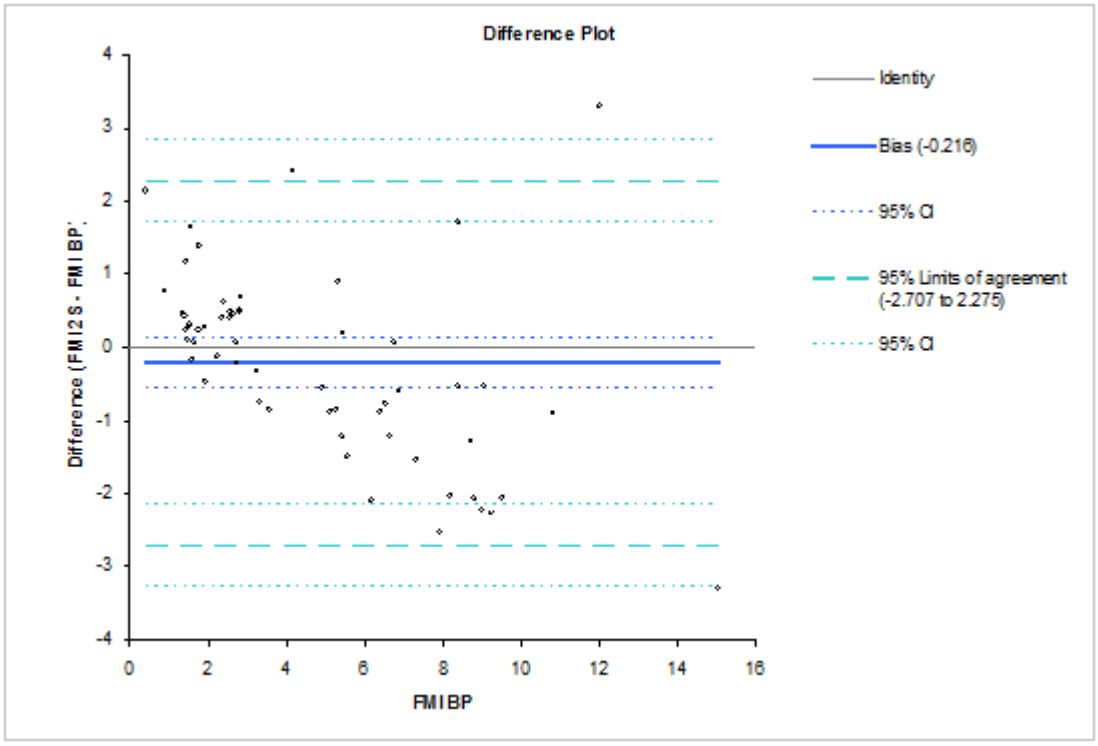

Figure 7: Comparison of Fat Mass Index (BODPOD [BP] vs. Two Skinfold Technique [2S]); Differences vs. FMI BP

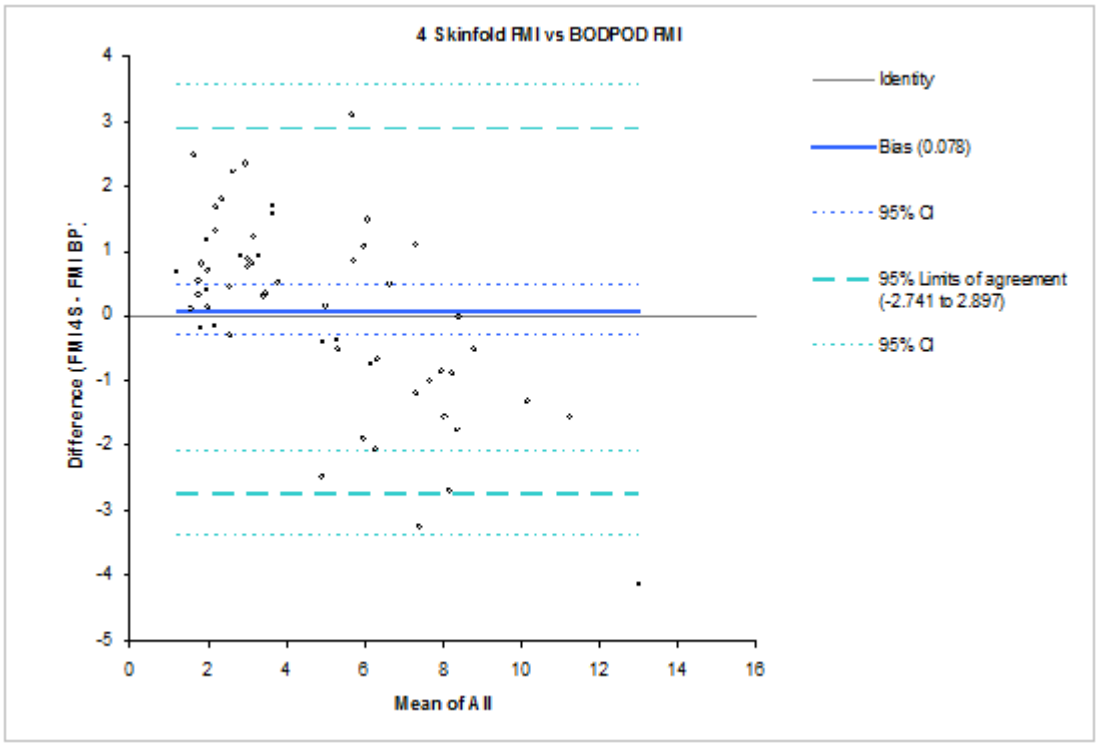

Figure 8: Comparison of Fat Mass Index (BODPOD [BP] vs. Four Skinfold Technique [4S]); Differences vs. Mean

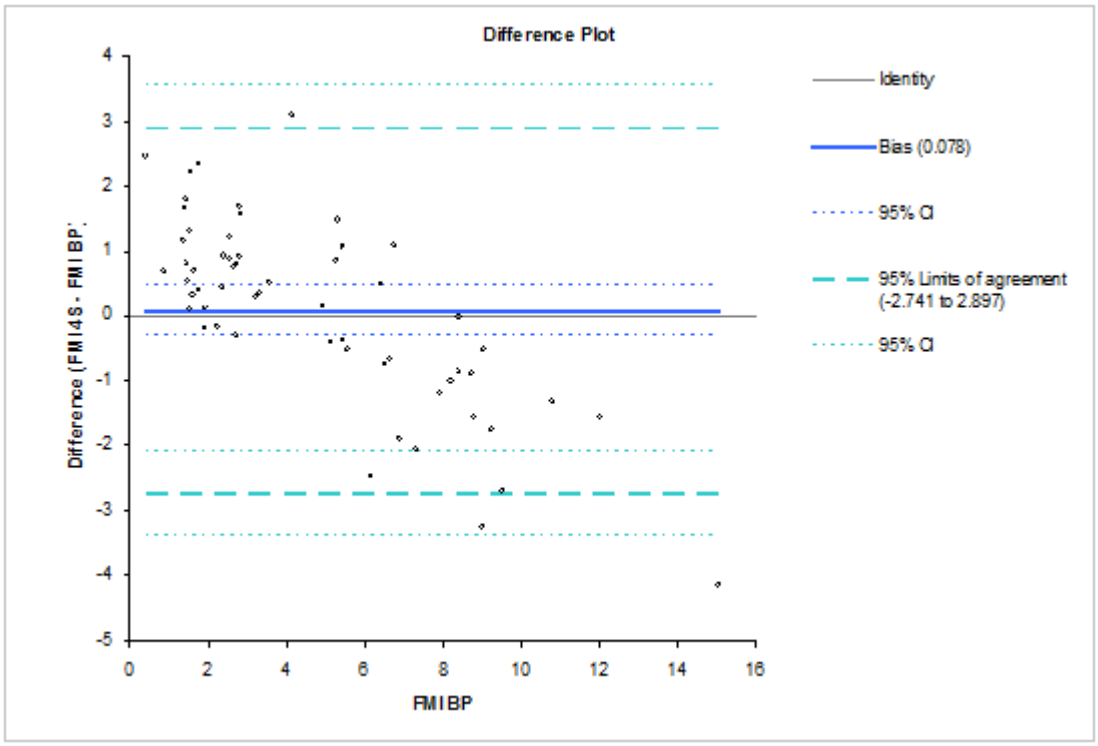

Figure 9: Comparison of Fat Mass Index (BODPOD [BP] vs. Four Skinfold Technique [4S]); Differences vs. FMI BP

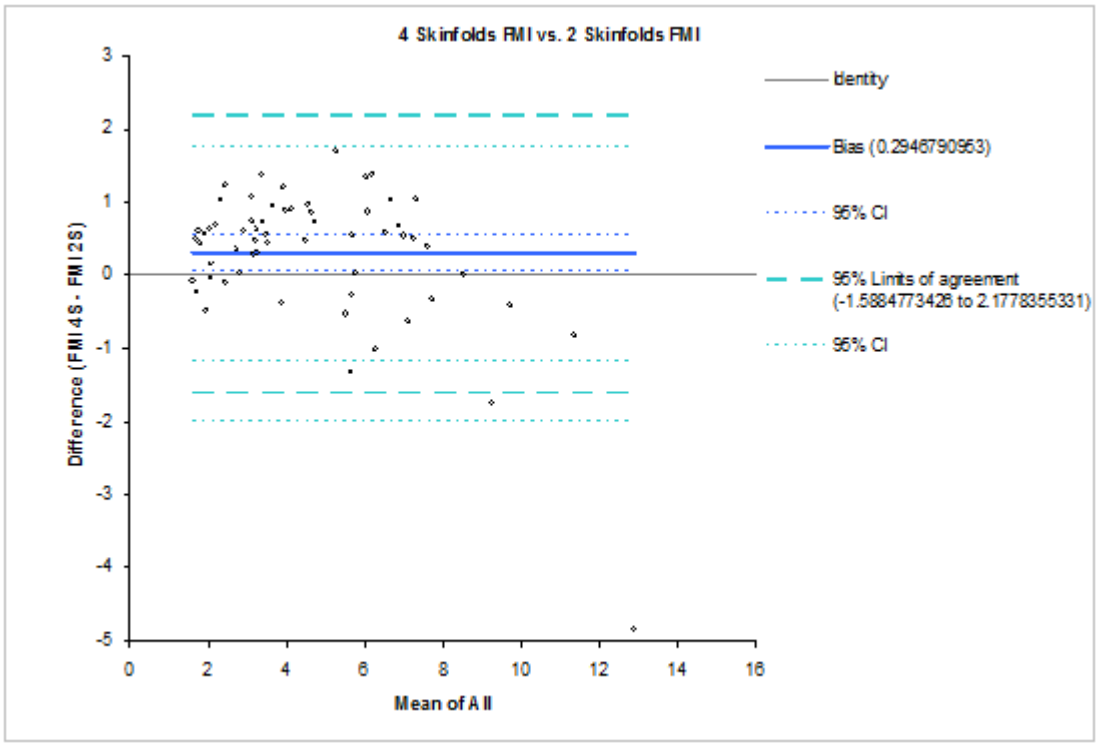

Figure 10: Comparison of Fat Mass Index (Two Skinfold [2S] vs. Four Skinfold Technique [4S]); Differences vs. Mean

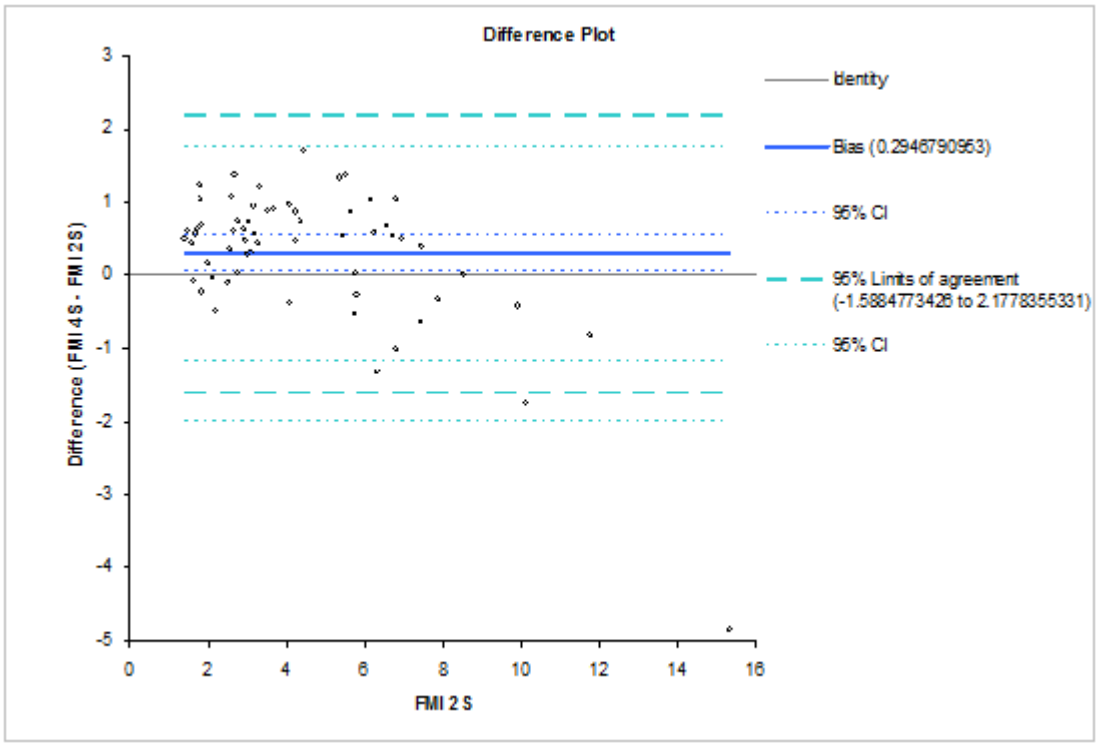

Figure 11: Comparison of Fat Mass Index (Two Skinfold [2S] vs. Four Skinfold Technique [4S]); Differences vs. Two Skinfold method

The Bland-Altman plots of the test differences plotted against the means, for skinfold measurement compared to BODPOD (figures 6 & 8) suggest that, when compared to the BODPOD, both skinfold-measurement techniques produce a slightly higher FMI than that from the BODPOD. However, the line of equality lies within the 95% confidence intervals for the bias (mean of the differences) suggesting overall there is no mean difference. More significantly, both skinfold techniques also trend towards 'over-reading' relative to the mean when the FMI is low and then 'under-reading' when the FMI is higher. This is shown by the marked downslope of the data trend across the line of equality from positive to negative differences, on both plots, as the FMI increases. This appearance is highly suggestive of systematic error in one of the methods used.

The use of quadratic elements within the skinfold equations which generate body density or percentage body fat (originating during the generation of the equations in the original research) may be a cause of this. As the two-skinfold (2S) equations are not reliant on the equations of Lohman or Siri (and also the BODPOD uses these) it does not seem likely that the error is within these conversions, as the same pattern is also seen in the four-skinfold data (which does use Lohman/Siri).

The other possibility is that within the algorithms for predicting thoracic volume by BODPOD, as used in this study, there is a similar error: if BODPOD overestimates lung volume in lean individuals it will produce a positive difference in the plot for lower *actual* FMI when compared to either skinfold method. A smaller residual body volume for measured body weight equates to higher density, thus lower fat content in the model, lower FMI and thus underestimate (positive difference) when compared to the other methods. To produce the linear error seen, BODPOD would also have to underestimate lung volume in obese individuals in order to underestimate body density (give higher FMI readings) in those with a higher FMI. As the FMI is indexed against height and the BODPOD uses height as a predictor in estimated thoracic gas volume, it is also possible that there could be a confounding interaction generated here.

When the plot (figure 10) comparing both skinfold methods is reviewed, the same trend towards over- and under-estimate is suggested, albeit in a less pronounced way. The mean of the differences is altered towards a positive mean difference bias, but the

line of equality is not found within the confidence intervals of this bias. This suggests that overall, the four skinfold technique under-reads density relative to the two skinfold thus giving a higher FMI and points to the error being in the skinfold equations rather than BODPOD volume estimates.

When examining the limits of agreement for these three plots, it is clear that the agreement between methods is clinically significantly different outwith a narrow range of FMI where the data trend crosses the line of equality. As would be expected, the two skinfold techniques are in closest agreement with each other (1.5 to 2.2kg/m^2) either side of the line of equality from the mean of the differences). When compared to FMI as measured by BODPOD, the limits of agreement are much wider (between 2.5 and 2.7kg/m^2). When the group data is examined, the data can be seen to be skewed in all groups with the median fat mass ranging from 3.43 to 4.47kg/m^2 [range 1.37 to 10.82kg/m^2]. In this group of individuals, therefore, a difference between techniques of 2.5kg/m^2 is a large potential difference. Correlation analysis against FMI measured with BODPOD using Spearman's correlation generated a rho value of 0.898 (4S)/0.923(2S) [$p<0.001$], but when the measured differences between the methods are considered proportionally to what is being measured, they are too great to say anything other than that the tests are not suitable to be used interchangeably in a research setting.

Figures 7, 9 and 11 differ from the three discussed above as they compare the difference between methods to the 'reference method'. For this, we chose BODPOD as the reference method as it directly measures body volume rather than extrapolates a density or body fat percentage from a proxy measure. Based on the fact that the two-skinfold method stratifies the calculation by pubertal status and other factors (thus is perhaps more tailored within a given population) it was chosen as the reference method for the comparison between the two skinfold methods. Using this method did not alter any of the findings outlined above.

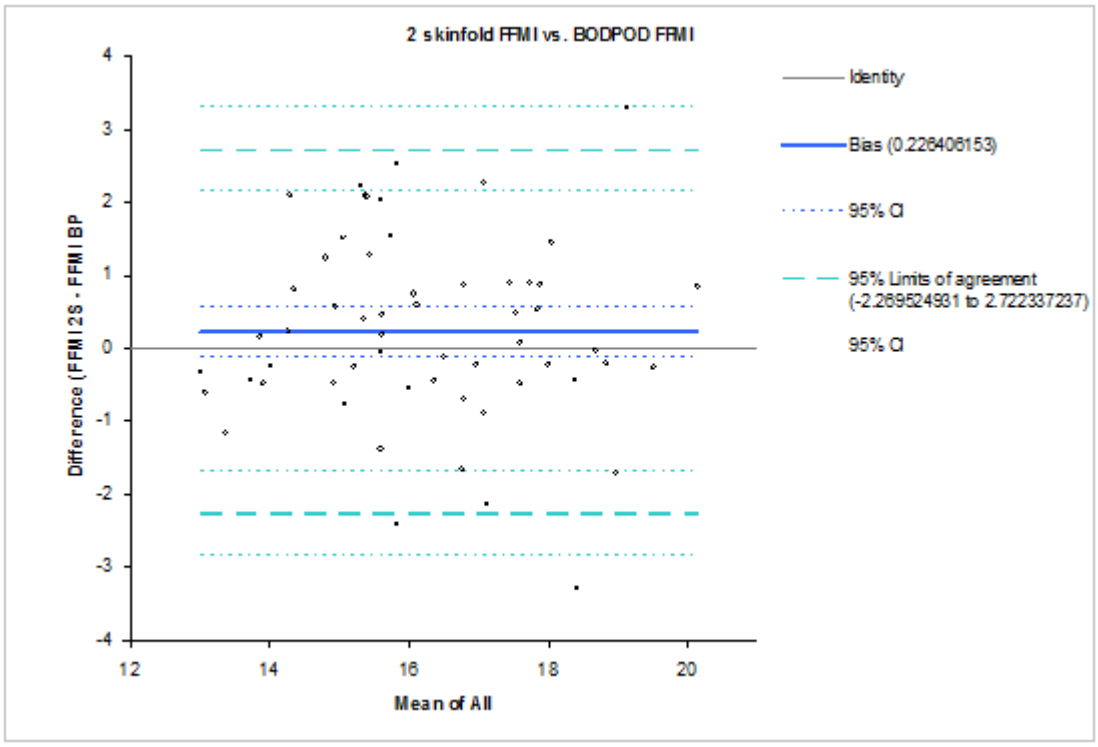

Figure 12: Comparison of Fat Free Mass Index (BODPOD [BP] vs. Two Skinfold Technique [2S]); Differences vs. Mean

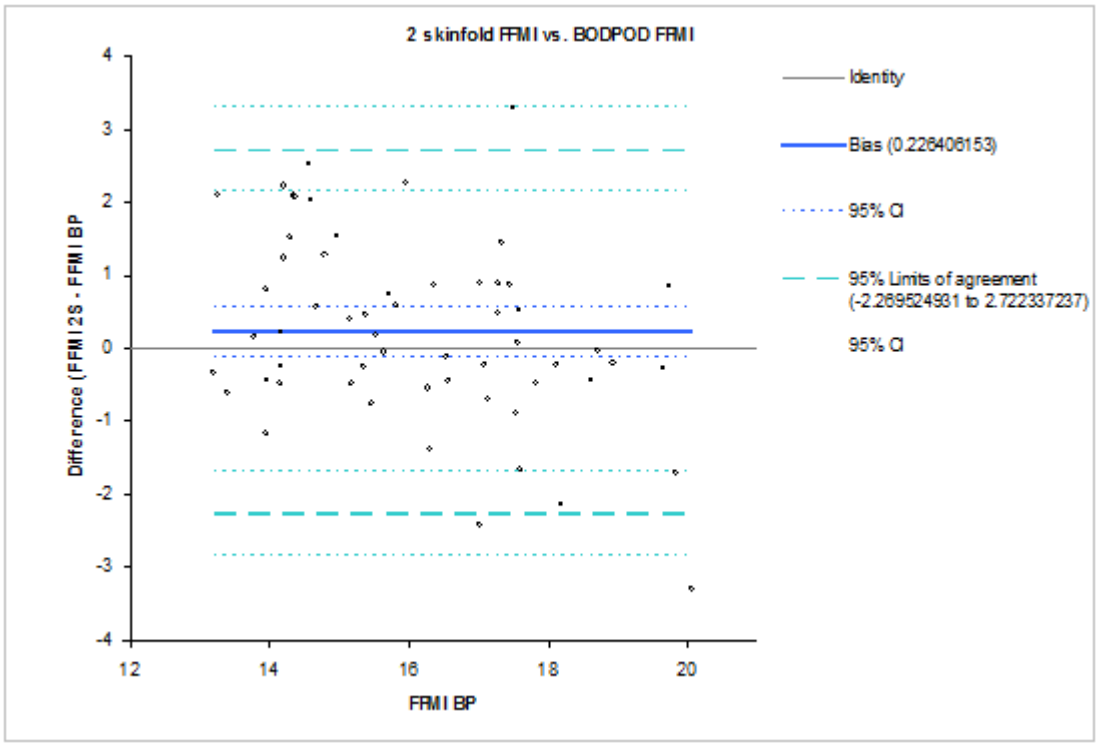

Figure 13: Comparison of Fat Free Mass Index (BODPOD [BP] vs. Two Skinfold Technique [2S]); Differences vs. FMI BP

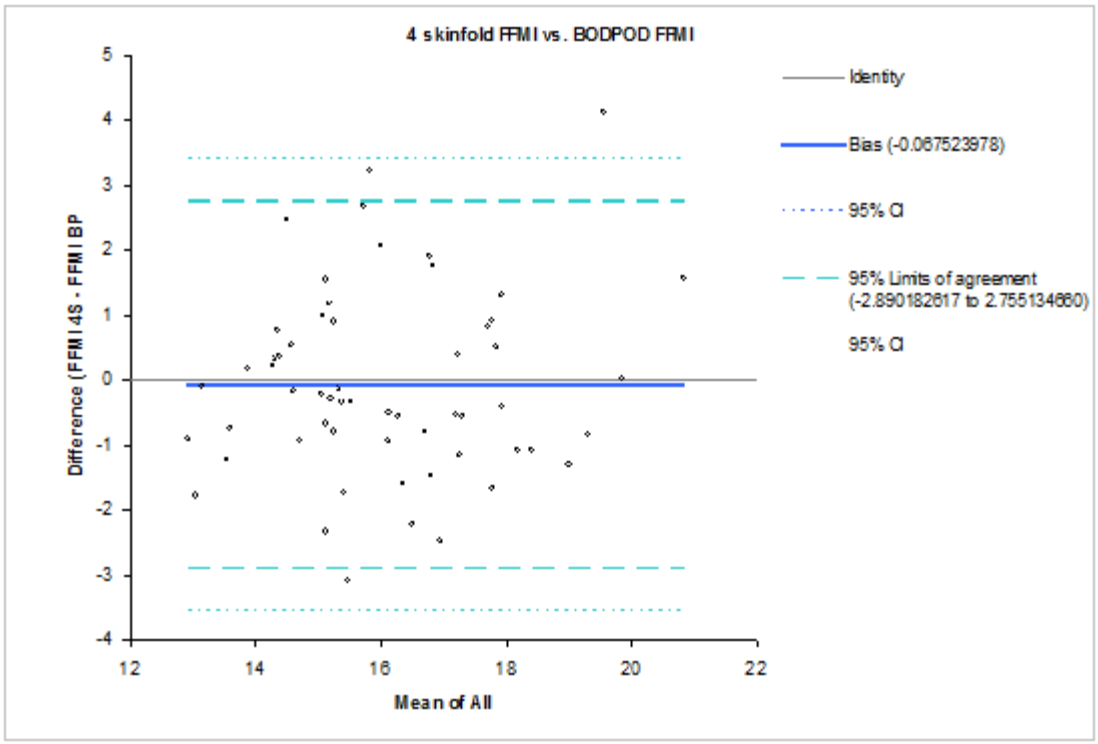

Figure 14: Comparison of Fat Free Mass Index (BODPOD [BP] vs. Four Skinfold Technique [4S]); differences vs. Mean

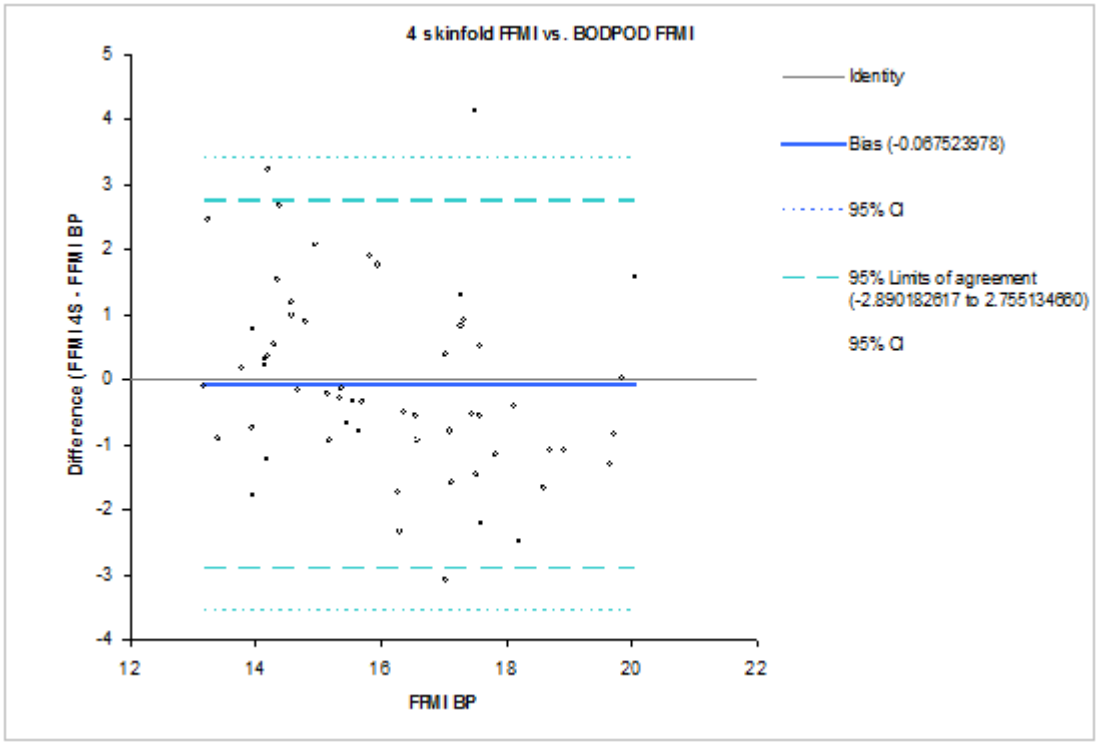

Figure 15: Comparison of Fat Free Mass Index (BODPOD [BP] vs. Four Skinfold Technique [4S]); Differences vs. FMI BP

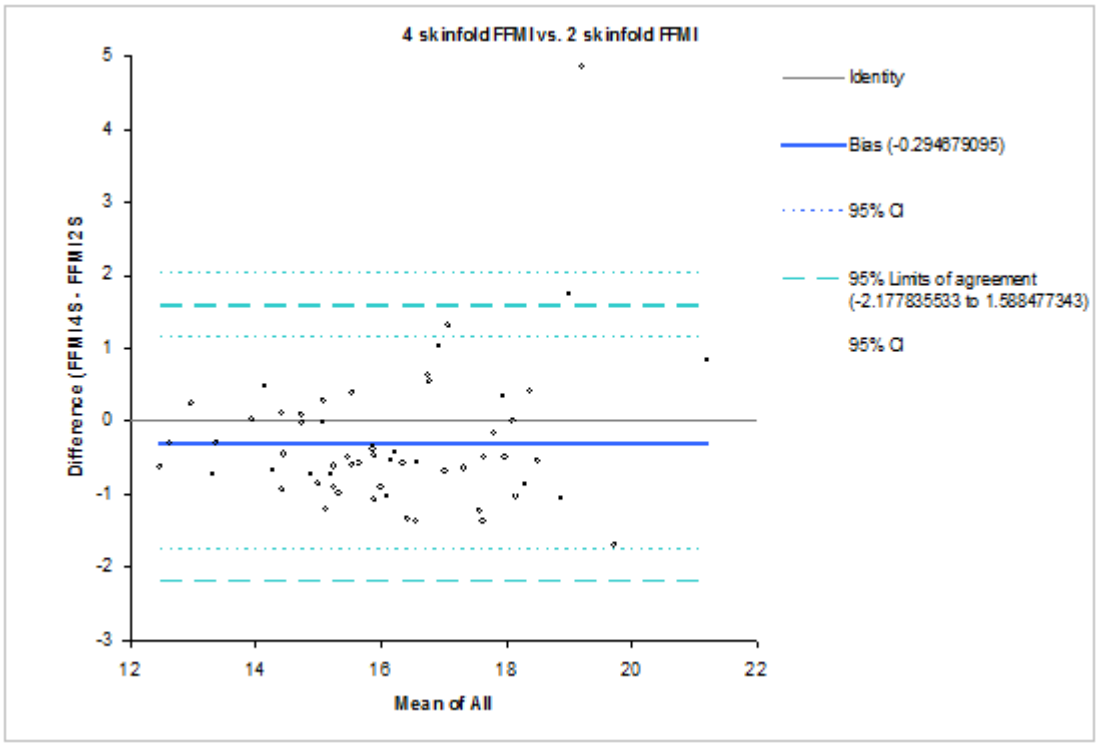

Figure 16: Comparison of Fat Free Mass Index (Two Skinfold [2S] vs. Four Skinfold Technique [4S]); Differences vs. Mean

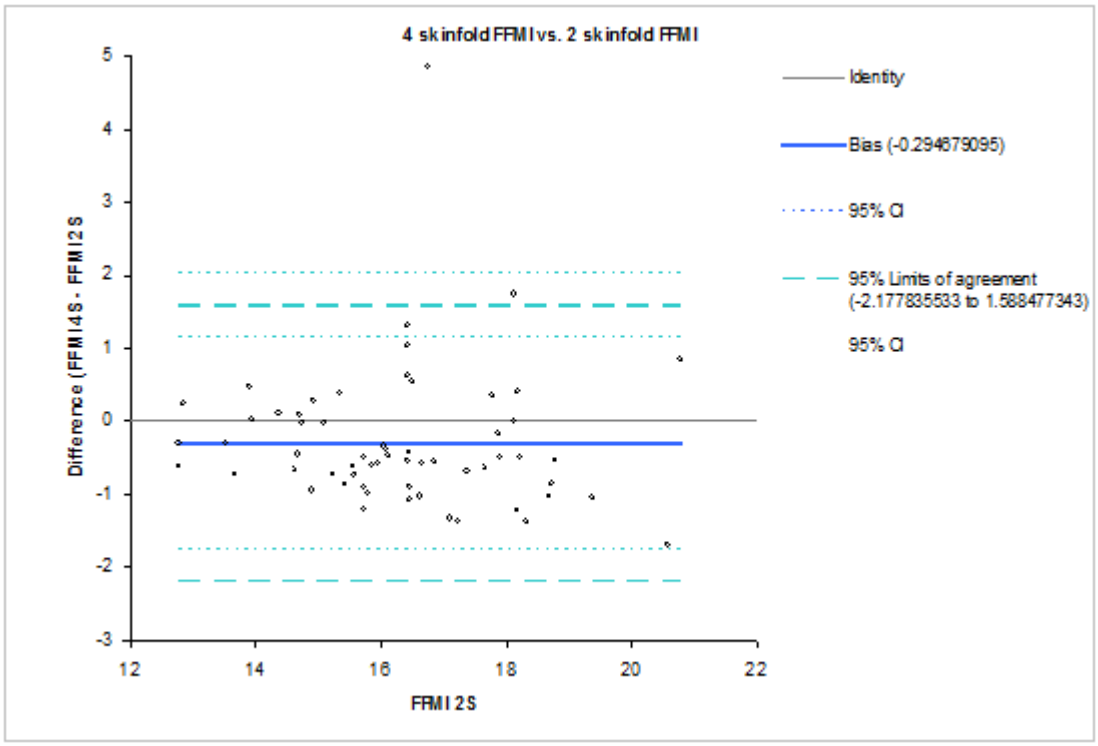

Figure 17: Comparison of Fat Free Mass Index (Two Skinfold [2S] vs. Four Skinfold Technique [4S]); Differences vs. Two Skinfold method

The same analysis was used to examine the techniques in the prediction of Fat-Free Mass Index (FFMI). Unsurprisingly, the bias directions were reversed but the pattern of distribution was more scattered and did not seem to show the same linear trend as seen in the FMI plots. For all three comparisons the 95% limits of agreement were similar in magnitude (kg/m^2) as the FMI plots *but* because the FFMI is a larger number (as most humans are more lean than fat) the relative error compared to the measured FFMI was reduced. However, the fact that the maximum FFMI generated was around 20kg/m2 (with the median between 15 and 16) the percentage error is still potentially (at best) 12.5% for any given measurement. As such, it is not possible to say that these tests could be used interchangeably in future studies.

3.2.2 Lipid compartments and content measured using MRS and Dixon methods

As discussed in my introduction, evidence from the literature suggests that the compartmentalisation of lipids is important in determining their eventual physiological effect. During the study the percentage of intrahepatic lipid present was measured directly using MRS, as were visceral/subcutaneous adipose tissue (at a single abdominal level) and serum lipids, in addition to the anthropometric body composition measures. We also had a measure of dietary energy, carbohydrate and fat intake from the SCRAN24 data. In order to examine the relationship of these variables correlation analysis was used, and then a model to examine associations was constructed using the significant variables.

Table 11 shows the measured variables that were thought to be likely to be related clinically to intrahepatic lipid content. Spearman's correlation analysis was used initially for this analysis as there is no requirement for normality in the distribution of the dataset. Once this was done, a second correlation analysis was done using univariate regression. For this, any non-normally distributed data were transformed to a normal distribution where possible. Pre- and post-transformation, the variables were tested for normality using the Shapiro-Wilk test of normality. The analysis showed that the percentage of intrahepatic lipid was most strongly associated with current body habitus (measured by current weight SDS, BMI SDS, FMI and percentage body fat), visceral adiposity, and serum lipids (most strongly circulating triglycerides). There is also a strong association with raised ALT levels: raised ALT is biochemical marker of

hepatocyte damage which can happen in the presence of raised intrahepatic lipid, but the lipid theory as discussed previously would not suggest it is a cause of raised IHL. In fact, when examined using linear regression, the results suggested that the ALT increased with lower levels of IHL, which is not physiologically explicable other than within the majority of the population studied there is a generally low level of intrahepatic fat and normal variation of ALT (within normal laboratory limits). Thus the association is likely to be spurious (type 1 error).

Using combinations of the variables with the strongest association to IHL, models were then tested using multivariate linear regression to assess their associations against IHL (as the dependent variable). Sequential removal of non-significant terms was carried out until a final model was left. Care was taken to avoid using closely related terms within the final model (e.g. FMI generated by BP was used but not that generated by skinfold measurement). The models used to generate the final model (A-C) are outlined below (table 12 to 14).

Table 11 (next page): Spearman's Correlation Analysis of measured variables against percent IHL (correlation coefficient) and Correlation using univariate linear regression [*p<0.1; **significant (p<0.05)] Note: Birth weight SDS was not transformable to a normal distribution

Measurement (vs. IHL)	**Corr.Coeff. (Rho)**	**P value**	**R^2**	**P value**
Birth Weight SDS	0.02	0.88	N/A	N/A
Gestational age	-0.18	0.16	0.02	0.32
Age at study visit	-0.10	0.44	0.01	0.56
Current weight SDS	0.22	0.09*	0.14	0.004**
Percentage Body Fat	0.30	0.02**	0.10	0.02**
Percentage Lean	-0.30	0.02**	0.11	0.01**
Fat Mass Index (BODPOD™)	0.29	0.03**	0.10	0.02**
Fat Mass Index (4 Skinfold)	0.18	0.17	0.05	0.10*
Fat Mass Index (2 Skinfold)	0.28	0.03**	0.11	0.01**
Body Mass Index SDS	0.23	0.07*	0.14	0.004**
Waist circumference (cm)	0.25	0.06*	0.17	0.001**
Mid-arm circumference (cm)	0.07	0.59	0.03	0.20
Visceral adipose tissue (VAT)	0.21	0.11	0.23	<0.001**
Subcutaneous adipose tissue (SAT)	0.25	0.05*	0.10	0.02**
VAT/SAT ratio	-0.03	0.82	0.01	0.58
VAT index (cm^2/m^2)	0.22	0.09*	0.21	<0.001**
SAT index (cm^2/m^2)	0.26	0.05**	0.06	0.06*
Tanner Stage	-0.19	0.14	N/A	N/A
Serum Cholesterol	0.25	0.10*	0.19	0.003**
Serum Triglyceride	0.33	0.02**	0.24	<0.001**
Low Density Lipoprotein-C	0.27	0.08*	0.20	0.002**
Serum Alanine Transferase	0.32	0.03**	0.25	<0.001**
Total Energy intake (in 24 hours)	0.09	0.49	0.002	0.77
Carbohydrate intake (in 24 hours)	0.09	0.49	0.005	0.58
Fat intake (in 24 hours)	0.04	0.75	0.0001	0.93
Protein intake (in 24 hours)	0.0017	0.99	0.001	0.79

Variable	Model 1	Model 2	Model 3	Model 4
Triglyceride	0.022	0.018	0.001	<0.001
LDL-C	0.467	0.056	-	-
Cholesterol	0.666	-	-	-
Tanner St 1-3	-	-	-	>0.05
Tanner St 4	-	-	-	0.007
Tanner St 5	-	-	-	0.04
R^2	0.31	0.30	0.24	0.39
Adjusted R^2	0.25	0.27	0.22	0.31
p value (for model)	0.002	<0.001	<0.001	0.001

Table 12: Multivariate model (A): Serum markers and significance in model where IHL is the dependent variable (*p* value of each component of model shown). Adjusted model for Tanner stage shown: Likelihood ratio testing of model excluding Tanner Stage shows Tanner Stage is significant in the complete model (*p*=0.04).

Variable	Model 1	Model 2	Model 3	Model 4	Model 5
Waist Circumference	0.18	0.03	0.03	0.001	<0.001
% Bodyfat (BODPOD)	0.54	0.54	0.81	-	-
FMI (BODPOD)	0.56	0.57	-	-	-
BMI SDS	0.89	-	-	-	-
Tanner St 1-3	-	-	-	-	>0.1
Tanner St 4	-	-	-	-	0.02
Tanner St 5	-	-	-	-	0.04
R^2	0.17	0.17	0.17	0.17	0.27
Adjusted R^2	0.11	0.12	0.14	0.15	0.20
p value (for model)	0.04	0.02	0.007	0.001	0.004

Table 13: Multivariate model (B): body composition measurements and significance in model where IHL is the dependent variable. Adjusted model for Tanner stage shown: Likelihood ratio testing of model excluding Tanner Stage shows Tanner Stage is **not** significant in the complete model (*p*=0.09)

Variable	Model 1	Model 2	Model 3	Model 4
Visceral Adipose Tissue (VAT)	0.35	0.03	<0.001	<0.001
Subcutaneous Adipose Tissue	0.68	0.64	-	-
VAT index	0.74	-	-	-
Tanner St 1-5	-	-	-	>0.1
R^2	0.23	0.23	0.23	0.26
Adjusted R^2	0.19	0.20	0.22	0.19
p value (for model)	0.002	<0.001	<0.001	0.005

Table 14: Multivariate model (C): Lipid compartmentalisation and significance in model where IHL is the dependent variable. Adjustment for Tanner stage is not significant

Variable	Model 1	Model 2	Model 3
Triglyceride	0.008	0.005	0.001
VAT	0.05	0.009	0.017
Waist Circumference	0.85	-	-
Tanner St 1-3	-	-	>0.05
Tanner St 4	-	-	0.007
Tanner St 5	-	-	0.03
R^2	0.35	0.35	0.47
Adjusted R^2	0.31	0.32	0.40
p value (for model)	0.0004	0.0001	0.0002

Table 15: Multivariate model (D): Significant variable from models A-C and significance in final model where IHL is the dependent variable. Adjusted model for Tanner stage shown: Likelihood ratio testing of model excluding Tanner Stage shows Tanner Stage is significant in the complete model (p=0.047)

Models A to C show that compared to IHL, serum triglyceride is the most strongly associated of the serum measures, waist circumference is the most closely associated body composition/habitus measure and visceral adipose tissue is the most strongly associated direct adipose tissue measure (not indexed for height).

The final model (D; table 15) shows that a combination of serum triglyceride level and visceral adipose tissue explain at least 32% of the variance seen in IHL levels. Adjustment for Tanner Stage did not show any reduction in statistical significance in the variables most closely associated with IHL deposition (they remained $p<0.05$), but was found to be significant when added to the model in association with triglyceride level (model A) thus also in the final model (models D and E). The correlation coefficients for Tanner staging were consistently negative (i.e. the higher the stage, more negative the association with IHL). There was no evidence of a significant contribution from Tanner staging to the association between VAT and IHL or waist circumference and IHL.

Further analysis using Spearman's test of correlation showed that there was no significant direct correlation between Tanner Stage and any of the serum variables (triglyceride, LDL-C, cholesterol or ALT: all $p>0.1$), body composition assessments or measured adipose tissue (VAT, SAT, VAT Index, SAT Index, FMI, % body fat: all $p>0.1$) but there was a correlation with waist circumference ($P=0.005$), mid arm circumference ($p<0.001$) and BMI SDS ($p= 0.007$). In the multivariate analysis there was a suggestion that the effect of Tanner Stage was strongest at self-assessed Stage 4 and 5. A Kruskall-Wallis test was therefore used to assess variables by staging (Tanner St 1 to 5) to see if these individual groups would show a difference. There was no demonstrable difference when stratified by these groups for IHL ($p=0.36$), VAT ($p=0.91$) FMI (0.44) or triglyceride ($p=0.83$). When the stages were grouped further (Tanner St 1-3 [pre-pubescent] vs. 4[pubescent] vs. 5[post-pubescent]) and tested using the same test, there was no significant difference in the groups in IHL ($p=0.3$), VAT ($p=0.76$) or triglyceride ($p=0.68$) though BMI SDS, mid-arm circumference and waist circumference were all still significantly different between the groups ($p<0.05$ for all).

When analysing the final model, it was found that there was significant heteroskedasticity in the data (i.e. it was not possible to say confidently that IHL and VAT/triglycerides were independent from a statistical perspective). The data was therefore reanalysed after transforming IHL to normalise the data (though theoretically this should not have affected the original model as IHL was the dependent variable). Transformation removed the heteroskedasticity and while it altered the *p* values for the individual terms, did not change the overall effect (see table 16). Including Tanner Staging did strengthen the model, as before.

With these findings in mind, the three preliminary models (A-C) were re-analysed but the three variables chosen for the final model all remained the most significant in re-analysis and therefore were used in the revised model. The original models (A-C) have been presented here as the use of a non-normally distributed independent variable is acceptable in the tests used.

Variable	**Model 1**	**Model 2**
Triglyceride	0.01	0.001
VAT	0.069	0.113
Tanner St 1-3	-	0.08
Tanner St 4	-	0.008
Tanner St 5	-	0.004
R^2	0.26	0.45
Adjusted R^2	0.23	0.35
p value (for model)	0.002	<0.001

Table 16: final model recalculated (E) and adjusted to remove heteroskedasticity by transformation of dependent variable to normal distribution (IHL). Adjusted model for Tanner stage shown: Likelihood ratio testing of model excluding Tanner Stage shows Tanner Stage is significant in the complete model (*p*=0.01).

3.2.3 Discussion

It is clear from the correlation analysis done between the auxological and body composition measures that there is a good correlation between them, i.e. all the measures tested report adiposity when it is present. It is also clear that they are well correlated with the visceral and subcutaneous fat deposition as directly measured in the study, but not so well with the intrahepatic lipid status. This raises the possibility that deposition of lipids in some compartments within the body is 'linear' in relation to overall adiposity (thus predictable from indirect measures of adiposity such as BMI or waist circumference) but (in the study cohort at least) the deposition of intrahepatic lipid may not be linear, and may only occur after a certain metabolic or adiposity threshold has been reached.

The comparative analysis using Bland Altman plots to further interrogate the body composition measurements showed that, in this population, the accuracy of the skinfold equations in predicting body fat percentage was not within small enough limits of agreement to be able to say that they could be used in a clinic setting in a bigger study monitoring an intervention. This study, based on the literature has assumed that the BODPOD is the gold standard as it reportedly has good reliability (in reproducibility of results) and the estimate thoracic volume has been shown to be reliable in the age group which we were testing (Fields *et al.*, 2002; Fields *et al.*, 2004b). It also has the advantage of directly measuring body volume and therefore should be prone to less error and extrapolation when compared to measurement of skinfolds. Within the use of the BODPOD, we have made assumptions about hydration status being equivalent in all the children (as all our volunteers had fasted overnight) and as noted in the comparative plots, one explanation for the systematic error seen might an error in the estimate of thoracic gas volume. It also uses the equations of Siri and Lohman which will introduce a degree of assumption due to the nature of their two-compartment models. However, the skinfold measurements have a higher degree of mathematical extrapolation to arrive at a density and will therefore be prone to more error. In addition, the equations used for the two-skinfold technique has arbitrary cut-offs for age and pubertal status which will have an influence on their accuracy in any given population, whereas the BODPOD provides a measurement of density without categorisation at that stage. There is also the issue of population

specificity when using the skinfold techniques, which are now quite old. There has been a change in the levels of obesity in children in the last 30 years and as such, the original equations relating skinfold summation to a test population may well be out of date in relation to modern children by virtue of the original test population being very different to their modern counterparts. As such, air displacement plethysmography (using the BODPOD™) would have to be the recommended method for this type of assessment in a future intervention study in this age group. While correlation analysis shows some good correlations between IHL and auxometric measurements such as FMI, BMI and waist circumference the fat that none of these are predictively associated with IHL at regression suggest that if, in a future study, intrahepatic lipid is the measurement of interest, then it will need to be measured using MRI. While correlated, none of the other methods offer a suitably proxy measurement.

When considering the measurement of intrahepatic lipid, the results which the study produced are largely in line with the literature published about metabolic outcomes in adolescents and older, namely that during this time any measurable effect of prematurity becomes harder to detect and influences from the environment seem to become predominant(Tinnion *et al.*, 2014). It is only for a few specific adult health outcomes such as blood pressure and arterial wall stiffness that there is clear origin and persisting contribution from preterm birth, in the context of a structural change in the body (e.g. nephron number)(Parkinson *et al.*, 2013). In this study, there was no demonstrable association between IHL and either birthweight SDS or gestational age. While these are crude measurements of the potential effects of preterm birth, any proportional programming events might be expected to be related directly to degree of prematurity *or* relative size at delivery. Their absence in this study does not preclude more subtle or 'one-off' programming events (or critical windows) but does suggest that variation in the cohort may be more as a result of postnatal environment.

The current literature regarding visceral adipose tissue and intrahepatic lipid in children and adolescents covers a variety of measurement techniques both in imaging modality (CT/MRI/ Ultrasonography/DEXA) and in vertebral level at which the image is taken (in those that use axial imaging)(Suliga, 2009). When looking at parameters derived independently of imaging, and associations which might explain the variance in VAT (in regression analysis), different authors have found that waist

circumference(Asayama *et al.*, 2002; Asayama *et al.*, 2005; Benfield *et al.*, 2008) and trunk skinfolds(Fox *et al.*, 2000) have been strongly, positively associated with VAT. Neither, individually, fully explained the variance. Some analyses extending data across different ages of child and ethnic sub-groups are less convincing about how predictive WC and BMI are for predicting VAT, though they correlate well at all ages with total adiposity(Bouchard, 2007; Katzmarzyk and Bouchard, 2014). There is also disagreement in the literature as to whether the site of measurement of waist circumference influences how well it correlates as a proxy for visceral adipose tissue(Bosy-Westphal *et al.*, 2010) or cardiometabolic risk(Harrington *et al.*, 2013). Visceral adipose tissue has been positively associated with triglyceride levels in obese adolescents(Caprio *et al.*, 1996) as well as basal insulin levels(Caprio, 1999) but other fat compartments were not. As might be expected, some of the variation seen in VAT levels and distribution between populations can be explained by genetic and ethnic differences (summarised by Suliga (Suliga, 2009)). Work by Goran and co-authors(Goran *et al.*, 1997; Goran *et al.*, 1998; Goran, 1999; Goran and Gower, 1999) sought to characterise some of the life course changes that might affect visceral fat and suggest that (as would be expected) age, level of development and pubertal status all have an influence on VAT, as well as those influences that are modifiable (also known as 'lifestyle' factors). The influence of gender and puberty is variable across ethnic origin(Staiano *et al.*, 2013). However, when considering the contribution to disease it has to be remembered that in the majority of *children* the absolute levels of VAT remain small in comparison to subcutaneous adipose tissue(Benfield *et al.*, 2008).

Intrahepatic lipid is most often studied in the context of disease, especially non-alcoholic fatty liver disease (NAFLD). In those born preterm, Modi's group from London (UK) has demonstrated with MRI/MRS that, even as outwardly healthy adults, those born preterm have a higher level of intrahepatic lipid than their terms-born peers(Thomas *et al.*, 2008; Thomas *et al.*, 2011). The group have also suggested that intrahepatic lipid levels are primarily correlated to neonatal illness severity (rather than growth or nutrition) and this, therefore, has potential implications for all babies born preterm who require intensive care. A cohort of Danish children, including lean and obese subjects, aged 8 to 18 years, showed that the prevalence of raised intrahepatic lipid was 31% in the obese group (vs 3% in the lean) and that where there

was hepatic steatosis, it was strongly associated with dyslipidaemia and increased glycosylated haemoglobin(Fonvig *et al.*, 2015). Della Corte's cohort of 60 children compared hepatic steatosis in children with NAFLD (n=30) and children with familial hypobetalipoproteinaemia (n-=30)(Della Corte *et al.*, 2013). The degree of steatosis was similar between the two groups but the NAFLD group had worse insulin sensitivity, suggesting that the presence of lipid per se (like in skeletal muscle) is not necessarily the only factor in subsequent pathological processes within the liver. Similar associations have been shown in targeted, obese, paediatric populations (defined variably as BMI >90 or 95th centile for the local population). Bille's cohort of those aged 6 to 20 years old who were obese demonstrated increased total body fat and high levels of liver enzymes on serum testing (suggesting hepatocyte damage) and of these, gamma-glutaryltransferase and alanine transferase were predictive of high levels of hepatic lipid(Bille *et al.*, 2012). VAT was also shown to be strongly associated with hepatic steatosis. Similarly, Duarte showed a significant positive association with waist circumference and hepatic lipid in a cohort of 77 children aged between 2 and 13 years old(Duarte and Silva, 2011). Though there is, therefore, evidence suggesting that there is a link between adverse metabolic phenotypes and hepatic lipid deposition, it is not yet clear how, exactly, hepatic lipid seen in children might then move on to cause problems in adulthood. While it is clear in adults with newly diagnosed T2DM that imposing a fasting diet has a marked effect in improving insulin sensitivity(Lim *et al.*, 2011a), and the authors of the research into increased IHL/VAT in children suggest that these are a sensible target for reduction during childhood to try to start adulthood with a 'clean slate', the studies to show sustained improvement in adult health outcomes by intervention in children with increased IHL/VAT have not yet been done.

One theory which might help to explain why some populations are susceptible to lipid deposition in the liver and subsequent (or concurrent) metabolic dysregulation is the unfolded protein response (UPR) theory(Henkel and Green, 2013). Endoplasmic reticulum stress provokes the UPR as a protective mechanism. It in turn has a role to play in regulating lipid metabolism (especially as ectopic lipid deposition is a source of endoplasmic reticulum stress). Thus a defect in, or dysregulation of, the UPR may lie at the root of unwanted hepatic lipid deposition and form part of a vicious cycle therein. It is worth noting that (as discussed previously) inflammation and endoplasmic

reticulum stress may be contributory to worsened insulin sensitivity and it is possible, therefore, that such a reduced sensitivity might be present in parallel with hepatic lipid deposition. In addition, deposition of lipid and a direct effect on basal insulin sensitivity (i.e. the lipid induction theory) through interruption of expression of glucose transporters might also be present alongside these processes as a contributor to the cellular stress (as glucose deprivation also worsens endoplasmic reticulum stress).

This study, of a cohort of adolescents born preterm, has shown that circulating triglyceride levels and visceral adipose deposits are positively associated with increasing levels of IHL in these children who were born preterm. Visceral adipose tissue levels do not appear to be directly associated with either resting insulin sensitivity of glucose disposal (see 3.4.4). It is not possible to say whether the circulating triglycerides are a causal agent or a symptom of hepatic lipid overload as some authors suggest. The presence of visceral adipose tissue in association with hepatic lipid deposition has already been shown in other study populations (the 'portal drainage' theory (Bjorntorp, 1990)). This study shows that it may also exists in the Growmore cohort, and regardless of aetiology, it importantly suggests a potential target for intervention. It is interesting that there is a strong association of a measured area of visceral adipose tissue at a single cross-sectional abdominal level, but when indexed to try to correct for body height, this association is less strong. These results sit alongside strong correlations seen between overall measures of obesity (e.g. waist circumference and BMI SDS) and amount of intrahepatic lipid, which do not translate into a direct association when using regression analysis. O'Connor(O'Connor *et al.*, 2015) found a strong association with waist circumference and visceral adipose tissue using the same vertebral level in females as we did, but slightly different in males (L1-2). It is possible this reflects that overall obesity is not important when considering deposition of lipid in the liver (i.e. only the visceral compartment is active in contributing to intrahepatic lipid) but that where there are auxological markers for obesity there is increased risk of visceral lipid deposition. This may also reflect a genetic component (either inherited or epigenetic) which predisposes some individuals to visceral lipid deposition in the absence of marked overall obesity, and others to non-visceral adiposity with relatively lipid-free organs.

In this population of adolescents pubertal status clearly makes a contribution to the level of IHL seen. The association is a negative one such that IHL decreases as puberty progresses through to completion. The data suggest that including Tanner Stage improves the strength of the models when considering IHL deposition, but there was not a clear correlation with other variables to suggest a simple mechanism through which this occurring (i.e. in isolation, Tanner stage did not clearly correlate with any of the key variables that were associated with IHL levels in the regression analysis). As might be expected, Tanner stage was associated with overall habitus, but only improved model strength in the regression models which had serum triglycerides as a variable. It may be that pubertal status is working through a mechanism related to lipid metabolism and adipose tissue elsewhere in the body through puberty (not viscerally) thus is related to triglyceride levels rather than IHL per se. However, as expected, increased circulating triglyceride levels are associated with increasing amounts of IHL which may be cause, effect, or both, in relation to hepatic lipid deposition.

The data did not show any link between dietary intake of total calories nor, specifically, fat/carbohydrate intake as measured by SCRAN24, and IHL. This probably reflects that, in this group of individuals, the fasting prior to examination as part of the study protocol means that the liver will have returned to a 'baseline' amount of lipid in it. It could be speculated that this is probably more representative of an individual's long term environment or diet than it is of the intake in the preceding 24 hours. Less metabolically flexible individuals would be expected to have higher baseline amounts of IHL compared to their metabolically health peers. However, other authors have noted a lack of correlation between visceral adipose tissue and diet/nutrition(Bouchard *et al.*, 1993).

3.3 Directly Measured Physical Activity, Skeletal Muscle Kinetics and Vitamin D

After each study visit the participants were offered the opportunity to wear accelerometers for three days to objectively measure their physical activity. This was done to allow comparison between the physiological measurements of muscle mitochondrial oxidative function taken by 31P-MRS and how active the volunteers were in the non-laboratory setting. In addition, Vitamin D was measured on any fasting serum samples taken, in order to factor this into the results, as it has been shown that vitamin D is associated with the muscle kinetics seen by this method (Sinha *et al.*, 2013).

3.3.1 Objectively measured physical activity

Accelerometers were used as described in the methods to measure physical activity during a three day period after the study. The cut-points used for counts per minute allowed the activity to be classified into sedentary, light or moderate-to-vigorous (Evenson *et al.*, 2008) and for each, a sub-classification of daily mean percentage time or daily mean number of minutes in that degree of activity. Figure 18 clearly shows that the cohort as a whole were not achieving the nationally recommended 60 minutes per day of moderate-to-vigorous activity (Strong *et al.*, 2005; Basterfield *et al.*, 2008).

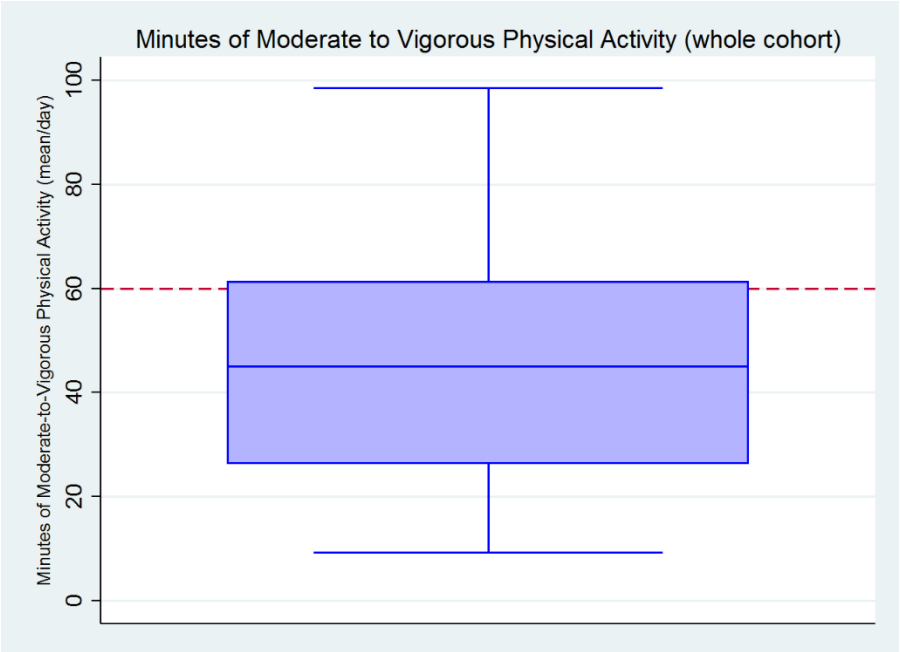

Figure 18: Moderate to vigorous activity (daily mean) in the cohort. National recommended minimum shown by red line.

	Male vs. Female	Growth vs. Protein study
Total Minutes Activity (mean/day)	0.95	0.76
MVPA (mean minutes)	**0.07***	0.31
MVPA (mean % total activity)	**0.07***	0.29
Sedentary activity (mean minutes)	0.28	0.76
Sedentary activity (mean % total)	**0.02****	**0.09***
Light activity (mean minutes)	0.15	0.25
Light Activity (mean % total)	**0.02****	0.11
Mean Step-count/day	0.14	0.43

Table 17: Within-cohort analysis of directly measured activity using accelerometers. Table shows *p*-values for Mann Whitney U test [*p<0.1; **p<0.05]

Valid results (>6 hours per day wear-time) from the cohort were obtained in a total of 44/60 who took the devices home. Reasons for not meeting the criteria were diary-validated wear-time too short, failure to return the device, periods of unexplained low level activity which did not correspond with the written diaries where it was not possible to confirm that the device was being worn.

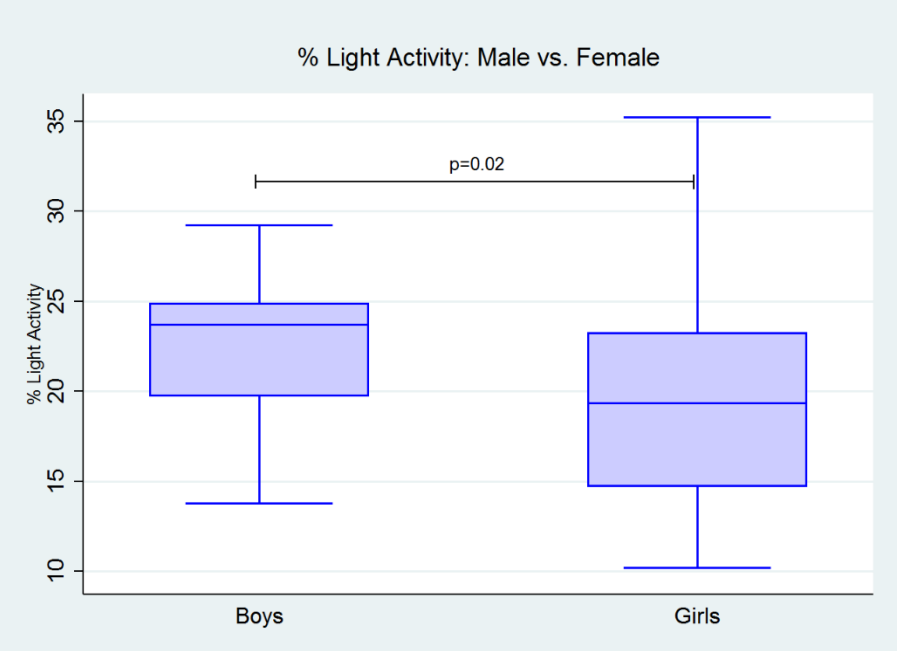

Figure 19: Percentage of time in light activity (daily mean) in the cohort: comparison of girls vs. boys.

Table 17 shows the main activity categories assessed for differences between sexes and shows that the boys in the cohort were more active than the girls (see figures 19 and 20) but this was only significant for light activity and sedentary activity. There was a trend observed for MVPA but this was not significant. There was no statistical significance between those individuals recruited into either the original growth study or the protein study.

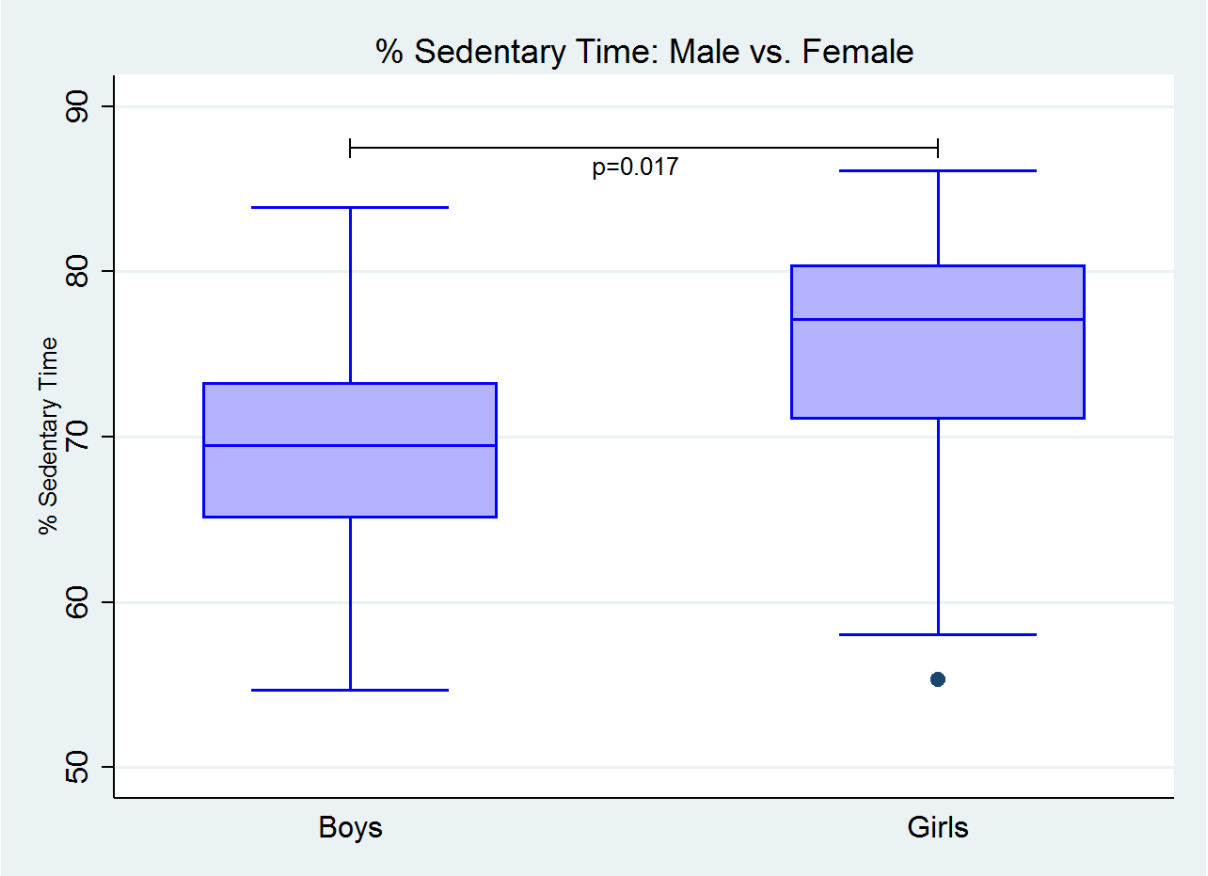

Figure 20: Percentage of time in sedentary activity (daily mean) in the cohort: comparison of girls vs. boys.

Table 18 presents a correlation analysis between the different categories of activity, total activity and mean daily step count, and other variables which it was thought could influence the activity. As these are associations, there is no comment on causality and though the exercise measures are presented as the dependent variables, if causality exists, they may be the independent variable. [*p<0.1;**p<0.05]

	Total Mins (mean/ day)	MVPA (mean mins)	MVPA (mean %)	Sed[y] act. (mean mins)	Sed[y] act. (mean %)	Light act. (mean mins)	Light act. (mean %l)	Mean Step-count/ day
Birth weight SDS	0.49	0.68	0.52	0.86	0.39	**0.08***	**0.06***	0.75
Gestational age at birth	0.83	**0.09***	0.17	0.90	0.50	0.78	0.79	0.24
Age at Study visit	0.76	**0.07***	**0.07***	0.78	0.13	0.63	0.38	0.17
Height SDS	0.26	0.47	0.66	0.25	0.99	0.94	0.85	0.77
Current Wt SDS	0.33	0.59	0.99	0.11	0.30	0.45	0.22	0.72
Current BMI SDS	0.89	0.99	0.71	0.46	0.24	0.39	0.21	0.55
Visceral Adipose Tissue (cm^2)	0.42	0.84	0.47	0.18	0.60	0.88	0.97	0.78
Subcutaneous Adipose Tissue (cm^2)	0.89	0.23	0.16	0.33	**0.05***	0.23	**0.09***	0.15
VAT/SAT ratio	0.73	0.49	0.42	0.39	0.11	0.27	**0.05***	0.44
VAT Index (cm^2/m^2)	0.61	0.84	0.55	0.35	0.79	0.75	0.86	0.92
SAT Index (cm^2/m^2)	0.93	0.31	0.21	0.34	**0.07***	0.29	0.11	0.22
Fat-free mass index (BODPOD)	0.45	0.46	0.72	0.56	0.52	0.49	0.37	0.93
Fat mass index (BODPOD)	0.34	0.27	0.26	0.84	0.10	0.18	0.13	0.18
Waist Circumference	0.62	0.34	0.24	0.45	**0.05***	**0.09***	**0.08***	**0.09***

Mid-arm Circumference	0.75	0.37	0.21	0.23	**0.053***	0.21	**0.08***	0.12
HOMA%S	0.36	0.84	0.63	0.51	0.98	0.40	0.48	0.77
Matsuda Index	**0.09***	0.51	0.68	0.50	0.12	**0.01****	**0.02****	0.11
Serum Vitamin D	0.95	0.25	0.18	0.30	**0.09***	0.20	**0.09***	0.15
Tanner Stage[1]	0.57	0.25	0.43	0.99	0.34	0.22	0.28	0.22
Q1 vs. Q4 Matsuda Index[2]	**0.10***	0.50	0.63	0.57	0.10	**0.02****	**0.02****	**0.07***
Q1 vs. Q4 HOMA%S[2]	**0.051***	0.56	0.43	0.17	0.85	0.14	0.44	0.70
Season (March to Sept vs. Oct to Feb)[2]	0.43	0.79	0.73	0.23	0.55	0.93	0.58	0.82

Table 18: Correlation analysis of directly measured physical activity using accelerometers, (Spearman's Correlation Analysis unless stated). Table shows *p*-values. Note: within cohort analysis shown for Tanner Stage, quartile and season comparison.[1]: Kruskall-Wallis Test; [2]: Mann Whitney U test [*p≤0.1;**p<0.05]

Total Activity Time

This measure of activity was most closely associated with the Insulin Sensitivity (IS) measures, in particular the Matsuda Index which is an estimation of glucose disposal. More daily activity would fit with a better baseline metabolic flexibility, but in those volunteers who achieved a minimum of six hours wear-time it is not possible to say whether they were worn for the full duration of the waking hours. The near-significant correlation (figure 21) with the HOMA modelling quartile analysis must also be viewed with caution for this reason, though the analysis showed that those in Q1 (least IS) had fewer total minutes of activity per day than those in Q4 (most IS).

Moderate to Vigorous Physical Activity

None of the variables examined in association with measured activity was significant for MVPA, but there was a trend towards age at study being associated: the younger the individual, the greater the time spend in MVPA. This finding is well described in other groups of children (Basterfield *et al.*, 2012). One recent study found an increase in fat-free mass index (in children who were younger and born small for gestational age at term) with increased moderate activity(Kennedy *et al.*, 2013), but this is not seen in our cohort.

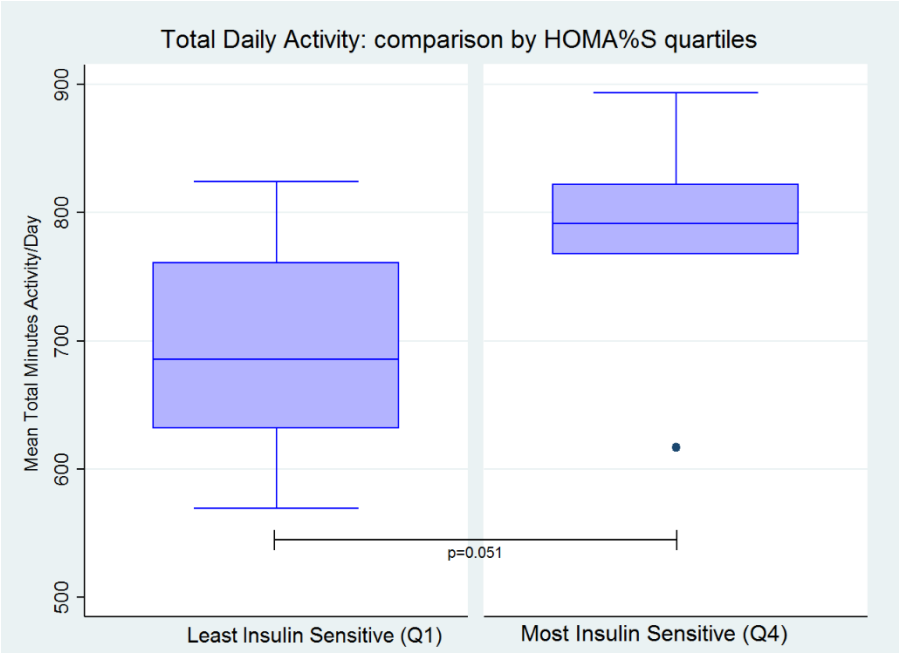

Figure 21: Total daily activity (mean minutes/day): comparison of most and least insulin sensitive by HOMA modelling (% sensitivity shown).

Sedentary Activity

The correlation analysis showed that there was a trend towards increased percentage time in sedentary activity associated with some markers of adiposity (Subcutaneous Adipose Tissue (SAT) Index, waist circumference, mid-arm circumference). This is consistent with other childhood cohorts (Basterfield *et al.*, 2012).

Light Physical Activity

The most striking finding in the analysis for light activity was the association with the measured Matsuda Index (see figure 22). For both percentage of time in light activity and the daily minutes in light activity the association was statistically significant

(p<0.05) suggesting that those who did more light exercise were also more insulin sensitive when given a glucose load. The absence of this association for IS measured using the HOMA method suggests that it is specifically related to metabolic flexibility as described by Kelley (Kelley, 2005) rather than 'baseline' fasting IS. This is discussed more in section 3.4. Trends towards a reduction in adiposity (SAT, waist circumference and mid-arm circumference) are also seen with an increase in time in light activity. Surprisingly, birthweight SDS score also seems to trend towards an association with time in light activity: time spent in light activity increases with lowered birthweight SDS. It is possible this is not a true effect as there is no association with gestational age at birth which is highly correlated with birthweight in terms of maturity (i.e. the birthweight SDS decreases with increased gestational age due to recruitment criteria from the original studies).

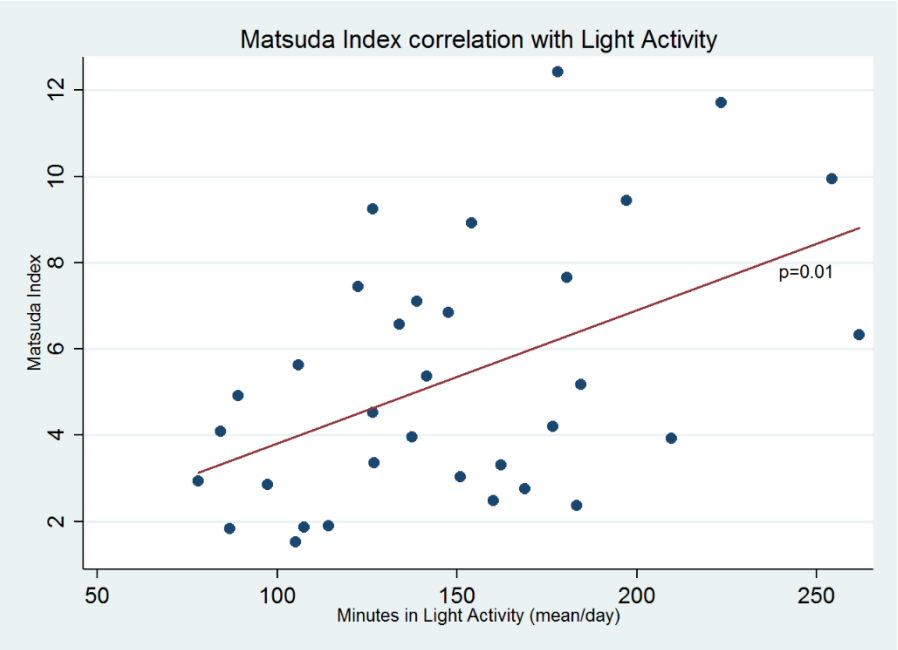

Figure 22: Significant correlation between Matsuda Index and light activity measured using accelerometry

Total Step Count (mean/day)

In line with the findings for light activity, there were trends seen associating increasing total step count/day with a reduction in waist circumference. The difference between highest and lowest quartiles for Matsuda Index when comparing total step count

within the cohort also showed a trend towards increased IS with higher daily step counts.

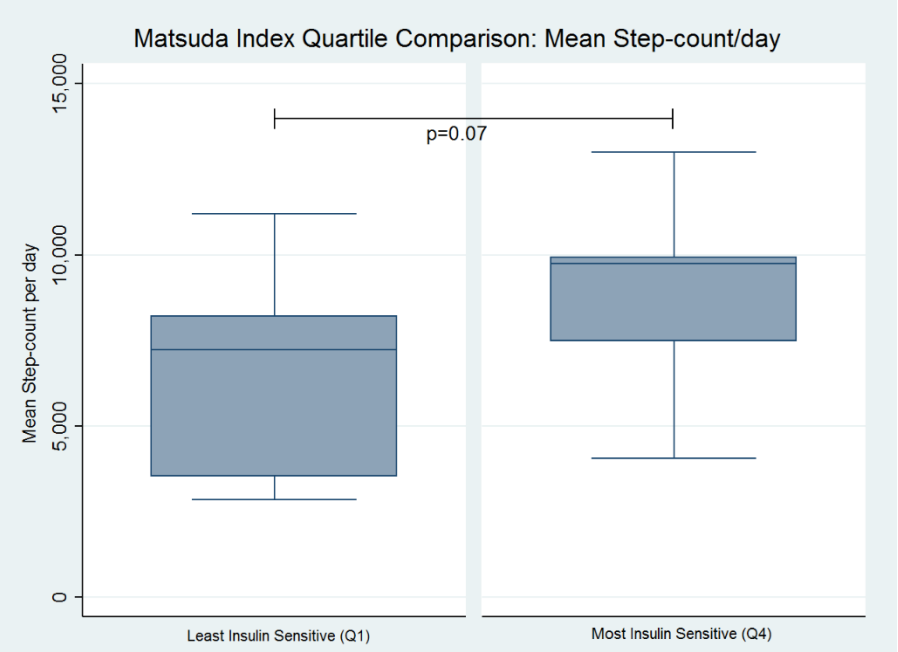

Figure 23: Trend towards increased daily step count between most and least insulin sensitive (Matsuda Index quartiles)

3.3.2 Skeletal Muscle Kinetics and mitochondrial oxidative capacity measured by 31P-MRS

One of the main investigative techniques used in this study was Magnetic Resonance Spectroscopy (MRS). To assess in-vivo mitochondrial function, 31P-MRS was used. A total of 59 of the cohort had an MRI scan and raw data was obtained from all of these. The raw data was subject to post-collection analysis as outlined in the methods and tables 19 to 21 present this data. There are two measures for phosphocreatine recovery (τ½PCr) which is the measure used here as a direct assessment of mitochondrial oxidative capacity in skeletal muscle. The better the oxidative capacity (measured by PCr recovery), the shorter the half time seen. Both datasets have both been processed using the Iotti correction to adjust the half times around the mean pH of the group, in order to allow within group comparisons (Iotti *et al.*, 1993). For this group, those participants who did not fully deplete their PCr store during the exercise were removed from analysis (leaving n=50). The τ½ADP is also presented. This measure gave a reciprocal measure of the process ongoing during the exercise and

also gave information about the quality of the MRS not always available by looking at the PCr results. The ADP curves were sensitive to inorganic phosphate splitting where the voxel crossed across two muscle groups, which then recovered at different rates. While this gave an overall τ½PCr, it could not necessarily be considered as accurate as those individuals who had no splitting (n=38). Therefore, the affected individuals were removed to give the data set presented here as 'Iotti-adjusted PCr recovery (τ½PCr) adjusted for non-split τ½ADP'.

Tables 19 and 20 show that there was no significant within cohort difference for either boys vs. girls, original study enrolment or the extremes of measured insulin sensitivity. Spearman correlation analysis of recorded minimum pH during standardised exercise showed no correlation between nadir pH and insulin sensitivity measured by either HOMA%S (Rho: -0.10; p=0.64) or Matsuda Indices (Rho: 0.07; p=0.75). Serum triglyceride levels were also analysed with respect to the measured muscle kinetics and no correlation was found (p=0.65).

	Male vs. Female	**Growth vs. Protein study**
Iotti-adjusted PCr recovery (τ½PCr)	0.26	**0.09***
Iotti-adjusted PCr recovery (τ½PCr) adjusted for non-split τ½ADP	0.48	0.63
τ½ADP	0.36	0.99

Table 19: Comparison of 31P-MRS measured muscle kinetics by group within the cohort. (Mann-Whitney U test); Table lists p-values for the comparisons. *p<0.1

	HOMA%S Q1 vs. Q4	**Matsuda Index Q1 vs. Q4**
Iotti-adjusted PCr recovery (τ½PCr)	0.62	0.85
Iotti-adjusted PCr recovery (τ½PCr) adjusted for non-split τ½ADP	0.85	0.44
τ½ADP	0.41	0.12

Table 20: Comparison of 31P-MRS measured muscle kinetics by groups within the cohort: comparison of most (Q4) and least (Q1) insulin sensitive. (Mann-Whitney U test); Table lists p-values for the comparisons.

	Iotti-adjusted PCr recovery (τ½PCr)	**Iotti-adjusted PCr recovery (τ½PCr) adjusted for non-split τ½ADP**	**τ½ADP**
Birth weight SDS	0.59	0.97	0.87
Gestational age at birth	**0.079***	**0.054***	**0.07***
Age at Study visit	0.26	0.69	0.67
Tanner Stage	0.12	0.45	0.71
Height SDS	0.46	**0.09***	0.15
Current Wt SDS	0.64	0.99	0.87
Current BMI SDS	0.62	0.52	0.75
Fat mass index (BODPOD)	0.47	0.55	0.80
Fat-free mass index (BODPOD)	0.37	0.58	0.92
Visceral Adipose Tissue (cm^2)	0.58	0.41	0.29
Subcutaneous Adipose Tissue (cm^2)	0.91	0.91	0.74
VAT/SAT ratio	0.66	0.75	0.44
VAT Index (cm^2/m^2)	0.41	0.25	0.24
SAT Index (cm^2/m^2)	0.67	0.60	0.93
Total Minutes Activity (mean/day)	0.59	0.82	0.92
MVPA (mean minutes)	0.52	0.33	0.25
MVPA (mean % total activity)	0.52	0.47	0.34
Sedentary activity (mean minutes)	0.44	0.91	0.74
Sedentary activity (mean % total)	0.15	0.38	0.39
Light activity (mean minutes)	0.29	0.34	0.57
Light Activity (mean % total)	0.12	0.43	0.55
Mean Step-count/day	0.57	0.49	0.46
Wt SDS change [term to 2yr]	0.85	0.12	**0.097***

Waist Circumference	0.85	0.91	0.78
Mid-arm Circumference	0.63	0.87	0.94
Serum Vitamin D	**0.03****	0.44	0.26

Table 21: Spearman Correlation analysis for muscle kinetics (τ½: half time for recovery; PCr: Phosphocreatine; ADP: adenosine diphosphate). MVPA: moderate-to-vigorous physical activity. For activity measures, mean (either minutes or % of total activity) is calculated over 3 days total data collection with minimum of 6 hours per day recorded. Wt. SDS: weight standard deviation (z) score. *p<0.1; ***p*<0.05

Table 21 shows correlation analysis of non-transformed variables using Spearman's correlation in relation to τ½PCr and τ½ADP. Univariate linear regression was also performed for these variables and yielded similar results, but required extensive transformation of the variables to normalise the data for regression. For this reason, the univariate regression data is therefore not presented here. There is a consistent association with gestational age and the length of half time during recovery (p=0.05 to p=0.07) with longer times seen in those individuals who were more preterm when born (see figure 24). In the Iotti-adjusted PCr recovery (τ½PCr) dataset, vitamin D is also significantly associated with the half time: the greater the vitamin D level, the quicker the half-time recorded (figure 25). This is not seen when those individuals with inorganic phosphate splitting are removed.

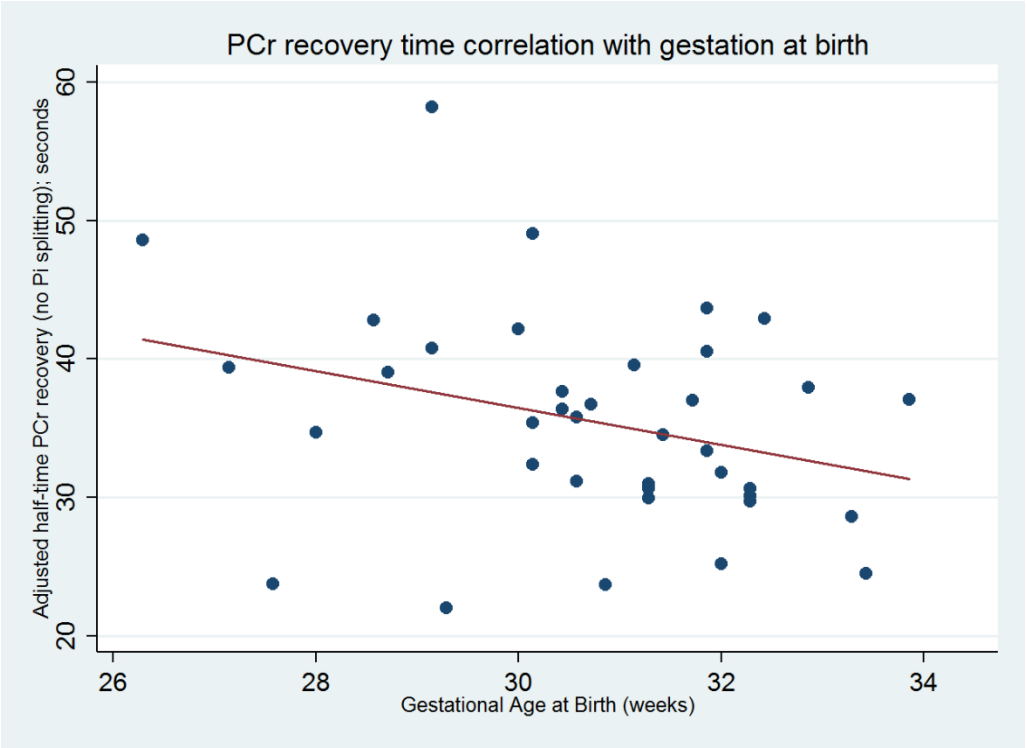

Figure 24: correlation between τ½PCr and gestational age at birth.

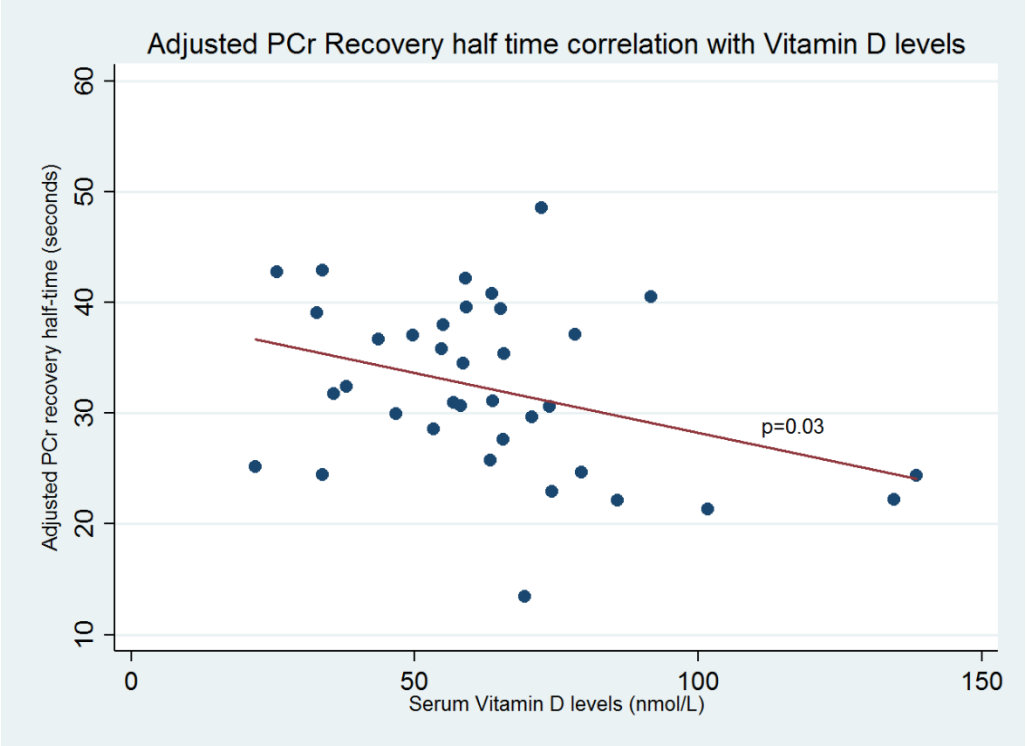

Figure 25: correlation between τ½PCr and Serum Vitamin D levels

Vitamin D

As other work has shown (Sinha *et al.*, 2013), vitamin D is associated with muscle kinetics measured with 31P-MRS and potentially a reversible cause of worsened mitochondrial oxidative capacity. Therefore, analysis was done to assess what factors might be influencing serum Vitamin D levels. In line with findings published in the literature, the Vitamin D levels in the cohort were significantly negatively associated with measures of adiposity (see table 22) and there was a similar negative association trend with sedentary activity. There was a trend towards a positive association with the percentage of time spent in light activity, and vitamin D was strongly positively associated with improved insulin sensitivity by both measures used in the study (see section 3.4). Unsurprisingly, serum vitamin D levels correlated with the month of the year, and time of year when considered as a dichotomous variable. These results are shown in figures 26 to 29. Multivariate analysis was attempted to refine causation for Vitamin D levels but none of the models generated were valid as the terms used were not found to be independent. This is perhaps in keeping with the fact that vitamin D has many effects across many body systems (Girgis *et al.*, 2013).

	Serum Vitamin D level
Male vs. Female[1]	1.11; 0.27
Growth vs. Protein study[1]	-0.44; 0.66
Tanner Stage[2]	1.66; 0.80
HOMA%S[3]	**0.34; 0.02****
Matsuda Index[3]	**0.42; 0.005****
Season (March to Sept vs. Oct to Feb)[1]	**3.24; 0.001****
Month of Visit[2]	14.5; 0.07*
Birth weight SDS[3]	-0.20; 0.19
Gestational age at birth[3]	-0.025; 0.87
Age at Study visit[3]	0.002; 0.99
Height SDS[3]	-0.20; 0.18
Current Wt SDS[3]	**-0.34; 0.02****
Current BMI SDS[3]	**-0.30; 0.04****
Fat mass index (BODPOD)[3]	**-0.27; 0.07***
Fat-free mass index (BODPOD)[3]	-0.13; 0.39
Waist Circumference[3]	**-0.28; 0.06***
Visceral Adipose Tissue (cm^2)[3]	**-0.36; 0.02****
Subcutaneous Adipose Tissue (cm^2)[3]	**-0.35; 0.02****
VAT/SAT ratio[3]	0.08; 0.59
VAT Index (cm^2/m^2)[3]	**-0.33; 0.03****
SAT Index (cm^2/m^2)[3]	**-0.33; 0.03****
Total Minutes Activity (mean/day)[3]	-0.01; 0.95
MVPA (mean minutes)[3]	0.20; 0.25
MVPA (mean % total activity)[3]	0.23; 0.18
Sedentary activity (mean minutes)[3]	-0.18; 0.30
Sedentary activity (mean % total)[3]	**-0.29; 0.09***
Light activity (mean minutes)[3]	0.22; 0.19
Light Activity (mean % total)[3]	**0.29; 0.086***

Mean Step-count/day[3]	0.25; 0.15

Table 22: Analysis of factors associated with serum Vitamin D levels and within cohort groups. [1]: Mann Whitney U test (z value; *p*); [2]: Kruskall-Wallis Test (Chi-Squared Value; *p*); [3]: Spearman Correlation (Rho; *p*) [*p<0.1; **p<0.05]

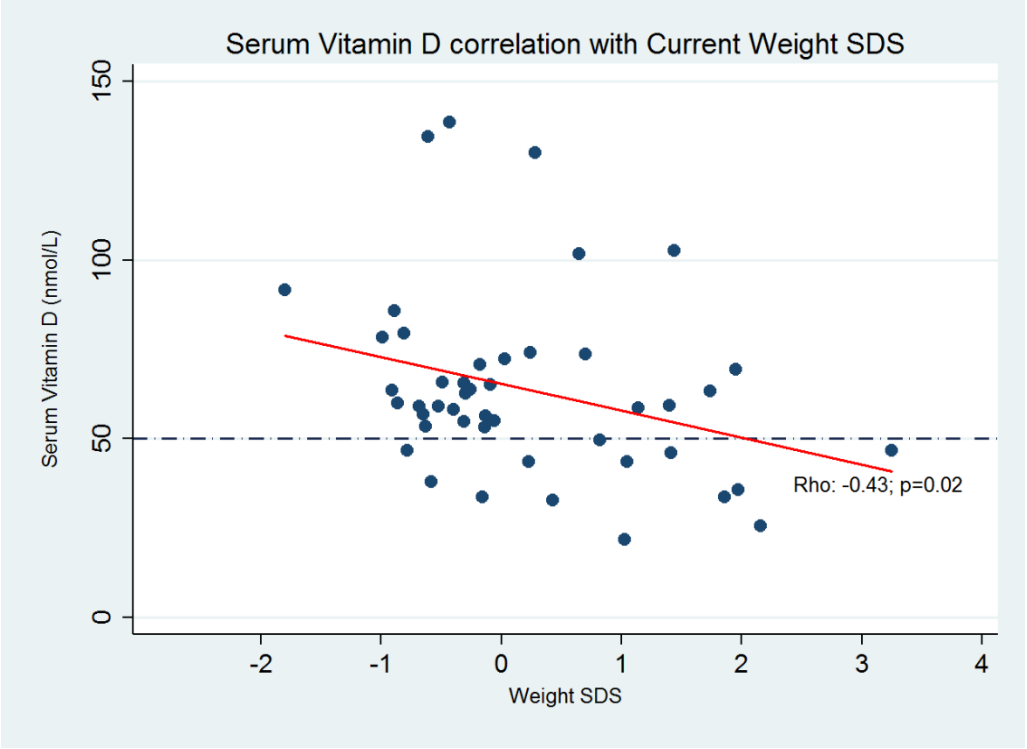

Figure 26: correlation between current weight SDS and Vitamin D

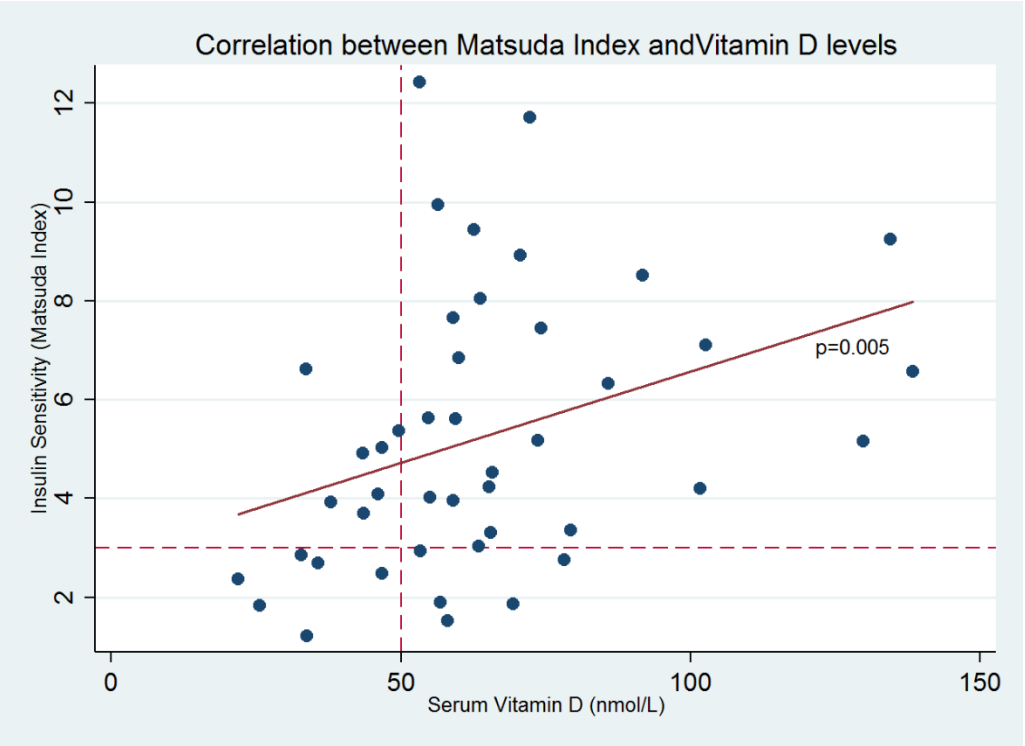

Figure 27: correlation between vitamin D and glucose disposal estimate as representation of IS (measured by Matsuda Index). Dashed lines represent accepted thresholds for Vitamin D deficiency (50nmol/L) and reduced IS (Matsuda Index <3)

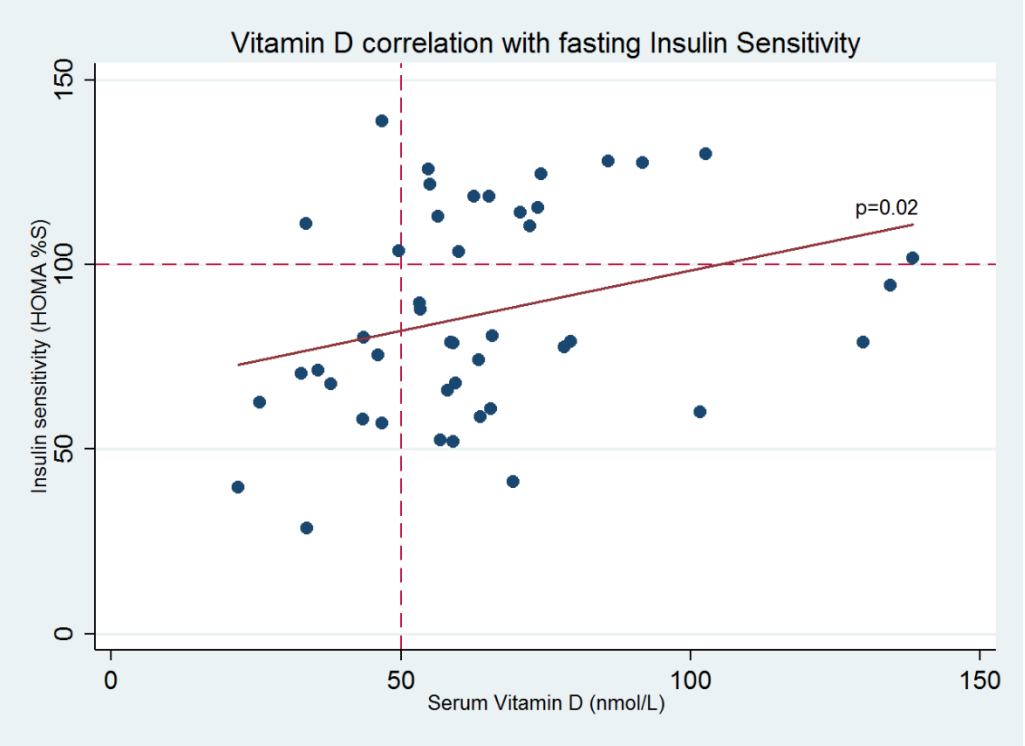

Figure 28: correlation between vitamin D and fasting IS (measured by HOMA modelling). Dashed lines represent accepted thresholds for Vitamin D deficiency (50nmol/L) and arbitrary reduced IS (HOMA%S<100)

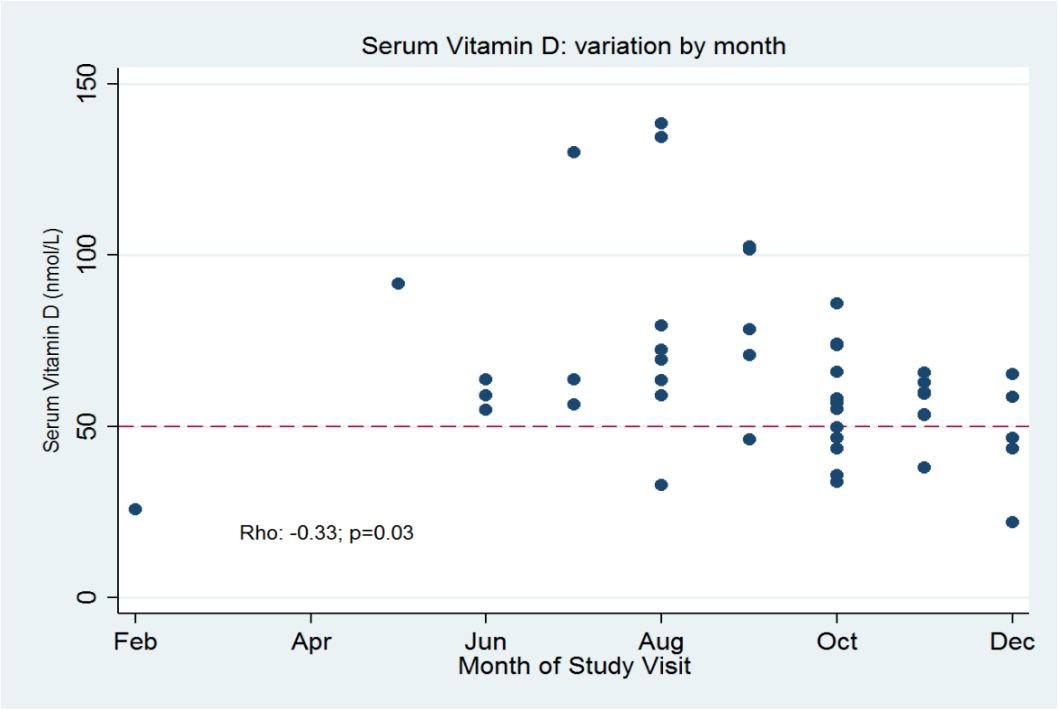

Figure 29: correlation between month of study visit and measured serum Vitamin D levels

3.3.3 Multivariate Analysis

Multivariate analysis was performed with both τ½PCr datasets to try to establish whether there were any strong associations that might explain some of the variance seen in the results. The larger dataset was used as it would have more power by virtue of increased numbers, but the smaller dataset was theoretically more accurate in terms of what was occurring. With both (tables 23 to 28), variables for activity measures and IS were considered separately before inclusion into the final models. Early life growth (see section 3.4) was also included in the analysis in light of gestational age being a potentially important variable. In the final models, both were considered using the different measures of IS. For each final model, non-significant terms were removed until the minimum number of significant variables remained.

For both Iotti-adjusted τ½PCr datasets, the final models showed that gestational age at birth was the most significant predictive factor for mitochondrial oxidative capacity. The earlier the gestational age at birth, the longer the half time for recovery seen. The two other factors that were predictive were fasting insulin sensitivity (measured by HOMA%S) and vitamin D status, with improved (shorter) half times where insulin sensitivity was higher and vitamin D levels higher.

	Model 1	Model 2	Model 3	Model 4	Model 5
Sedentary activity (mean % total)	0.40	0.33	0.325	0.098	0.009
Mean Step-count/day	0.11	0.14	0.03	0.04	0.03
Light Activity (mean % total)	0.40	0.33	0.33	0.69	
MVPA (mean % total activity)	0.41	0.33	0.33		
MVPA (mean minutes)	0.59	0.92			
Total Minutes Activity (mean/day)	0.83				
Sedentary activity (mean minutes)	0.81				
Light activity (mean minutes)	0.98				
R^2	0.26	0.22	0.22	0.20	0.19
Adjusted R^2	0..04	0.09	0.12	0.12	0.14
p value (for model)	0.35	0.17	0.09	0.07	0.03

Table 23: Multivariate regression analysis (model A) for Iotti-adjusted PCr recovery (τ½PCr) with directly measured activity subgroups. Non-normal data was log transformed before regression analysis.

	Model 1	Model 2
HOMA%S	0.368	0.166
Matsuda Index	0.852	
R^2	0.06	0.057
Adjusted R^2	0.000	0.03
p value (for model)	0.385	0.166

Table 24: Multivariate regression analysis (model B) for Iotti-adjusted PCr recovery (τ½PCr) with directly measured insulin sensitivity. Non-normal data was log transformed before regression analysis.

	Model 1	Model 2	Model 3	Model 4	Model 5
Gestational age at birth	0.053	0.178	0.04	0.009	0.005
Serum Vitamin D	0.225	0.084	0.20	0.124	0.045
Sedentary activity (mean % total)	0.196	0.175	0.19	0.479	
Mean Step-count/day	0.19	0.151	0.26		
HOMA%S	0.291	0.259			
Wt SDS change [term to 2yr]	0.45				
R^2	0.80	0.44	0.41	0.37	0.28
Adjusted R^2	0.64	0.30	0.30	0.29	0.24
p value (for model)	0.03	0.03	0.02	0.01	0.004

Table 25: Multivariate regression analysis (model C) for Iotti-adjusted PCr recovery (τ½PCr), Final model; testing for heteroskedasticity showed that the final terms (Vitamin D and Gestational Age) were independent. Inclusion of Tanner Staging was not significant by likelihood ratio testing of this model with or without Tanner Staging included. Substitution of Matsuda Index for HOMA%S did not improve the model, leaving only gestational age as the final term and increased heteroskedasticity (p=0.07 with Matsuda Index i.e. less independence of terms; p=0.59 with HOMA%S). Non-normal data was log transformed before regression analysis.

	Model 1	Model 2		Model 1	Model 2
Total Minutes Activity (mean/day)	0.305		HOMA%S	0.436	0.115
MVPA (mean % total activity)	0.087	0.088	Matsuda Index	0.497	
Sedentary activity (mean % total)	0.086	0.088			
Light Activity (mean % total)	0.086	0.088			
Mean Step-count/day	0.029	0.041			
R^2	0.31	0.27	R^2	0.12	0.10
Adjusted R^2	0.13	0.12	Adjusted R^2	0.04	0.07
p value (for model)	0.18	0.16	*p* value (for model)	0.25	0.12

Table 26: Multivariate regression analysis (model D) for Iotti-adjusted PCr recovery (τ½PCr) adjusted for non-split τ½ADP. Directly measured activity subgroups and insulin sensitivity. Non-normal data was log-transformed before regression analysis.

	Model 1	Model 2	Model 3	Model 4
HOMA%S	0.566	0.037	0.008	0.103
Gestational age at birth	0.002	0.012	0.007	0.002
Wt SDS change [term to 2yr]	0.174	0.063	0.085	
Serum Vitamin D	0.112	0.391		
Mean Step-count/day	0.855			
R^2	0.99	0.69	0.67	0.43
Adjusted R^2	0.96	0.59	0.60	0.37
p value (for model)	0.006	0.004	0.002	0.002

Table 27: Multivariate regression analysis (model E) for Iotti-adjusted PCr recovery (τ½PCr) adjusted for non-split τ½ADP, Final model; including HOMA%S as the IS term. Testing for heteroskedasticity showed that the final terms (HOMA%S and Gestational Age) were independent. Inclusion of Tanner Staging was not significant by likelihood ratio testing of this model with or without Tanner Staging included.

	Model 1	Model 2	Model 3	Model 4
Gestational age at birth	0.008	0.002	<0.001	0.001
Serum Vitamin D	0.08	0.327	0.16	0.25
Mean Step-count/day	0.495	0.70	0.65	
Matsuda Index	0.48	0.96		
Wt SDS change [term to 2yr]	0.69			
R^2	0.99	0.65	0.66	0.41
Adjusted R^2	0.98	0.51	0.59	0.36
p value (for model)	0.02	0.02	0.002	0.003

Table 28: Multivariate regression analysis (model F) for Iotti-adjusted PCr recovery (τ½PCr) adjusted for non-split τ½ADP, Final model; Including Matsuda Index as the IS term. Testing for heteroskedasticity showed that the final terms (serum Vitamin D and Gestational Age) were independent. Inclusion of Tanner Staging was not significant by likelihood ratio testing of this model with or without Tanner Staging included.

3.3.4 Discussion

The results obtained by objective measurement of daily activity and 31P-MRS interrogation of in-vivo muscle kinetics was interesting, if for no reason other than there appeared to be no direct correlation between the two. It might reasonably have been expected that habitual activity levels would be associated with the oxidative capacity of the mitochondria if it were very modifiable by training the muscles with exercise. However, absence of such a link may reflect the fact that the standardised test is designed to deplete the PCr stores completely to measure recovery, and thus is perhaps more extreme than usual low-level aerobic exercise experienced at the cellular level. There were, however, three key predictive variables which were exposed in multivariate testing, relating to the PCr half-time results.

The finding that gestational age at birth was consistently predictive of τ½PCr is important because it is most likely a variable that will not be modifiable by intervention later in life. Work by Uauy and colleagues previously (Bertocci *et al.*, 1992) has shown that infants born preterm have a smaller PCr signal than their term born counterparts, using a similar MRS technique to that used in this study. The authors

speculated that these hypotonic preterm infants may have had limited phosphate reserves such that when challenged by even a small increase in activity they depleted their reserves very quickly and took a long time to recover. However, this would not explain persistence of this effect once the infants become ambulant, active children and adolescents. Using other methods to measure oxidative capacity (aerobic fitness score), Rogers and colleagues (Rogers *et al.*, 2005) demonstrated that at age 17, adolescents born preterm have reduced oxidative capacity and are more likely to become fatigued. The other finding that may be relevant in these children comes from post-mortem specimens from the skeletal muscle of infants born preterm which demonstrate that in preterms, there is a predominance of type 2 (low-oxidative) muscle fibres which have, relatively, a low density of mitochondria (Keens *et al.*, 1978; Schloon *et al.*, 1979; Vogler and Bove, 1985). In these studies which looked at muscles such as the diaphragm and intercostal muscles, it was demonstrated that the normal development towards term is to increase the density of type 1 (high oxidative) fibres. However, in surviving preterm infants who have experienced the normal preterm course of difficulty with deposition of lean tissue, there may be arrest or unchangeable alteration in the density of type 1 fibres. A permanent reduction in mitochondrial density may then be detectable in a prolonged PCr recovery. Aside from optimising protein nutrition in the preterm infant, which will always be attempted as long as it does not conflict with neuroprotective nutritional strategies, it does not suggest that this could be modified with intervention from term-equivalent age onwards.

The second variable shown to be important in determining τ½PCr is insulin sensitivity; specifically fasting IS as measured by HOMA (% Sensitivity) modelling. % Sensitivity was specifically chosen as it reflects the physiological truth that IS is a continuum: insulin resistance is more analogous with a clinically defined quantity that allows physicians to quantify when a person may need intervention by defining a 'normal' cut-off. Physiologically, reduced IS leading to increased τ½PCr could be explained by reduced transit of glucose into the myocyte secondary to reduced expression of the insulin receptor and glucose transporting receptors at the myocyte membrane. This was demonstrated elegantly by Lim and colleagues using similar imaging techniques during a hyperglycaemic hyperinsulinaemic clamp study in diabetics (Lim *et al.*, 2011c). In this study, the thing that does not fit with this possibility is that the Matsuda Index, which

is a better measure of glucose disposal than HOMA does not show any kind of relationship to τ½PCr. Reviewing the multivariate modelling, therefore, leads to the third, important variable: Vitamin D.

In our study, as seen in Sinha's work (Sinha *et al.*, 2013), there is a relationship between decreasing serum vitamin D levels and increasing recovery times for τ½PCr. This effect is independent to that seen from gestational age at birth in our cohort. Vitamin D has many effects in many different tissues but it is not clear that it has a direct effect on muscle at the myocyte membrane. It is possible that it could have an effect, as a hormone-like substance, at the mitochondrial genome level upregulating either the cellular mechanisms and machinery needed to improve oxidative function, or more directly influencing ATP synthesis (Psarra *et al.*, 2006). Vitamin D may also play a role in mitochondrial uptake of Calcium, needed for effective muscle function but also potentially important in stimulating ATP synthesis by the mitochondria. Sinha proposes this as potential mechanism of vitamin D action and cites work on vitamin D deficient chick muscles (Mukherjee *et al.*, 1981) as an example where the muscle does not appear able to retain calcium.

Our study also showed a very strong association between vitamin D and IS (see section 3.4). It is possible, therefore, that the association seen in some of the multivariate models between HOMA sensitivity and mitochondrial oxidative capacity is, therefore, a statistical flaw which has occurred because of the underlying association between Vitamin D and IS. Thus HOMA sensitivity appears in the model in place of vitamin D due to the nature of the test, rather than because IS has a direct effect on τ½PCr.

In his study, Sinha replaced vitamin D in his subjects and demonstrated an improvement in clinical, and MRS muscle kinetic, outcomes. For our cohort, therefore, it would be a potential intervention to supplement the cohort, and potentially those born preterm elsewhere, with vitamin D in order to try to improve mitochondrial oxidative function. Mize and colleagues cite an example of a child with muscle hypotonia and rickets where supplementation with vitamin D, calcium and phosphate improved muscle kinetics measured by MRS (Mize *et al.*, 1988). We did not measure serum phosphate or calcium, but if vitamin D was used as a targeted intervention in our cohort, these other serum biochemical measures might also need checking. Intervention with supplementation also has to be considered in the clinical context: in

Sinha's study he had objective measures of fatigue and weakness in his population where we have none. The muscle kinetics are not associated with measured of adiposity and therefore no clinical impact of the τ½PCr results can be assumed. In addition, our findings were that of a linear association: supplementing only those who were clinically vitamin D deficient (<25nmol/L or <50nmol/L depending on clinical threshold) would not necessarily encompass all of those who had relatively slow τ½PCr recovery. We would, therefore, not (in this study cohort) have any grounds on which to recommend supplementation, beyond the usual clinical thresholds. The data do show clearly, however, the potential need for supplementation through the winter months in the North East, though this might well readily apply to any age group. If in future studies there is evidence that there are clinical symptoms related to oxidative function impacting on quality of life, in children or adolescents similar to those in our cohort, then vitamin D supplementation may well be a good potential intervention to try to ameliorate these symptoms. However, Rogers' work suggests that these adolescents do not perceive their differences in exercise capacity from term-born peers as impacting on their quality of life (Rogers *et al.*, 2005).

The data from the cohort from directly measured activity was, broadly speaking, similar to the data seen in the millennium cohort who are another North-East based cohort who have had accelerometric recording of their activity levels (Basterfield *et al.*, 2011b; Basterfield *et al.*, 2012). In our cohort, as theirs, we saw that boys were more likely to be physically active than girls (Jimenez-Pavon *et al.*, 2010) and that as the cohort aged, the amount of MVPA trended down. We also saw that increased time in sedentary activity was associated with a trend towards increase in some markers of adiposity. This has specifically not been found in some other authors work (Kennedy *et al.*, 2013). The most striking new finding from the activity data was good evidence that light activity is most strongly associated with IS measures. This has been discussed in more detail in section 3.4. While there was no direct association between τ½PCr and any of the activity measures, it is possible that light activity may have an indirect effect on muscle oxidative function via either its positive association with IS, or via IS to vitamin D, which is associated with τ½PCr, as outlined above. Importantly, increasing light exercise seems to show a trend for reducing some of the markers of increased adiposity (waist circumference, mid-arm circumference, subcutaneous adipose tissue)

which we have shown are the things that might influence deposition of visceral and hepatic lipids. In terms of preventing progression of metabolic disease, light activity might be more acceptable as an intervention (less daunting and perhaps more sustainable than MVPA interventions) and therefore these are encouraging findings with respect to modifying the metabolic outcomes of those born preterm.

3.4 Insulin sensitivity

One of the key determinants of metabolic outcomes in 'pre-diabetes', the metabolic syndrome and in type 2 diabetes is thought to be reduced insulin sensitivity (also described in the literature as insulin resistance). If there is a link between prematurity and later metabolic ill health, insulin sensitivity (IS) may be an important determinant or element which changes early in the process before actual ill-health occurs. It was therefore important to examine in the current study cohort.

Two measures of insulin sensitivity were used. Firstly % Sensitivity calculated from the HOMA2 model (HOMA%S) was used as a measure of resting insulin sensitivity (it is calculated from fasting insulin and glucose levels). This was used in preference to the more often quoted HOMA IR (insulin resistance) measure, which is the reciprocal of %S: HOMA-IR = 100/%S. The choice was because it reflected the likelihood that in this relatively young cohort it is more appropriate to talk about a change in sensitivity to insulin (along a continuum) rather than an insulin 'resistance' which invites a cut-off or threshold assumption which may not apply. 100% sensitivity would be the expected sensitivity for any given individual, which allows some sub-group analysis if required. The second measure of IS used was the Matsuda Index which, as outlined in the methods section, is an index which was formulated to be closely related to glucose disposal as measured by a clamp study. It is one which lends itself to our methodology (i.e. an oral glucose tolerance test) and is different to the HOMA%S measure in as much as it looks at the body's response to a glucose challenge. It is, therefore, useful in assessing current metabolic flexibility, which may not be reflected by a single HOMA%S value.

As my literature review of factors influencing IS in the infant-to-adult time-course demonstrated, growth may be an important a factor in later IS. Therefore, the longitudinal data available for the cohort was examined to see which periods of growth could be included in the analysis.

3.4.1 Growth in the cohort

Examining the longitudinal cohort data (for the 60 adolescents involved in the current study), it was clear that the most data points available per participant were for the weight standard deviation scores (Wt. SDS). It was therefore possible to examine

change in Wt. SDS as a measure of growth velocity over time period as a proxy for growth. While this does not control for factors such as height, and includes no measure of lean tissue vs. fat deposition, it is a reasonable measure as it is indexed to the population mean for the age at the time of measurement.

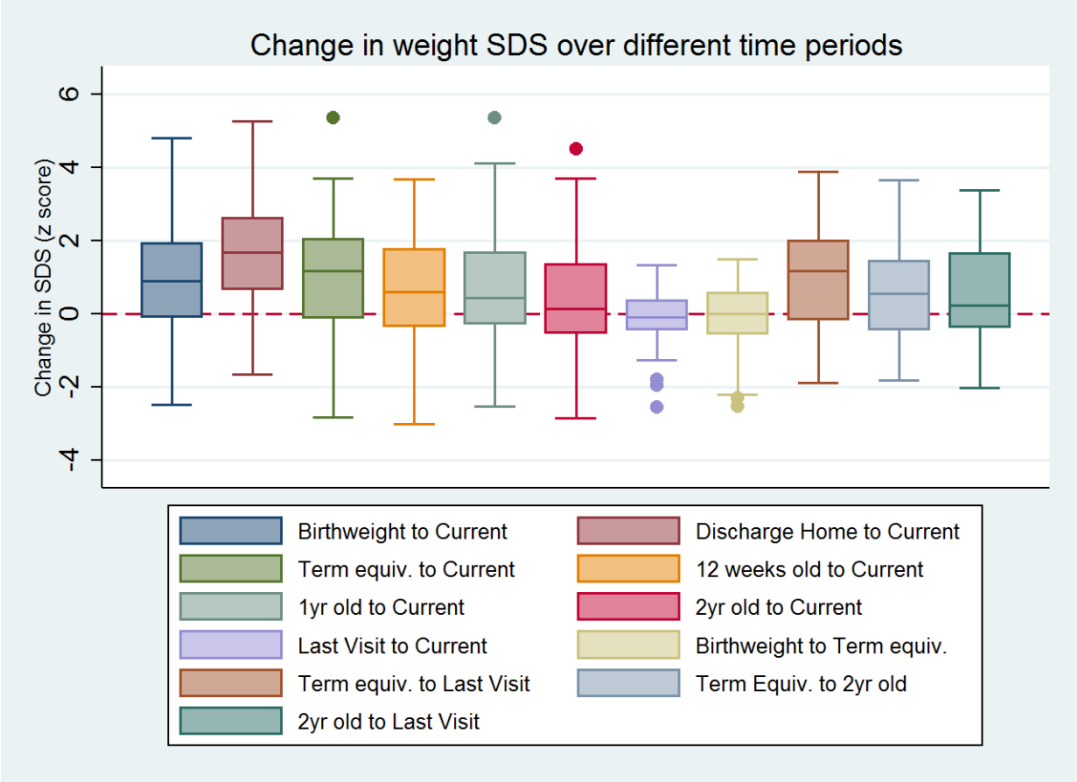

Figure 30: Changes in weight SDS over different time period in the current study cohort. 'Last visit': cohort aged 10-12yrs old. Box-plots show median, IQR.

Figure 30 shows different epochs of growth within the current study cohort. It was clear from this that to look at the cohort in terms of changing growth velocities, it would be important to assess change *between* calculated Wt. SDS change in epochs, in a longitudinal fashion rather than from any given time-point to the current study, in order to capture accurately any periods of rapid growth. Figure 31 shows this graphically and suggests that the major periods of growth are from term equivalent to 2 years and from 2 years to the last visit. However, there is little difference between the changes in the SDS score either between birth-to-term-equivalent, or from the last visit-to-current (p=0.89).

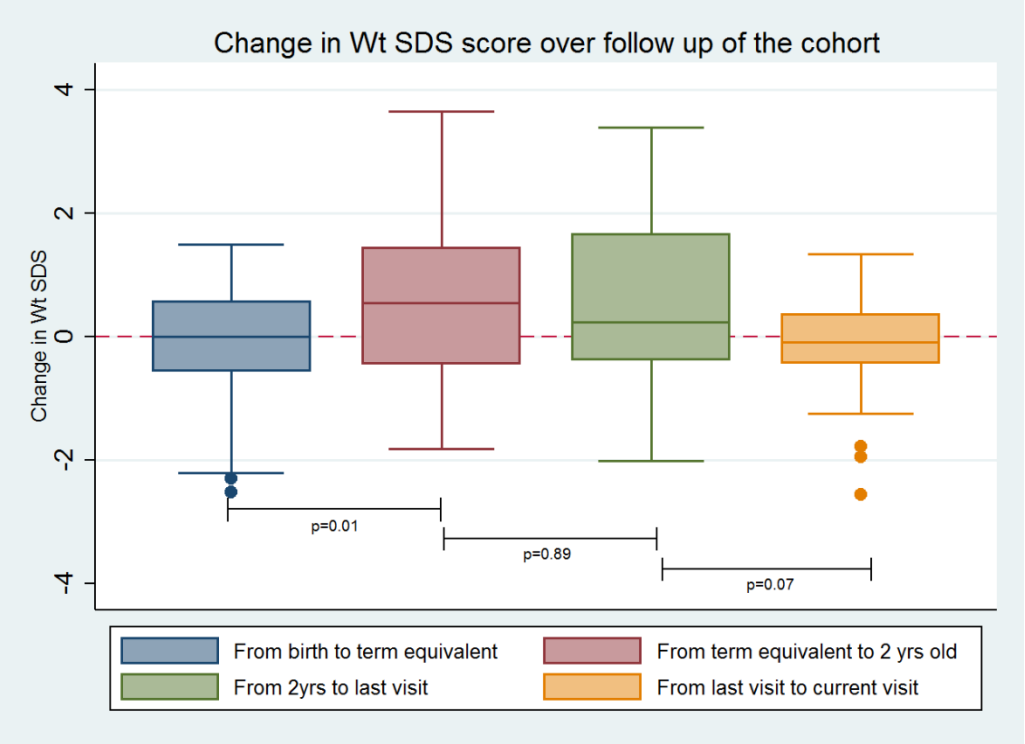

Figure 31: Change in weight SDS longitudinally in study cohort from recruitment to current study. Boxplot shows Median, IQR. *P* values are Mann Whitney U tests between epochs for SDS change across each epoch.

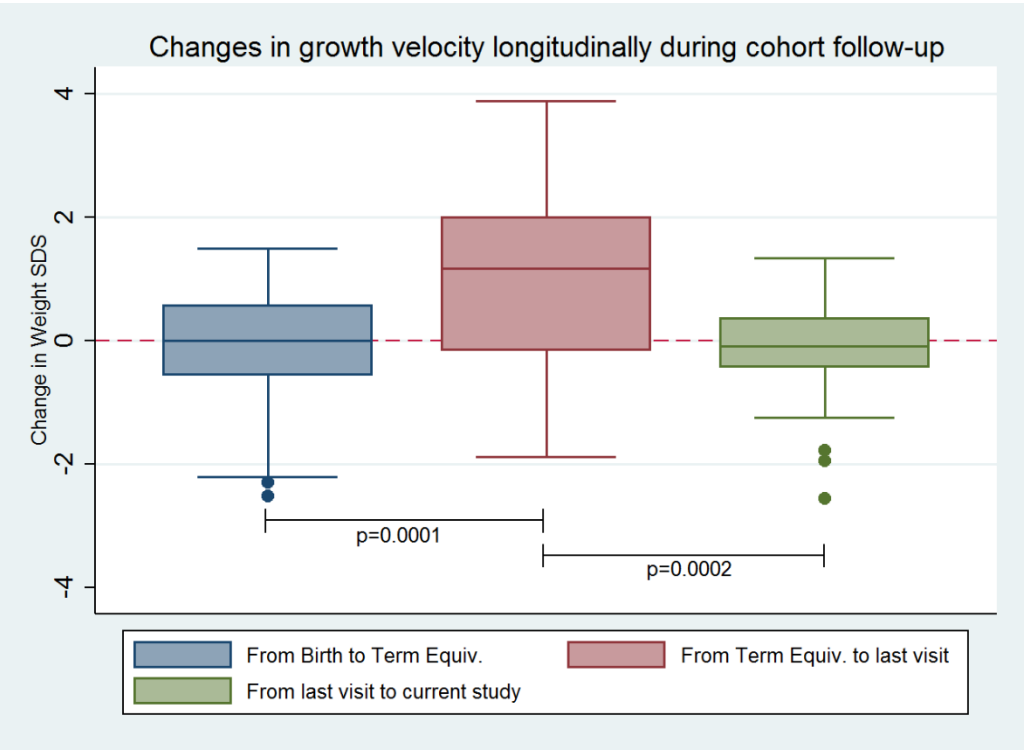

Figure 32: Change in weight SDS longitudinally in study cohort from recruitment to current study. Boxplot shows Median, IQR. *P* values are Mann Whitney U tests between epochs for SDS change across each epoch.

Figure 32 illustrates the same longitudinal change but combines the middle two epochs to give a 'term equivalent to last visit' epoch. From the data it was not possible to say a-priori whether the change in Wt. SDS between term-equivalent to 2 years old would be more important to IS measurement than the change from term equivalent to the last visit. As the change seen between 2yrs old to last visit was not significant when compared to the change from last visit to current visit, the suggestion was that the initial change in Wt. SDS from term equivalent to 2 years would be most likely to be important, but for completeness, term equivalent-to-last visit was also used in the analysis of IS measurements.

3.4.2 Insulin Sensitivity: correlation analysis

From the 60 participants in the study, 46 had blood taken to allow serum analysis. In 46, there was sufficient blood obtained to do a fasting HOMA %S calculation, and in 45 there was sufficient to get a 2-hour sample to allow calculation of the Matsuda index. One participant (study number GM01) was excluded due to an extremely high calculated sensitivity which was thought to be due to an erroneous insulin measurement, using the HOMA2 model. Thus 45 results were available for the HOMA%S and 44 for the Matsuda Index analyses.

Table 29 shows the results of Spearman's correlation analysis for both HOMA%S and the Matsuda Index in the study cohort. This was done to assess whether there were any significant correlations between insulin sensitivity and potentially clinically significant variables in the general areas of: auxological measurement; overall body composition (BODPOD™); measured lipid compartments; serum measurements; dietary intake; and directly measured activity. Unsurprisingly, Spearman correlation for HOMA%S against Matsuda index was highly significant (Rho: 0.66; $p<0.001$) as both use the fasting insulin/glucose measurements in their calculations (figure 33).

For correlation analysis against HOMA%S it was clear that, as with the data from the last study visit, the fasting insulin sensitivity was strongly correlated to current body composition (BMI SDS, p=0.01; Wt. SDS, p=0.02; total body fat, p=0.02; Fat Mass Index, p=0.01). HOMA%S was also seen to be associated with: body habitus (waist circumference, p=0.02); subcutaneous adipose tissue (cm^2 and index, cm^2/m^2; p<0.01);

serum lipids (cholesterol, triglycerides, LDL-C; all p<0.05); and serum vitamin D (p=0.02).

	HOMA % Sensitivity (Rho; *p*)	**Matsuda Index (Rho; *p*)**
Iotti-adjusted PCr recovery (τ½PCr)	0.15; 0.38	0.09; 0.61
Birth weight SDS	-0.03; 0.83	-0.17; 0.26
Gestational age at birth	-0.02; 0.88	-0.01; 0.96
Tanner Stage	-0.10; 0.52	-0.07; 0.67
Current Wt SDS	-0.34; **0.02****	-0.29; **0.06***
Wt SDS change [term to 2yr]	-0.37; **0.046****	-0.15; 0.43
Wt SDS change [term to last visit]	-0.19; 0.28	-0.11; 0.55
Intrahepatic Lipid (%)	-0.11; 0.49	-0.11; 0.50
Total body fat (%)	-0.36; **0.02****	-0.36; **0.02****
Fat mass index (BODPOD)	-0.39; **0.01****	-0.37; **0.01****
Fat-free mass index (BODPOD)	-0.09; 0.52	-0.08; 0.58
Waist Circumference	-0.34; **0.02****	-0.31; **0.04****
Mid-arm Circumference	-0.09; 0.55	-0.17; 0.28
Current BMI SDS	-0.37; **0.01****	-0.39; **0.008****
Visceral Adipose Tissue (cm^2)	-0.12; 0.45	-0.25; 0.11
Subcutaneous Adipose Tissue (cm^2)	-0.46; **0.002****	-0.41; **0.005****
VAT/SAT ratio	0.38; **0.01****	0.24; 0.12
VAT Index (cm^2/m^2)	-0.13; 0.41	-0.25; 0.11
SAT Index (cm^2/m^2)	-0.46; **0.002****	-0.43; **0.004****
Serum Cholesterol	-0.31; **0.04****	-0.15; 0.34
Serum Triglyceride	-0.50; **<0.001****	-0.49; **<0.001****
Serum LDL-C	-0.38; **0.01****	-0.19; 0.21
Serum Vitamin D	0.34; **0.02****	0.42; **0.005****

Dietary Energy (in 24 hours)	0.10; 0.52	0.24; 0.12
Carbohydrate intake (in 24 hours)	0.12; 0.43	0.23; 0.13
Fat intake (in 24 hours)	-0.01; 0.96	0.14; 0.38
Protein intake (in 24 hours)	0.21; 0.17	0.35; **0.02****
MVPA (mean minutes)	0.05; 0.79	0.12; 0.51
MVPA (mean % total activity)	-0.02; 0.90	0.07; 0.68
Sedentary activity (mean minutes)	0.19; 0.29	0.12; 0.50
Sedentary activity (mean % total)	-0.08; 0.66	-0.27; 0.12
Light activity (mean minutes)	0.25; 0.15	0.44; **0.01****
Light Activity (mean % total)	0.19; 0.28	0.41; **0.02****

Table 29: Spearman Correlation analysis for variables and either HOMA %S or Matsuda indices. LDL-C: low density lipoprotein C; MVPA: moderate-to-vigorous physical activity; for activity measures, mean (either minutes or % of total activity) is calculated over 3 days total data collection with minimum of 6 hours per day recorded. [*p<0.1; **p<0.05]

Those with measurements suggesting that they were overweight, or that had increased lipid deposition, had lower IS. There was no measurable association with intrahepatic lipid content (p=0.49) or visceral adipose tissue (p=0.45) which was surprising when considering the lipid hypothesis for reduced IS. For both, the direction of the correlation coefficient (Rho) did suggest that a lower lipid content (either IHL or VAT) was in that of improved insulin sensitivity but neither result was strong enough to consider a trend formed. Importantly, growth as indicated by change in Wt. SDS from term equivalent age to 2 years old was significantly correlated with measured IS (by HOMA%S, p=0.046) with the correlation coefficient suggesting that the smaller the change in Wt. SDS (i.e. the closer the SDS score was to staying the same between time-points) the more insulin sensitive the participant would be.

For Matsuda Index, there were significant changes in the profile of associated variables from that seen with HOMA%S. Current body composition variables were less significant or trending towards significance compared to HOMA%S (current Wt. SDS, p=0.06; total body fat, p=0.01; waist circumference, p=0.04; BMI SDS p=0.008) and

only serum triglycerides and Vitamin D levels were significant. Subcutaneous adipose tissue was also very significantly associated with the measured Matsuda Index and unlike the HOMA%S correlation, protein intake in 24 hours and light exercise also reached significance ($p<0.05$). Directly measured skeletal muscle oxidative capacity (adjusted τ½PCr) did not correlate with either measure of insulin sensitivity.

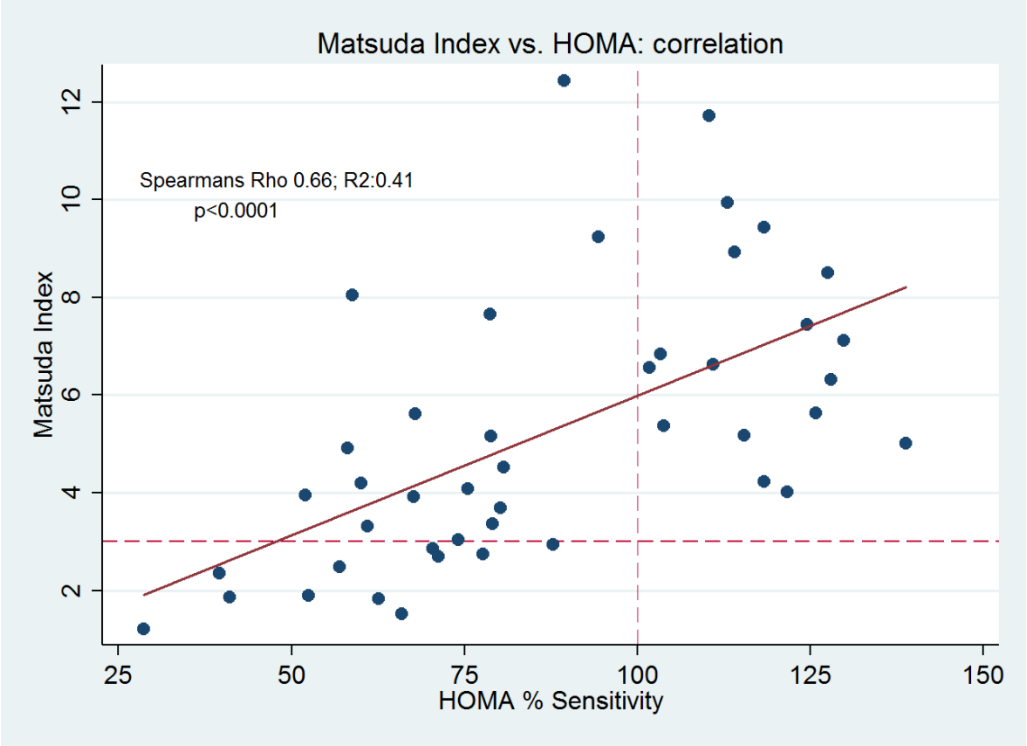

Figure 33: HOMA%S results plotted against Matsuda Index.

3.4.3 Insulin Sensitivity and Intrahepatic Lipid content

The lack of correlation between either IS measurement and intrahepatic lipid (IHL) content was an unexpected result. In order to examine this further, both measures of IS were plotted against intrahepatic lipid content (figures 34 to 36).

The plot of HOMA%S against IHL showed that while the majority of the cohort had clinically normal levels of intrahepatic lipid (IHL<5%) and a range of insulin sensitivities, there was subgroup of five individuals who appeared to have a high IHL content and low IS. When the same graph was plotted using the Matsuda Index, it could be seen that two of the low-IS/high-IHL individuals seemed to have a normal IS in terms of glucose disposal as measured by the Matsuda Index (i.e. an index >3). It was also clear that in the rest of the cohort there were some individuals who had reduced IS

measured by HOMA%S but normalised Matsuda Indices. This suggested that the resting IS did not necessarily equate to metabolic inflexibility in terms of coping with a glucose load and perhaps suggested that resting reduction in IS precedes reduced capacity to dispose of glucose in development of metabolic disease. In order to examine possible differences between these five low-IS/High-IHL individuals, subgroup analysis was completed (using Mann Whitney U tests) between the groups defined as IS<100%/IHL>5%, IS<100%/IHL<5% and IS>100%/IHL<5%.

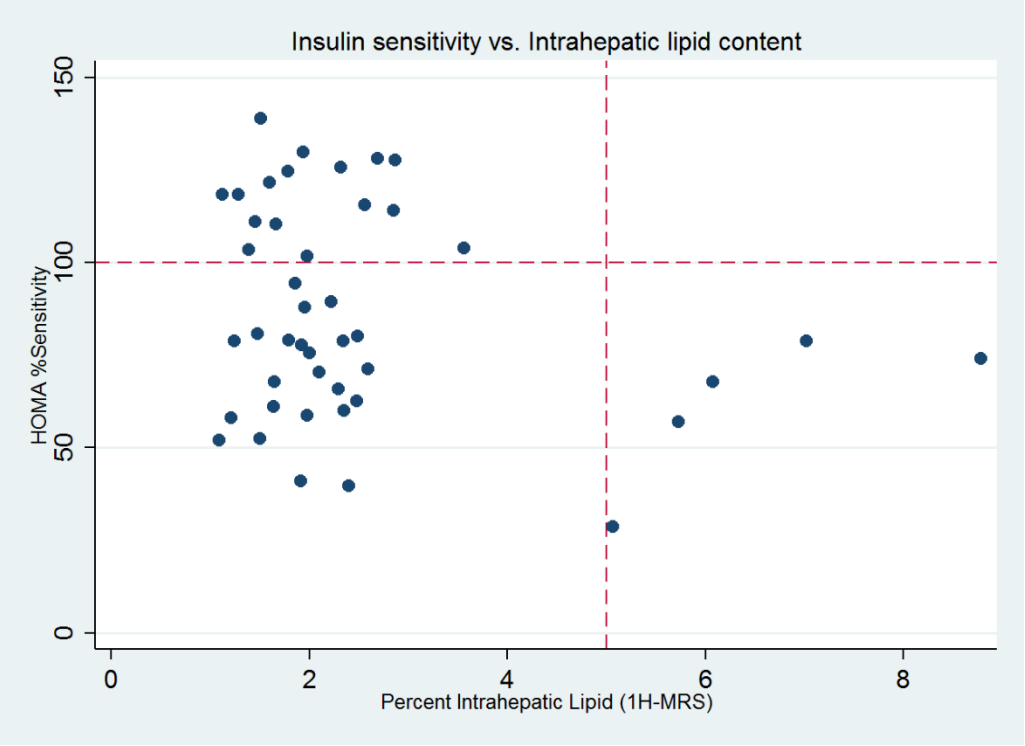

Figure 34: HOMA%S vs. Intrahepatic Lipid content

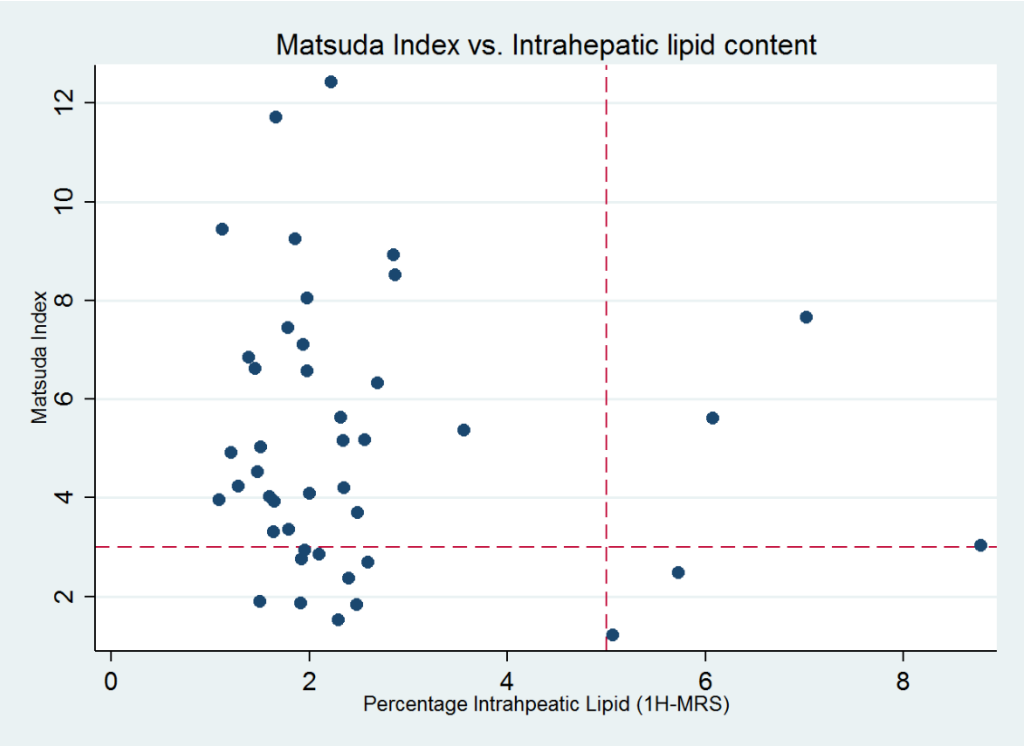

Figure 35: Matsuda Index vs. Intrahepatic lipid content

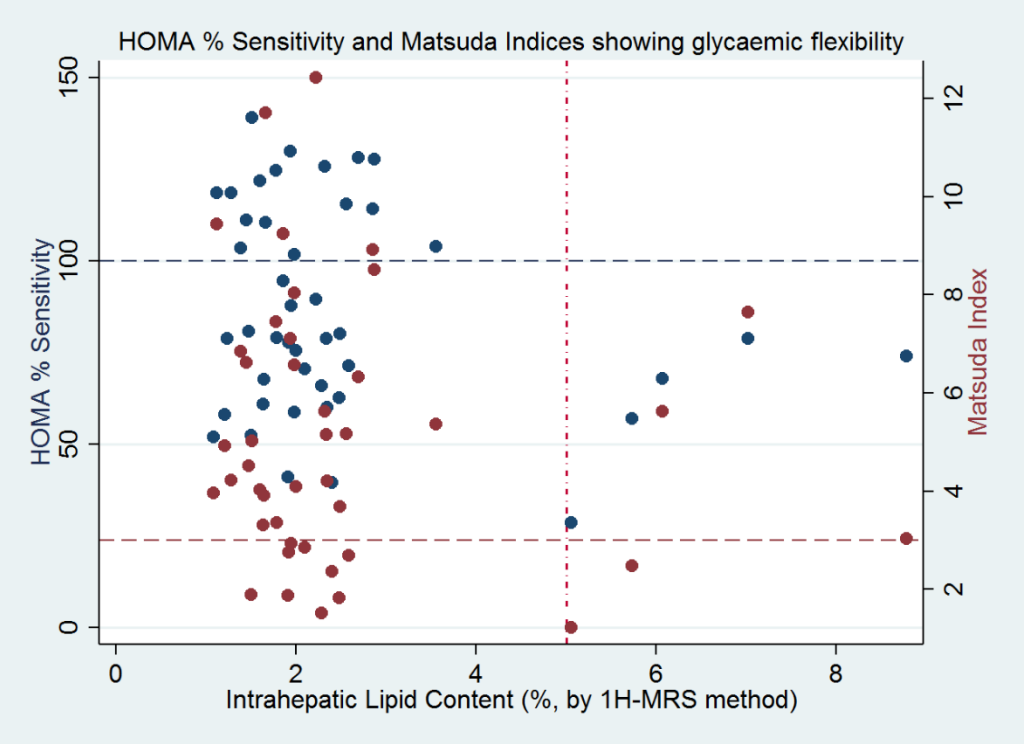

Figure 36: Matsuda Index and HOMA%S vs. Intrahepatic lipid content to show changes from low insulin sensitivity to normal Matsuda indices after administration of a glucose load

Within the 5 individuals who had IHL>5%/IS<100%, the two who 'normalised' their Matsuda Indices were at the opposite ends of the pubertal spectrum (1 and 5) and the remaining 3 were stages 3, 4, and 5. As there were only 3 remaining who were consistently of low IS (by both HOMA%S and Matsuda index) the subgroup analysis was done using the HOMA%S values to see if there were any particular differences between the outlying individuals (n=5) and the remainder of the cohort. The results of the subgroup analysis are listed in table 30. The subgroup analysis also included the comparison of all individuals with IHL<5% divided into two groups based on IS >100% or <100%.

Compared to the remainder of the cohort as a group, the 5 individuals with IHL>5% had universally raised serum lipids, higher indices for overweight/obesity (raised BMI SDS, waist circumference and Wt. SDS, $p<0.05$), evidence of higher lipid deposition across compartments (especially visceral adipose deposition, $p=0.007$) but were not significantly different with respect to vitamin D status or Tanner Stage. These findings are in line with the analysis looking at IHL and visceral adiposity presented in section 3.2.

When the five individuals with IHL>5% are compared to the cohort subgroup with IS>100% and IHL<5% there is the same pattern of results seen as when comparing them to the remainder of the cohort as a whole, but the significance is more marked. The five individuals, compared to this 'healthy' subset have increased lipid deposition measured by both auxological and body composition techniques (FMI, BMI [SDS], waist circumference, Wt. SDS) and direct measurement of lipid deposition (visceral [$p=0.017$] and subcutaneous [$p=0.02$]). They also have higher serum lipid content ($p\leq0.03$).

	IS<100%/IHL>5% vs. rest of cohort	**IS<100%/IHL>5% vs. IS<100%/IHL <5%**	**IS<100%/IHL>5% vs. IS>100%/IHL<5%**	**IS<100%/IHL<5% vs. IS>100%/IHL<5%**
Iotti-adjusted PCr recovery (τ½PCr)	0.61	0.74	0.64	0.39
Birth weight SDS	0.96	0.53	0.77	0.98
Gestational age at birth	0.37	0.22	0.36	0.90
Tanner Stage	0.79	0.85	0.70	0.61
Current Wt SDS	**0.03****	0.11	**0.03****	0.40
Wt SDS change [term to 2yr]	0.62	0.59	0.70	**0.046****
Wt SDS change [term to last visit]	0.71	0.79	0.71	0.58
Total body fat (%)	0.08	0.27	**0.04****	**0.02****
Fat mass index (BODPOD)	0.06	0.23	**0.02****	**0.02***
Waist Circumference	**0.02****	**0.04****	**0.02****	0.27
Mid-arm Circumference	0.19	0.25	0.28	0.62
Current BMI SDS	**0.02****	**0.07***	**0.02****	0.22
Height SDS	0.11	0.22	0.16	0.54
Visceral Adipose Tissue (cm^2)	**0.007****	**0.02****	**0.02****	0.97
Subcutaneous Adipose Tissue (cm^2)	**0.04****	0.14	**0.02****	**0.01****
VAT/SAT ratio	0.70	0.35	0.46	**0.02****
VAT Index (cm^2/m^2)	**0.008****	**0.04****	**0.01****	0.82

SAT Index (cm^2/m^2)	**0.07***	0.29	**0.02****	**0.008****
Serum Cholesterol	**0.02****	**0.05****	**0.03****	**0.01****
Serum Triglyceride	**0.002****	**0.004****	**0.002****	**0.01****
Serum LDL-C	**0.01****	**0.03****	**0.007****	**0.01****
Serum Vitamin D	0.34	0.79	**0.09***	0.12
MVPA (mean minutes)†	**0.053***	0.11	0.31	0.24
MVPA (mean % total activity)†	**0.04****	**0.06***	**0.07***	0.53
Sedentary activity (mean minutes)†	**0.04****	**0.06***	**0.02****	0.13
Sedentary activity (mean % total)†	**0.02****	**0.01****	**0.05***	0.24
Light activity (mean minutes)†	0.29	0.17	0.59	**0.03****
Light Activity (mean % total)†	**0.03****	**0.01****	**0.10***	0.19

Table 30: Comparisons between groups within the cohort defined by Insulin sensitivity (HOMA % Sensitivity) and Intrahepatic Lipid levels. Small groups (n=3) defined by IS (<100%, >100%) or IHL (<5%, >5%). Mann Whitney U test used to assess differences between groups. †Activity measures: it should be noted that for comparisons involving the IS<100%/IHL>5% group, there are only n=3 out of 5 possible datasets that could be used (vs. up to n=41 for the rest of the cohort). [*P<0.1**P<0.05]

The final analysis was comparison between the halves of the cohort with IHL<5% who were IS>100%, and <100%, to see if there were any differences that could be detected as possible precursors for developing low HOMA%S. In this comparison the group with IS<100% had significantly higher fat mass index (p=0.02) and higher subcutaneous adipose tissue deposits (SAT index [cm^2/m^2], p=0.008), as well as higher circulating lipids. In this comparison, early growth again seemed to be important with those who were less insulin sensitive having had a higher change in Wt. SDS between term-equivalent and 2 years old (p=0.046). Unlike the general Spearman correlation analysis for the HOMA%S measures, in this subgroup analysis, it was also shown to be significant that those who have a higher IS are more likely to spend a greater percentage of their activity in light activity than those with lower IS (p=0.03).

Within the first three comparisons, table 30 lists the analysis of activity between the various groups, but there are only 3 out of the 5 with IHL>5% who completed wearing the accelerometers satisfactorily to produce comparable data. Thus, while the data for the comparison of IHL<5% but IS >100% vs. IS<100% is useable and produced a valid result for light exercise, the results for the other three comparisons cannot be meaningfully interpreted due to the tiny sample size.

For the final comparison (all those with IHL<5% and IS measured by HOMA%S), Fisher's exact test was also performed to see if the original study grouping (growth study vs. protein study) had any significant effect on whether the participants had HOMA%S above or below 100%. The two-tailed result showed p=1 and one tailed test had a p=0.59, showing that there were no significant effects in the study cohort with respect to HOMA%S, originating from their original study recruitment.

	HOMA % Sensitivity Q1 vs. Q4; *p* value	**Matsuda Index Q1 vs. Q4; *p* value**
Iotti-adjusted PCr recovery (τ½PCr)	0.62	0.85
Birth weight SDS	0.71	0.92
Gestational age at birth	0.90	0.65
Intrahepatic Lipid (%)	0.95	0.44
Tanner Stage	0.45	0.56
Current Wt SDS	0.13	0.14
Wt SDS change [term to 2yr]	**0.04****	0.24
Wt SDS change [term to last visit]	0.35	0.31
Total body fat (%)	0.12	**0.09***
Fat mass index (BODPOD)	**0.08***	**0.09***
Waist Circumference	0.11	**0.052***
Mid-arm Circumference	0.85	0.39
Current BMI SDS	**0.08***	**0.04****
Height SDS	0.23	0.31
Visceral Adipose Tissue (cm^2)	0.67	**0.07***
Subcutaneous Adipose Tissue (cm^2)	**0.04****	**0.04****
VAT/SAT ratio	**0.09***	0.38
VAT Index (cm^2/m^2)	0.67	**0.07***
SAT Index (cm^2/m^2)	**0.04****	**0.04****
Serum Cholesterol	0.28	0.67
Serum Triglyceride	**0.006****	**0.007****
Serum LDL-C	**0.07***	0.41
Serum Vitamin D	**0.045****	**0.005****
MVPA (mean minutes)†	0.56	0.51

MVPA (mean % total activity)†	0.44	0.63
Sedentary activity (mean minutes)†	0.17	0.57
Sedentary activity (mean % total)†	0.84	0.102
Light activity (mean minutes)†	0.14	**0.02****
Light Activity (mean % total)†	0.44	**0.02****

Table 31: Comparison between most (Q4) and least (Q1) insulin sensitive quartiles within the cohort for both HOMA % Sensitivity and Matsuda indices. Comparisons done with Mann Whitney U tests. [*p<0.1,**p<0.05]

The comparison excluding the five subjects with IHL>5% spanned a range of IS and did not include the Matsuda Indices. Therefore, to interrogate the extremes of the data further for potential clarification of factors which might predict IS, the HOMA%S and Matsuda data (excluding the five with IHL>5%) was split into quartiles to allow comparison of those subjects with the highest and lowest IS for both measures. Table 31 shows the comparisons of the most (Q4) and least (Q1) sensitive subjects for both HOMA%S and Matsuda Index compared to the other measured variables. Common to both measures of IS were differences between the extreme quartiles for body composition (FMI, BMI SDS), adipose tissue deposition (subcutaneous) and serum triglyceride levels. Between the extreme quartiles in these measures, a tendency for increased adiposity was associated with reduced insulin sensitivity. Vitamin D level were also significantly different between Q1 and Q4 in both indices (p=0.045). Specific to HOMA%S, serum LDL-C levels were different between the two groups (with a tendency to higher levels in Q1) and change in Wt. SDS was also significant, as it had been in comparison involving all with IHL<5%. Specific to the Matsuda Index measurements, light exercise (absolute minutes and % of total daily activity) was significant with higher % in light exercise and higher absolute minutes associated with the higher IS in Q4. Visceral adiposity and protein intake trended towards significance (p<0.1 >0.05) with those in Q1 having greater visceral adipose deposits and lower protein intake in their diet.

	HOMA % Sensitivity			Matsuda Index		
	Corr Coeff	**R^2**	***P***	**Corr Coeff**	**R^2**	***P***
Current Wt SDS	-11.6	0.19	**0.003****	-1.0	0.15	**0.01****
Wt SDS change [term to 2yr]	-7.3	0.09	0.105	-0.33	0.02	0.40
Wt SDS change [term to last visit]	-3.8	0.04	0.23	-0.13	0.005	0.70
Total body fat (%)	-17.2	0.16	**0.006****	-1.4	0.11	**0.03****
Fat mass index (BODPOD)	-15.0	0.17	**0.004****	-1.3	0.13	**0.02****
Waist Circumference	1288	0.14	**0.01****	118	0.12	**0.02****
Current BMI SDS	-10.1	0.16	**0.007****	-0.94	0.14	**0.01****
Visceral Adipose Tissue (cm^2)	-6.16	0.02	0.38	-1.24	0.09	**0.058***
Subcut' Adipose Tissue (cm^2)†	264	0.19	**0.003****	23.3	016	**0.009****
VAT/SAT ratio	16.3	0.17	**0.006****	0.86	0.05	0.16
VAT Index (cm^2/m^2)†	20.0	0.005	0.65	6.76	0.062	0.11
SAT Index (cm^2/m^2)	-15.9	0.21	**0.002****	-1.43	0.17	**0.006****
Serum Cholesterol	590.1	0.12	**0.02**	24.3	0.02	0.34
Serum Triglyceride	-34.4	0.28	**<0.001****	-3.1	0.25	**<0.001****
Serum LDL-C	-52.2	0.22	**0.001****	-2.2	0.04	0.19
Serum Vitamin D	25.9	0.13	**0.01****	2.7	0.15	**0.008****
Protein intake (in 24 hours)	3.74	0.06	0.10	0.57	0.15	**0.01****
Light activity (mean minutes)	0.18	0.11	0.06	0.03	0.24	**0.004****
Light Activity (mean % total)	1.14	0.05	0.20	0.25	0.19	**0.01****

Table 32 (previous page): Univariate linear regression analysis of variables found to be significant using Spearman's correlation. Any non-normally distributed data log-transformed for analysis, with exception that †: data normalised by inverse of SQRT before regression therefore direction of correlation coefficient reversed.
[*p<0.1,**p<0.05]

	Model 1	**Model 2**	**Model 3**
Fat Mass Index	0.19	0.20	0.24
Current weight SDS	0.19	0.20	0.16
BMI SDS	0.67	0.56	
Waist Circumference	0.74		
R^2	0.22	0.22	0.21
Adjusted R^2	0.14	0.16	0.17
p value (for model)	0.036	0.016	0.007

Table 33: HOMA % Sensitivity multivariate regression modelling (model A): body composition measures (table shows *p* values for individual components of model)

	Model 1
SAT index	0.001
VAT index	0.168
R^2	0.24
Adjusted R^2	0.21
p value (for model)	0.003

Table 34: HOMA % Sensitivity multivariate regression modelling (model B): measured abdominal fat, indexed against height (table shows *p* values for individual components)

	Model 1	Model 2
Cholesterol	0.67	
Triglyceride	0.02	0.02
LDL-C	0.10	0.04
Vitamin D (total)	0.03	0.03
R^2	0.43	0.42
Adjusted R^2	0.36	0.38
p value (for model)	<0.001	<0.001

Table 35: HOMA % Sensitivity multivariate regression modelling (model C): measured serum lipids and vitamin D (table shows *p* values for individual components of model)

	Model 1	Model 2	Model 3	Model 4	Model 5
Fat Mass Index	0.24	0.11	0.03	0.048	0.02
Triglyceride	0.009	0.004	0.001	0.002	0.001
Vitamin D (total)	0.10	0.10	0.09	0.10	
Wt SDS change [term to 2yr]	0.25	0.17	0.16		
LDL-C	0.31	0.28			
SAT index	0.69				
R^2	0.59	0.59	0.57	0.42	0.37
Adjusted R^2	0.47	0.50	0.50	0.37	0.34
p value (for model)	0.002	<0.001	<0.001	<0.001	<0.001

Table 36: HOMA % Sensitivity multivariate regression modelling (Final model [D]): Significant variables for predicting Insulin Sensitivity by HOMA % Sensitivity. Fat mass index used as significant body composition variable (table shows *p* values for individual components of model).

	Model 1	Model 2	Model 3	Model 4
Triglyceride	0.02	0.01	0.04	0.02
Vitamin D (total)	0.15	0.15	0.11	0.03
LDL-C	0.18	0.10	0.05	0.039
Current Weight SDS	0.54	0.17	0.11	
Wt SDS change [term to 2yr]	0.39	0.27		
SAT index	0.73			
R^2	0.57	0.58	0.46	0.42
Adjusted R^2	0.45	0.48	0.40	0.38
p value (for model)	0.004	<0.001	<0.001	<0.001

Table 37: HOMA % Sensitivity multivariate regression modelling (Final model [E]): Significant variables for predicting Insulin Sensitivity by HOMA % Sensitivity. Current Weight SDS used as significant body composition variable (table shows *p* values for individual components of model).

Once the analysis of the data using extreme quartiles was performed, the most significant variables (both as per analysis and in clinical terms) were examined using linear regression. Where necessary the variables were normalised with log transformation to allow analysis. The results of the univariate analysis are shown in table 32.

From these results, significant variables were modelled together for both the HOMA%S and Matsuda indices to give a final model for each. The process of modelling for HOMA%S is shown in tables 33 to 35. In these models, neither fat mass index nor current weight SDS reached significance as an individual term but both are clinically potentially important and so were included in final modelling (table 36, 37). When FMI is included in the model (table 36) for HOMA%S it is a significant term. With serum triglyceride, it accounts for nearly 40% of the variance seen in the measurement of HOMA%S after removal of non-significant co-variables. Importantly for our cohort, this suggests that intervention might be possible to modify HOMA%S through these

indices. For both FMI and serum triglyceride the correlation is negative, i.e. the lower the FMI or serum triglyceride, the better the IS.

As current Wt. SDS was also identified as significant in the earlier correlation analysis it was used as a variable in this multivariate modelling (table 37) but when the model is pared down removing non-significant variables, it is lost to a final model involving only serum measures. Interestingly Vitamin D is left in this model but disappears from the previous one, suggesting that it is associated with FMI and Wt. SDS, being more important than current Wt. SDS in IS, but less so than FMI. In the second model, therefore, it is almost a 'proxy' measure for FMI.

The same process was executed for the results from the Matsuda Index data, using modelling around similar variables to pick the most significant and then putting them into a single multivariate model, refined by removing non-significant terms (tables 38 to 41). The final models were then constructed using the significant variables and, as with the HOMA%S modelling, both FMI and current Wt. SDS were used (tables 41 and 42). Both processes left the same final model with serum triglyceride and mean minutes in light activity being most predictive of a glucose disposal (i.e. IS by Matsuda Index). The correlation coefficients showed that the more light exercise, the higher the Matsuda Index (IS) was, and the higher the serum triglyceride levels, the lower the IS as seen in the HOMA%S modelling.

	Model 1	**Model 2**	**Model 3**
Fat Mass Index	0.46	0.39	0.38
Current weight SDS	0.49	0.35	0.20
Waist Circumference	0.89	0.91	
BMI SDS	0.93		
R^2	0.16	0.16	0.16
Adjusted R^2	0.07	0.10	0.12
p value (for model)	0.13	0.07	0.03

Table 38: Matsuda Index multivariate regression modelling (model F): body composition measures (table shows *p* values for individual components of model)

	Model 1
SAT index	0.03
VAT index	0.79
R^2	0.17
Adjusted R^2	0.13
p value (for model)	0.02

Table39: Matsuda Index multivariate regression modelling (model G): measured abdominal fat, indexed against height (table shows *p* values for individual components)

	Model 1
Triglyceride	0.002
Vitamin D (total)	0.02
R^2	0.35
Adjusted R^2	0.32
p value (for model)	<0.001

Table 40: Matsuda Index multivariate regression modelling (model H): measured serum triglycerides and vitamin D (table shows *p* values for individual components of model)

	Model 1	**Model 2**
Light Activity Mean (Total Minutes/day)	0.17	0.006
Protein Intake (g in 24 hours)	0.08	0.08
Light Activity Mean (percent of all activity/day)	0.94	
R^2	0.31	0.31
Adjusted R^2	0.24	0.26
p value (for model)	0.01	0.004

Table 41: Matsuda Index multivariate regression modelling (model I): Activity and dietary intake (table shows *p* values for individual components of model)

	Model 1	Model 2	Model 3	Model 4	Model 5
Triglyceride	0.007	0.004	0.003	0.03	0.001
Vitamin D (total)	0.22	0.19	0.21	0.14	0.012
Light Activity Mean (Total Minutes/day)	0.09	0.08	0.02	0.01	
Fat Mass Index	0.32	0.35	0.33		
SAT index	0.57	0.66			
Protein Intake (g in 24 hours)	0.69				
R^2	0.55	0.54	0.56	0.54	0.50
Adjusted R^2	0.42	0.44	0.49	0.49	0.46
p value (for model)	0.004	0.002	<0.001	<0.001	<0.001

Table 42: Matsuda Index multivariate regression modelling (Final model [J]): Significant variables for predicting Insulin Sensitivity by Matsuda Index. Fat mass index used as significant body composition variable (table shows p values for individual components of model).

	Model 1	Model 2	Model 3	Model 4	Model 5
Triglyceride	0.008	0.006	0.003	0.003	0.001
Light Activity Mean (Total Minutes/day)	0.08	0.03	0.02	0.01	0.012
Vitamin D (total)	0.32	0.31	0.25	0.14	
Current Wt SDS	0.39	0.31	0.29		
Protein Intake (g in 24 hours)	0.60	0.67			
SAT index	0.74				
R^2	0.54	0.57	0.56	0.54	0.50
Adjusted R^2	0.41	0.47	0.49	0.49	0.46
p value (for model)	0.005	<0.001	<0.001	<0.001	<0.001

Table 43: Matsuda Index multivariate regression modelling (Final model [K]): Significant variables for predicting Insulin Sensitivity by Matsuda Index. Current Weight SDS used as significant body composition variable (table shows *p* values for individual components of model).

For both of the 'final' HOMA%S multivariate models and the single, final, multivariate model for Matsuda Index, the models were tested for heteroskedasticity before and after adjustment for Tanner Stage. Inclusion of Tanner Stage did not alter any of the final models (the difference between models with or without Tanner Stage was insignificant on likelihood ratio testing) and there was no heteroskedasticity, suggesting that the terms included in the final models were independent.

3.4.4 Discussion

When considering the analysis of IS, the overall aim was perhaps to try to discern both whether there was any detectable correlation with preterm birth that may have influenced IS measured later in life, and also what other factors associated IS were at work, with a view to potential therapeutic intervention. As the logic model generated from the literature review showed (Tinnion *et al.*, 2014), the suggestion is that the further on those who are born preterm are from infancy, the less the measurable effect of prematurity, low birthweight or rapid growth is. In the current cohort, there was no correlation at all between gestational age, birthweight SDS, and the original study enrolment (growth or protein study), and the measured IS by either method.

In this study the different correlation analyses showed that for both methods of IS measurement, current body habitus is an important determinant of IS: the more 'adipose' the body habitus (high fat mass, large waist circumference, high BMI SDS) the lower the insulin sensitivity. This was in keeping with the findings from the last study visit the cohort undertook (Wood *et al.*, 2013) and the wider literature which suggests their influence over IS increases with chronological age (Tinnion *et al.*, 2014).

It was unexpected that there was no correlation between IHL content and measured IS. The literature around the 'lipid hypothesis' of reduced IS would have suggested that hepatic lipid deposition would be related to measured IS. Examination of the correlation between IHL and IS identified a subgroup of adolescents with both above 'normal' IHL (clinically significant levels are considered to be >5%) and an IS of less than 100%. The majority of the cohort had low (<5%) and so this analysis allowed the opportunity to examine whether there was anything that could predict development of an IHL>5%/low IS status, as well as look for differences between those with normal IHL and high or low IS. The analysis showed that at low IS, the differences between the

groups with IHL above or below 5% were related mainly to lipid deposition as found earlier in section 3.2 (visceral adipose tissue and serum lipids, especially triglycerides). There was little difference in body composition. However, when comparing the five individuals with high IHL to the individuals with IS>100% and low IHL, this 'healthy' subgroup were leaner and had less subcutaneous fat, in addition to reduced visceral adipose tissue. They also had lower levels of serum lipids. These results all fit with the observation that 'acquired metabolic changes' over time have a big influence on IS. They also suggest that there may be opportunity for intervention during the childhood years that might move children towards a metabolically healthy phenotype and modify positively ongoing metabolic development.

When comparing those with different IS (by HOMA%S) but IHL<5% (both whole group and highest/lowest quartile for IS) similar trends were found as between the five 'outliers' and the 'healthy' subgroups, but the quartile analysis also allowed comparison within the Matsuda Index measurements. As HOMA%S is essentially a fasting state measurement and Matsuda Index is a measure of glucose disposal (in this study, over a two hour oral glucose tolerance test), it would be expected that there would be a difference in the factors influencing the measured IS. The initial graphing of IHL vs. IS suggested that even in those with high IHL and low IS, there was still a measurable degree of metabolic flexibility such that of the five individuals who had low IS by HOMA%S with IHL>5%, when given a glucose load, two recorded normal Matsuda indices suggesting an ability to cope with the glucose load. In the general cohort, this was also seen in a number of individuals with HOMA%S of <100% and IHL<5% who subsequently had normalised Matsuda Indices. This can be interpreted as a demonstration of functional metabolic flexibility despite reduced fasting IS. Importantly, when considering these results, there was no discernible effect from pubertal stage in any of the modelling. Examination of the highest and lowest quartiles showed some commonality of influence on IS between the two methods: both showed a trend towards significance for FMI and BMI SDS, and triglyceride was significantly associated with both.

Low vitamin D was also significantly associated with reduced IS by both methods. The significance of triglycerides was not surprising as there are documented physiological mechanisms by which they can directly affect IS. Vitamin D has also been shown to be

lower in obese individuals, and in those born preterm, and this might explain the correlation seen. In other populations there is a well-documented association between reduced vitamin D and markers of reduced IS (Chiu *et al.*, 2004; Forouhi *et al.*, 2008; Liu *et al.*, 2009a; Baz-Hecht and Goldfine, 2010)] Some studies with vitamin D replacement have claimed to improve insulin sensitivity (Belenchia *et al.*, 2013; Kelishadi *et al.*, 2014) and so there may be a direct mechanism at work between IS and Vitamin D levels in both the most and least insulin-sensitive individuals.

The question of whether there is an in-vivo link between vitamin D and insulin secretion and action is not yet resolved. The PROspective Metabolism and ISlet cell Evaluation (PROMISE) study(Kayaniyil *et al.*, 2011) reported associations in univariate analysis between baseline vitamin D status and progression to dysglycaemic states (diabetes, impaired fasting glucose or reduced glucose disposal) in an over-50 year old population, but not in longitudinal multivariate analysis. In an earlier paper(Kayaniyil *et al.*, 2010) the same authors had presented results to suggest that in a cross-sectional study of individuals at risk of T2DM there was an association with vitamin D levels and both fasting insulin sensitivity (measured by HOMA) and glucose disposal (measured by Matsuda index) even after adjusting for sociodemographic factors, physical activity, supplement use, parathyroid hormone, and BMI. Rat studies involving induction of diabetic-like states by destruction of islet cells (by streptozotocin administration in vivo), followed by dietary supplementation of vitamin D, found upregulated transcription and expression of glutamatergic receptors in those animals who had been given the vitamin D supplement(Jayanarayanan *et al.*, 2015). In rats, these receptors increased the calcium-mediated release of insulin and the authors postulate a role for them in the 'antidiabetic' actions of vitamin D seen in the experiment. Incubation of untreated, rat, islet cells with vitamin D for an hour resulted in increased glucose-stimulated insulin secretion in-vitro(Jeddi *et al.*, 2015). In humans, the presence of the vitamin D receptor in pancreatic tissue and the vitamin D response element in the insulin gene promoter region, with evidence of transcriptional activation caused by vitamin D(Maestro *et al.*, 2003) also strengthen the possibility that vitamin D may play an in-vivo role in insulin homeostasis.

The presence of individuals who were: insulin sensitive by both measures with IHL<5%; insulin sensitive by Matsuda indices but not by HOMA%S with IHL<5%; and three who

had low IS by both methods and IHL>5%; suggests that there may be a metabolic progression from 'normal' to 'functionally metabolically flexible' to 'metabolically inflexible'. The data do not give any answers as to what exact changes occur at each step, but they would suggest that the first changes are related to increased adiposity in body habitus, total fat and subcutaneous deposition of fat (which reduce resting IS), then an increase in serum lipids and visceral adipose tissue (which then leads to increased IHL). The fact that individuals could have IS above and below 100% despite IHL<5% would suggest that clinically high IHL is not a mandatory determinant of IS in these individuals and that the two may be 'uncoupled'. However, it is also possible that there is a threshold effect beyond which IHL begins to accumulate and then begins to contribute to overall IS. No individual had IHL>5% and resting IS>100% suggesting that IHL follows IS, not the other way round. The lack of direct association between IHL and IS is also, perhaps, a reason to revisit the possibility of looking at intra- and extra-myocellular lipid and seeing if there is a similar distribution to IHL, or whether it shows a relationship to IS in a more direct fashion.

There was also the finding that some individuals retained functional flexibility, when faced with a glucose load, to explain. In the quartile analysis, the data suggested that individuals with higher levels of light exercise and lower levels of visceral adipose tissue had higher Matsuda Indices and therefore it may be that this type of exercise is protective, even if there is IHL>5%. Similarly, adopting a lifestyle which reduces the likelihood of developing visceral adipose tissue deposition may help improve metabolic flexibility.

Quartile analysis also showed that early life growth was different between the most and least insulin sensitive individuals, measured by HOMA%S, with those who had greatest change in Wt. SDS from term equivalent age to 2 years old being least sensitive to insulin. This suggests that there is a degree of metabolic programming by early life growth involved in the difference between the most and least sensitive, but when introduced as a variable in multivariate modelling, it loses its significance compared to other contemporary variables. This univariate significance of early growth for IS becoming less significant through the life-course is in keeping with the published literature.

Multivariate analysis confirmed that IS was strongly related to fasting triglyceride levels in both HOMA%S and Matsuda calculations. The analysis produced two potential models for HOMA%S, one which included fat mass index and one based on serum measurements (triglyceride, LDL-C and Vitamin D), in addition to triglycerides. From a clinical perspective, the model with fat mass index is more relevant as it takes account of the changes in metabolic status leading to reduced IS that are suggested by the quartile and subgroup analysis. It is also more elegant because the process of generating the model with fat mass index involved inclusion of vitamin D and LDL-C (the other significant terms for the three-variable HOMA%S model) and fat mass index was more significant than either of the other terms. The multivariate model for Matsuda Index (consistently) suggested that the number of minutes in light activity was important in predicting the glucose disposal after loading, thus IS, alongside serum triglyceride levels: longer in light exercise was proportional to improvement in Matsuda Index (i.e. IS). This would provide a good potential target for intervention, and it is notable that it is light exercise that seems to have the beneficial effect: as such this might be more palatable as an intervention for health than one which required regular MVPA. Light exercise, which might be considered conditioning for skeletal muscle function, over longer periods, may have a specific effect on the skeletal muscle IS. This would explain why it is associated with disposal of glucose after a load (not the fasting IS) and also why two individuals with IHL>5% and IS<100% can have normal Matsuda Indices. In line with some of the research into type 2 diabetes which showed that reduced IS in skeletal muscle in type 2 diabetes was not secondary to a mitochondrial function issue (Lim *et al.*, 2011c) (but probably more to do with glucose uptake into the myocyte) there was no association between oxidative function and IS in this analysis.

Overall, the results for analysis of IS in the study cohort suggest that while there is no discernible link to prematurity or birthweight, early growth may have some bearing on an individual's fasting insulin sensitivity. However, when contemporary variables are taken into account, its significance is greatly reduced. This is in keeping with the published literature. In general, the two different measures of IS show that there are some body composition and habitus variables which influence IS across both measurement techniques, but there is a difference in the flexibility with which a

glucose load can be tolerated. As such, the influences on this ability are different to those governing the baseline IS in any individual. Aside from interventions designed to improve baseline IS by reducing circulating lipids, reducing deposition of adipose tissue and (possibly) optimising vitamin D levels, the results also suggest that targeting at-risk individuals with light activity interventions might help preserve metabolic flexibility even when baseline IS is reduced.

3.5 Future Studies: cohort opinion on lifestyle intervention

At the end of each study visit, the volunteers were given a questionnaire with ten options for interventions that could be used in a future intervention study. The intervention would be designed to be minimally intrusive to family life but be well adhered to, in order to try to improve lifestyle in children born preterm. In an intervention study, the current study protocol (or modified version thereof) would be used to assess progress over time. On the questionnaire, each intervention was scored on a Likert-style rating scale (from 0 to 10; 10 = most desirable; see page 162)

3.5.1 Questionnaire results

Option for intervention	Mean	SD	Mode	**Median**	**IQR**
'one to one' with dietician	6.89	2.19	8	**7.0**	**5.4 to 8**
Wii fit and weekly coach	7.06	2.28	8	**8.0**	**6.0 to 9**
Cooking lessons for whole family	7.88	1.95	9	**9.0**	**6.4 to 9**
Free access to local swimming pool	8.45	1.68	10	**9.0**	**8.0 to 10**
Weekend lifestyle and activity camps (children)	7.03	2.21	9	**7.0**	**5.0 to 9**
School holiday lifestyle and activity camps	7.24	2.32	8	**8.0**	**6.0 to 9**
Gym membership for 1 year	7.83	2.30	10	**9.0**	**7.0 to 10**
Space on an allotment and teaching how to grow your own vegetables	5.68	2.82	5	**6.0**	**3.0 to 8**
Organised walks to/from school	5.81	2.43	5	**5.0**	**4.0 to 8**
Regular coaching in team sports of child's choice	7.99	1.91	9	**8.0**	**7.0 to 9**

Table 44: Results of Lifestyle Intervention Questionnaire. Kruskall-Wallis test for difference between groups: Chi-squared: 74.2; $p<0.001$

The results from the questionnaire (table 44 and figure 37) showed that there was a wide spread of preferences for which intervention would be most acceptable. There

was significant difference across the groups with the option for space on an allotment being least popular and the most popular being free access to a swimming pool. This would be the recommendation for a set lifestyle intervention from this study, for future studies, as it would fit with the observation that light activity is probably most beneficial in metabolic health outcomes. We have no evidence to suggest that a particular dietary pattern in the data collected, nor that mandating MVPA, would be most effective in modifying the metabolic outcomes within the cohort. However, it would have to be mandated for a longer session, frequently undertaken, at a gentle pace. This would possibly require supervision or coaching until a habit was formed, with a diary record kept to ensure consistency in the intervention. This approach would also fit observations from other studies that long term aerobic training is best in improving mitochondrial oxidative function as measured by τ½PCr using 31P-MRS (McCully *et al.*, 1989).

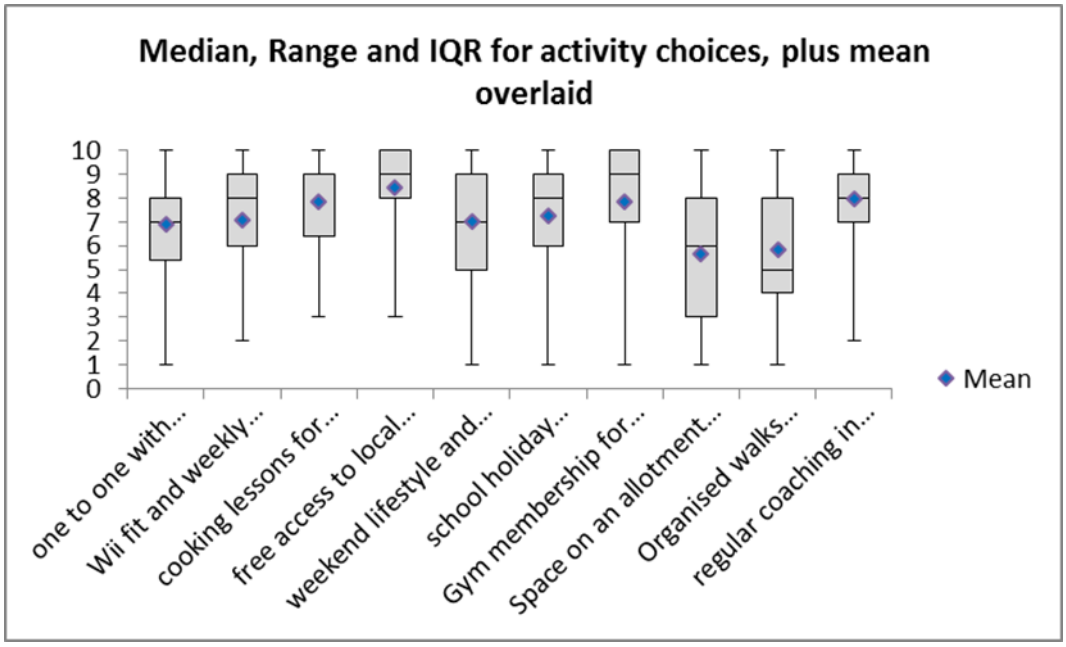

Figure 37: graphical representation of cohort preferences for lifestyle intervention.

Chapter 4. Study Strengths, Weaknesses and Confounding Factors

The current study had areas of both strength and weakness, some by its design, some which are related to the methods, and some because of the nature of the cohort.

4.1 Strengths

The fact that the study cohort was drawn from the larger, original cohort, who have a good track record for participating in research, meant that within the study period it was possible to achieve the target recruitment of 60 participants. The study itself was only one visit. Although the children had to be fasting for the morning visit, the single visit minimised inconvenience and disruption in this largely school-attending cohort, whilst still producing data. The way the children were recruited also was shown, by the data produced, to have produced a cohort representative of the larger cohort and one mostly unaffected by the original recruitment (into growth or protein studies).

The study used many different techniques to assess a wide range of metabolic outcomes. In practice this was challenging to arrange and program into one visit (requiring liaison with and training from with experts in various different fields). It required a lot of support in analysing the raw data from specialist techniques (e.g. MRS, nutritional recall and accelerometer) but this was achieved within a framework that would allow the study to be repeated in future studies. All of the methods used had been used in this age group before with the exception (locally) of the magnetic resonance protocol, which was unique in the centre's experience to date, while being familiar to them in its components in other age groups.

It is also a strength of the cohort that data from previous visits exists (in a longitudinal fashion). This allowed previous data, notably early growth patterns and changes in body composition (weight SDS score change) from the last visit to be used in analysis of the data from the study visits. The study design also meant that that other areas of both current (notably epigenetics) and potential future (metabolomic) research will have samples from this cohort visit to analyse in future and relate back to the cohort data. Although this research is not a feature in the analysis of this study, it adds to the strength of the overall data existing about this cohort.

It is hoped that this research will continue to be added to by analysis of samples stored from the cohort and ongoing analysis of existing data. Over time this will add to the knowledge of metabolic outcomes in ex-preterm infants. It is also improving knowledge of the normative reference data for this cohort of ex-preterm infants, which has not been collected before in a similar group of children.

4.2 Weaknesses and confounding factors

One of the most obvious weaknesses in the study design is that there are no control subjects. It would have been ideal to have had closely-matched, term-born adolescents undergoing the same procedures to provide comparative data. However, there were constraints of matching and budget to account for: to properly match to the cohort, we would have had to recruit 2:1 in favour of term-born adolescents, meaning that with the budget for 60 MRIs initially, we would have only gathered data on 20 of the unique individuals who make up the current cohort. To optimise resources, therefore, it was decided that it would be best to omit controls. It is also true that there is an inherent bias within the cohort regarding their original recruitment towards those recruited at more mature birth gestations being of relatively smaller birthweights. This was taken into account by using these data as part of the analysis. The cohort, however, are very representative of an important subset of preterm-born children: those who neonatologists would regard as 'intact survivors', in whom a normal childhood and subsequently fully participant adult life within society would be expected. Thus, any findings in their cohort which might be linked to early onset adult illness that could limit their quality of life, adversely influence the societal value of their ability to engage in work as an adult, and increase their need for long-term support from the nationalised health service are important. This is especially so, if these links could be modified in a positive way. While a lack of external controls prevents comparative statements about the cohort with respect to those born at term, knowing that the cohort are representative of a particularly important group of those born preterm means that the results are still generalizable to others who are born preterm and have similar neonatal outcomes.

The children in the cohort spanned the full range of pubertal stages as categorised by Tanner Stage self-assessment. It has been shown in the literature that children who are obese are more likely to enter puberty early (Kleber *et al.*, 2010) and once in

puberty, insulin sensitivity tends to decrease (Kurtoglu *et al.*, 2010; Sinaiko and Caprio, 2012). However, there is also some data to suggest that puberty may be delayed in girls born preterm but not in boys (Hui *et al.*, 2012). Self-assessment of Tanner Stage was used for several pragmatic reasons. Firstly the cohort were familiar with this method from the study visit previous to this one, and it is thought to be a reliable method of assessing pubertal stage (Tanner *et al.*, 1966a; Tanner *et al.*, 1966b). It avoided the embarrassment (in a group of volunteers who were giving up their time to help us) that a physician-based assessment would involve. There was also no female staff member who could have performed these assessments in the research team. Secondly, this method imposed no restriction on when the subjects could visit. In order to assess the hormonal status of participants otherwise, it would involve serum testing which immediately restricts the number of people who could be assessed. It would also require specific constraints around timing within an individual female participant's menstrual cycle (if they were of sufficient maturity) so that across the cohort, there was comparability of results in what are otherwise normally fluctuating hormonal levels. This study did not find any association or effect in multivariate modelling between Tanner-Stage and IS (measured by either of the study methods). It has to be considered as a possibility that this reflects type 2 error (if one considers the null hypothesis that 'pubertal stage has no effect on IS', based on the literature published). The source of such an error could be sample size: we had 45 individuals in whom we were able to calculate % sensitivity by the HOMA method and 44 for whom we could calculate the Matsuda Index. This may not have been enough to avoid error, as the sample size was calculated based on previous results. It could also be that the methods used to assess IS are not ones that are consistently accurate enough for use in the age group chosen. While the participants were also given instructions to fasted, and none reported deviating from this request, it is not possible to be absolutely sure of compliance. However, there are multiple examples of HOMA being used in children in the literature, as summarised in my review (Tinnion *et al.*, 2014), such that it can be reasonably expected that compared to similar groups it is an acceptable test to have used.

With regard to overall sample size, a cohort of sixty is a relatively small number of subjects. In each of the different aspects of the study there were some who did not

take part (with the exception of auxological measurements, n=60). There was also some loss of data through the technical challenges of both acquiring the data and the quality control measures taken during processing. An example of this was the 31P-MRS processing whereby only 38 of the cohort had data that showed no problem with the collection in the post-collection analysis. These factors may also have led to type 2 error being introduced into the dataset. Within the data, the cut-offs and inclusion criteria were kept strict (for example hours of activity recorded per day) and although this restricted eventual dataset sizes, it does mean that the data used was robust and reliable in quality. The presence of type 2 error may be represented in the results by the presence of numerous 'trends' in the data that do not reach significance. It would require a more extensive study with higher numbers of participants to assess this further.

4.3 Technique-specific confounding

4.3.1 Mitochondrial Oxidative Capacity

As discussed previously, there was evidence in the cohort data of inorganic phosphate splitting secondary to placement of the sampling voxel across two or more functionally different muscle groups, with different rates of recovery after exercise. In the analysis this was accounted for at the expense of sample size. Likewise, analysis of the τ½PCr data showed that not all of the participants were able to fully deplete their PCr stores and their data was also not useable. While neither of these, from the study power calculation, should have adversely affected the results, they do raise the issue that in a similar age cohort in future, the technique might need modification to improve reliability especially if the MRS was used as a repeated measure over time.

The τ½PCr results were also subject to post-collection correction by the Iotti method to validate within group analysis based on the mean minimum pH reached within the group during exercise. This method is based on the observation that adult τ½PCr can be influenced by pH (thought potentially to be a mechanism for stimulating mitochondrial ATP levels (Taylor *et al.*, 1997)) whereas in children there is generally higher oxidative capacity(Ratel *et al.*, 2002a; Ratel *et al.*, 2002b). Adolescents lie between these two groups and given the data, to allow within cohort comparison, it was felt appropriate to apply the correction. This makes direct comparison with other

studies difficult in terms of absolute half-times generated, but trends within the data should be comparable.

4.3.2 Vitamin D measurement

Vitamin D has been discussed earlier in this thesis and the mechanisms by which it acts are, in the wider picture, not fully understood. Therefore, it is a notable weakness of this study that serum calcium and phosphate were not measured in those who had vitamin D measured. We have no good evidence to comment either way about the calcium or phosphate status of the cohort, but calcium and phosphate have potential to interact with muscles and cell receptors alongside vitamin D and are related to vitamin D by bone metabolism. With hindsight it would have been interesting information to have in identifying whether vitamin D alone was key, as we found it in the study, or whether calcium and phosphate were also having an effect. In terms of missing data potentially confounding the results, this omission may well be the most significant. It should be noted, however that vitamin D has been shown in isolation to have an effect on muscle oxidative function: Sinha and colleagues did not find any difference in Ca/PO_4 levels between their vitamin D replete and deficient subgroups, yet found an effect on muscle (Sinha *et al.*, 2013).

4.3.3 Measurement of lipid compartmentalisation

As noted in the results, measurement of IHL produced three distinct subgroups when compared to IS. The majority of the cohort had very low levels of intrahepatic lipid and there were only five in the subgroup with IHL>5%. It is likely that this reflects the population from which this cohort were drawn, but it increases the possibility that the when the IHL content is <5%, small differences between individuals might have relatively large effects which are not possible to detect with the MRS measures. It is also true (as with IS) that the assumption of a fasting state at scan was made. If the participants had not been fasting then there is the possibility of confounding within the data due to temporary lipid deposition in the liver as part of the normal metabolic processes following a meal.

Similarly, the method of determining visceral/subcutaneous adipose tissue involved assessment of fat content of the tissues using 50% MRI signal intensity as the centre-point on an MRI-generated image, around which a dichotomous result (fat tissue vs

non-fat) was generated using software programmes. While this was effective in generating data, it is not necessarily a true reflection of the nature of metabolically active tissue: in any given voxel where the fat content was <50% (and labelled 'no fat') there may well have been metabolically active fat that was essentially undetected. While this does not seem to have influenced the results, it is possible that in future studies a refinement of the technique using more grey-scale intervals, might give more accurate results in this assessment.

4.3.4 Activity Measurement and Dietary Intake

The study used accelerometers to directly measure activity and the SCRAN24 computer software to assess dietary recall. The benefits of using these methods was outlined in the methods chapter of this thesis but both are subject to bias through participant use. Specifically, interpretation of the accelerometer data is helped by completion of a written activity diary which is subject to recall bias if not filled in contemporarily, and the accelerometers we used could not be worn in activity where they would get wet. Thus, where activities such as swimming were done, we had no way of assessing directly the nature or exact duration of the activity. Similarly the SCRAN24 software is a form of 24 hour recall diary and so is also subject to recall bias. The SCRAN24 diary data did not show any significant associations with any of the other variables which may reflect the possibility that the nature of influence that diet has on metabolism is a more long-term than a single 24-hour recall can elicit. In future studies, therefore, it may be necessary to use repeated measures of dietary intake over a longer time to accurately reflect dietary habits.

Chapter 5. Discussion

At the start of this study, four null hypotheses were proposed regarding the metabolic status of this cohort of adolescents who were born preterm. The results for the investigation of each of the three main outcomes (IS, mitochondrial oxidative function and activity, and intrahepatic lipid levels) have been discussed at the end of each section in chapter 3.

The study investigations showed that the null hypotheses were not entirely correct, but did identify some areas where unexpected results were found. The main associations found are outlined in table 45. In regard to mitochondrial oxidative function, no association was found with directly measured physical activity or adiposity but there was an association with vitamin D and fasting insulin sensitivity seen. A consistent and persistent association of τ½PCr in skeletal muscle after standardised exercise with gestational age at birth was also demonstrated such that the more preterm an individual at delivery, the lower their oxidative capacity was as measured using 31P-MRS in adolescence. The study demonstrated that the strongest predictors for raised IHL were raised serum triglyceride levels and visceral adipose tissue, but did not find any evidence to link IHL directly to insulin sensitivity in this cohort. When IS was investigated, it was shown that there were common factors which were strongly associated with both measures of IS (relating to adiposity and serum triglyceride levels) but unexpectedly there was also a strong association with serum vitamin D levels and both measures of IS. For IS measured by HOMA modelling, there was a linear association with change in weight SDS score between term equivalent age and 2 years old which was not seen in analysis with IS measured by Matsuda Index. Reciprocally, there was an association with light activity levels and IS measured by Matsuda Index which was not seen when looking at IS by HOMA modelling. The correlation analysis between auxological and body composition measures showed good agreement between themselves and also with the directly measured visceral/subcutaneous lipid measurements. They did not, however, correlate well with the intrahepatic lipid levels measured by 1H-MRS. Bland Altman analysis showed that the two skinfold-measurement techniques used to generate body composition measures were not closely enough correlated with the BODPOD™ measurements to allow them to be used as an alternative method in clinical practice.

Outcome (dependant variable)	Positive Associations	Negative Associations	Comments
Intrahepatic Lipid Content (% measured by 1H-MRS)	Visceral Adipose Tissue Deposition	Tanner stage influences multivariate modelling (increased TS is associated with reduced IHL) but in isolation it is not directly associated with IHL levels.	Where increased IHL was present, there were increased markers of adiposity (BMI, FMI, Wt. SDS, Waist Circ.) but these were not predictive of IHL deposition
Fasting IS (measured by HOMA % Sensitivity)	Vitamin D levels	Triglyceride levels Infant Growth (ΔWt SDS from term equivalent to 2 yrs) Subcutaneous lipid BMI SDS, FMI, waist Circumference, current weight SDS	
Glucose Disposal as measure of IS (measured by Matsuda Index)	Vitamin D levels Light Activity	Triglyceride levels Subcutaneous lipid BMI SDS, FMI, waist Circumference, current weight SDS	Protein Intake trends towards a positive association. A trend towards association of increased visceral adipose tissue with worsening IS was also seen
Mitochondrial Oxidative Function (measured by τ½PCr with 31P-MRS)	Fasting IS Serum Vitamin D	Gestational Age at Birth	

Table 45: Summary of associations: main outcome variables with other measured variables.

While these results are interesting as discrete, independent groups, the study has clearly shown that there are metabolic links between the main outcomes measured.

These are common factors which have an association with two, or more, of the outcomes studied, and may also be predictive when part of multivariate models.

The overall purpose of studying the cohort since birth has been to try to find out what the metabolic life course of these children is: where neonatal and early life care rightly focuses on optimising brain development via growth and nutrition strategies, the focus shifts as they get older. In adolescence and early adulthood, especially in 'intact' NICU survivors, there is a population-based argument for identifying those at risk of developing adult health issues earlier than their term peers, as soon as possible. In parallel, trying to identify those factors which, if modifiable, might have a beneficial effect on these metabolic health outcomes would therefore potentially allow targeted intervention in this population.

5.1 Common Metabolic Factors

Figure 38 shows a model for the connections between the main metabolic variables measured in the cohort, as determined by analysis in this study. The only direct link found between two of the main outcome variables was that between fasting IS and mitochondrial oxidative function but, as discussed earlier, it is not possible to say whether this is a real effect (representing a mechanism by which substrate entry into the myocyte is restricted) or whether it is secondary to an association between oxidative function and vitamin D. Research on adults with type 2 diabetes, as well as no association with the Matsuda Index measurement, has shown that it is very unlikely that the effect is a primary mitochondrial defect across the cohort (Lim *et al.*, 2011c; Lim *et al.*, 2011b). On the other hand, the evidence across the literature for an influencing role of vitamin D on mitochondrial oxidative function is increasing. Our cohort demonstrated the same association between lower vitamin D levels and longer half times for PCR recovery, thus reduced mitochondrial oxidative capacity, as seen in other cohorts (Sinha *et al.*, 2013).

The other variable with a consistently significant association to measured outcomes was serum triglyceride levels (and to a lesser extent serum LDL-C and total cholesterol levels). Increasing triglyceride levels were strongly associated with reduced IS by both measures and also strongly associated with increased measured intrahepatic lipid content and subcutaneous adipose tissue deposition. As discussed earlier, the

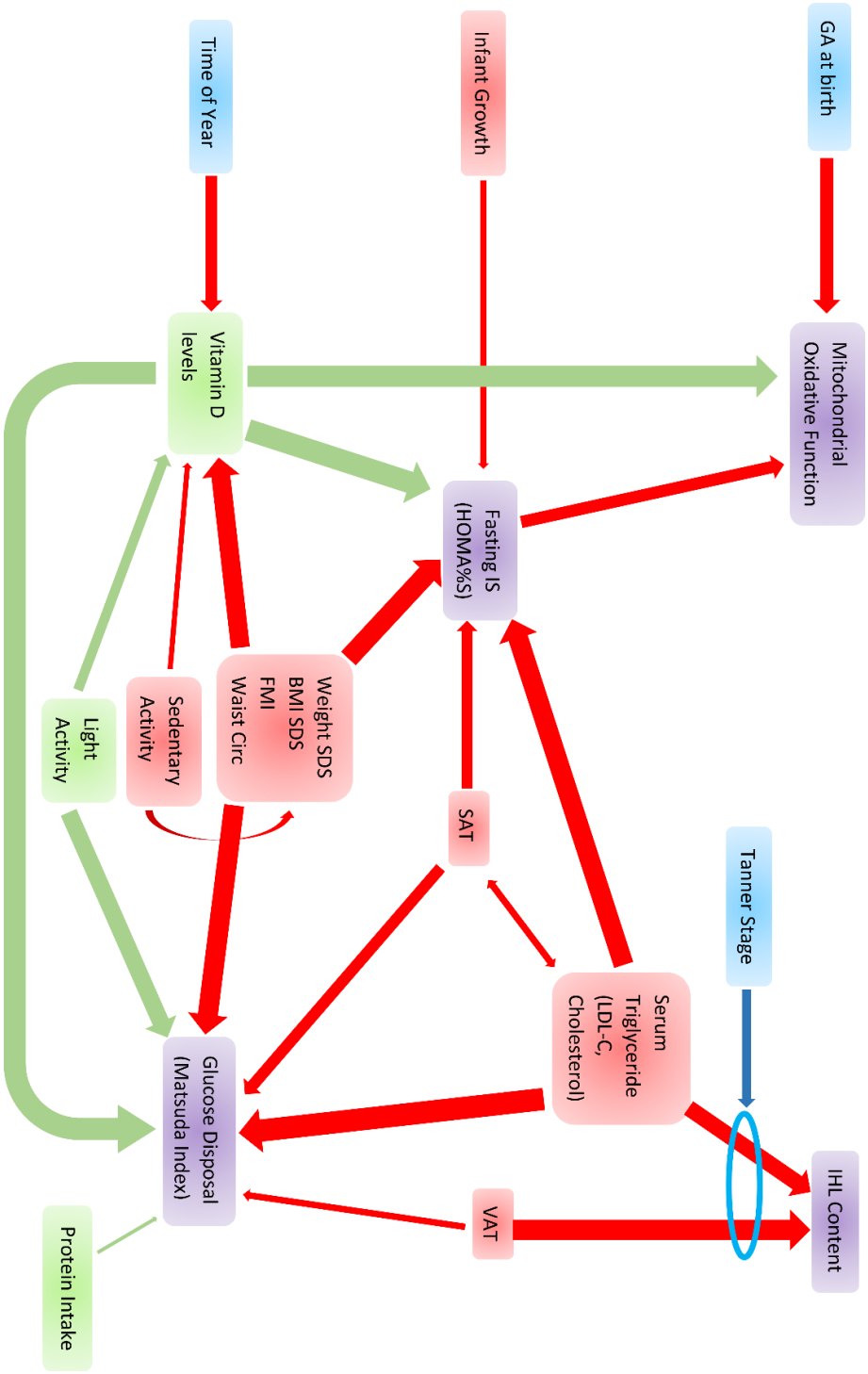

Figure 38: Summary model showing the metabolic interactions found in this study. Purple: study outcomes; Blue: unmodifiable influences; Green: modifiable influences which might have a positive effect on metabolic status; Red: potentially modifiable metabolic influences associated with worse outcomes in the study. Arrows show general direction of effect as demonstrated in the study. Size of arrow is representative of approximate effect size seen in study.

decrease in insulin sensitivity with increased triglyceride levels is as would be expected, and it is intuitive that they might be associated with increased IHL. However, while triglyceride levels are an independent predictor for raised IHL, the data cannot definitively answer the question as to whether raised triglyceride levels are the cause or an effect of raised IHL. However, a fasting lipid profile favouring higher triglycerides is clearly a risk for metabolic problems, and even if it is a marker rather than cause, it provides an opportunity for monitoring and potentially intervention. It is interesting that the auxological and body composition data show a correlation with visceral and subcutaneous lipid deposition but not IHL. The data analysis defined a subgroup of adolescents who had both high IHL and low IS. These data suggest that the development of raised IHL may post-date reduction in IS and that there may be a metabolic threshold beyond which clinically significant IHL begins to accumulate. However, while the mechanism for this may not be clear, it has been shown in other populations that calorie restriction and resistance exercise can reduce IHL and pancreatic lipid (Kleber *et al.*, 2010; Hallsworth *et al.*, 2011; Lim *et al.*, 2011a) and improve IS.

From the data collected it is also clear that other measures of adiposity (FMI, BMI, Weight SDS) increasing are negatively associated with IS and vitamin D levels. The multivariate modelling would suggest that these changes in body composition and auxology are markers rather than independent predictors for IS. At the same time there was a clear positive association between Vitamin D levels and IS measured by both methods used in the study. If future research elucidates a direct cellular mechanism of action for vitamin D on insulin secretion in humans it may offer an explanation for the observed association in this study of reduced adiposity associated with improved IS.

The evidence from the cohort does not necessarily support universal supplementation with vitamin D as this is only an observational study, not one looking at change after intervention. However, it would be reasonable to suggest that given its wide-ranging associations in this study, and the association with a negative trend through the winter months, optimisation of vitamin D levels where there was clinical deficiency would be advisable to help optimise metabolic status.

The final common factor of note is activity. In the study, we were able to measure this directly using accelerometry. The results showed that there was a trend towards association between sedentary activity and both auxological measures and vitamin D levels (with more sedentary time being associated with worse indices in both). Correspondingly, there was a positive association between light activity and both IS (Matsuda Index) and vitamin D levels. There was no association between any of the exercise variables and time of year to explain this association. It is possible that the association between light activity and vitamin D was mediated through increased activity time outside leading to increased endogenous vitamin D production (though we did not measure this) or it might have been related to subtle changes in body composition improved by activity which then influence circulating vitamin D levels. Similarly, the association between light activity and improved glucose disposal might be direct effects (for example aerobic conditioning improving overall metabolic flexibility) or via its association with vitamin D levels, improving IS by mechanisms discussed earlier. In either case it is only the light form of activity that has as positive effect. This has also been seen in animal models (Amaral *et al.*, 2015), older humans (Loprinzi *et al.*, 2014) and individuals at high risk of metabolic syndrome (Herzig *et al.*, 2014). In a group of adolescents it might be more possible to achieve changes in activity from sedentary to light activity more easily, and habitually, than it would introducing a programme of activity aimed at increasing MVPA. This then has potential for best gains in health at least perceived disruption of lifestyle.

5.2 Non-modifiable factors

The model shown in figure 38 suggests that there are two important non-modifiable factors with respect to the cohort. The first is gestational age at birth. This is unmodifiable by virtue of the fact that the causes for preterm delivery are legion and while obstetricians try to avoid it if possible, there will always be a clinical tension between safely delivering a baby early, to a live mother, and risking either maternal or infant death, or both by trying to prolong gestation. The second, and very applicable to this cohort, is puberty. During the transition to adulthood, humans will go through puberty and we have made attempts to adjust our data set to account for this. Our results showed that it appears to have an effect when considering IHL content such that as the cohort progressed through puberty, the IHL content decreased. As there

are other modifiable factors that also affect this IHL, there is still opportunity to intervene to modify outcome where it is thought an adolescent may be at risk, and the general trend is that progressing to a later pubertal status is beneficial.

With respect to the current cohort, infant growth (as represented by Δ weight SDS from term equivalent to 2 years old) is effectively unmodifiable and, as discussed, the nutritional strategies relevant to infants before and in this period are those to protect and promote neurodevelopment. As we have shown, the magnitude of persistence of an effect into adolescence is reduced by other modifiable variables through childhood and adolescence. However, its detectable presence at all suggests that those tailoring neonatal nutrition, for similar cohorts in the future, should perhaps do so with one eye on later metabolic health. Where excessive growth can be limited in this period, without compromising neurodevelopment, this has potential for the best adult health outcomes.

5.2.1 Consideration of the study results within the Developmental Origins Hypothesis.

As discussed previously, the Developmental Origins of Health and Disease Theory (DOHaD) suggests that by interruption of normal development, or exposure of those normal processes to environment during critical windows, things can be changed (perhaps by mechanisms such as epigenetic modification of the genome) which have profound effects in later life. Those born preterm are at a higher risk of metabolic compromise in their life course (Parkinson *et al.*, 2013) and this study raises two possible situations where this process may reflect the DOHaD theory. Firstly, the strong and persistent finding that gestational age is related to mitochondrial oxidative function (perhaps by a developmental arrest or restriction in skeletal muscle differentiation, as discussed earlier). Secondly, the association of early growth, though weakened by later influences, could be an example of metabolic programming. The other factors associated with outcome in the cohort would not, at face value, appear to be directly related to early life events. However, programming is such that it could be exerting effects via situation-specific intermediaries such as vitamin D. This is only speculation as there were no control subjects in the study.

5.3 Potential Interventions to improve metabolic health in the cohort.

As outlined in this chapter, the results show associations which could be viewed as potential targets for intervention to improve metabolic health.

These would be:

1. *Optimisation of vitamin D status*. This would potentially help with both IS and mitochondrial oxidative function, though within the study results, it would not be possible to recommend universal supplementation beyond correction of clinical deficiency, if detected. It should be noted that where clinical vitamin D deficiency was found in individuals in the study, a letter was sent to them and their GP recommending supplementation be provided, if the participant wanted it. This was done based purely on serum level and with no knowledge of the participant's corresponding IS or oxidative function results.
2. *Encouragement of light activity with associated calorie restriction*. While the study data showed that there might be benefits from engaging in regular, prolonged, increased light activity periods (for both IS and vitamin D levels) we had no direct evidence of any association with calorie intake 9measured over a 24 hour period), either benefit or harm, and any of the measured outcomes. It could be argued that calorie restriction cannot therefore be recommended, but there are limits on the dietary data collected (see chapter 4) and good evidence to suggest that reducing calorie intake can reduce IHL (Lim *et al.*, 2011a). It is also the case that triglyceride levels will be related to longer term dietary habits, and with other substance such as fructose being linked to adverse metabolic outcomes (Bremer *et al.*, 2012; Lustig, 2013), inclusion of dietary change alongside activity recommendations is unavoidable. Conversely, sedentary period should be discouraged. Section 3.5 outlines the type of interventions the cohort might engage with, and swimming (which could be tailored fit the light activity mould) was most favoured.

5.4 Future Research

Based on the findings of the current study cohort and the longitudinal data that exists in the cohort, there are two directions that should be considered for future research. Firstly, if possible, the cohort should be followed up in early-to-mid adulthood in order

to try to assess what *actual* adult health outcomes have occurred and then related these back to the longitudinal data that exists. Secondly, consideration should be given to repeating the research as done in the current cohort, with a bigger (possibly) multicentre cohort with the current generation of neonates.

In the time since the original research started with recruitment and enrolment of the NPTBGS cohort, neonatology has progressed in keeping extremely preterm babies alive and is continually trying to improve their neurological outcomes (Wood *et al.*, 2000; Costeloe *et al.*, 2012; Moore *et al.*, 2012). Alongside these increased survival rates, more information regarding the fact that moderate-to-late preterm infants are not the same as their term born peers is emerging (Ananth *et al.*, 2013; Scheuchenegger *et al.*, 2014; Walsh *et al.*, 2014; Kelly *et al.*, 2015). In both groups, an understanding of how intervention and life-course affects metabolic outcome will be essential in ensuring todays preterm infants become tomorrow's healthy adults.

Total Word Count: 56228

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CHILDREN'S INFORMATION SHEET

1. Study title

Growth and metabolic outcome in children born preterm (GROW-MORE study)

2. Invitation paragraph

You are being invited to take part in a research study. This leaflet tells you about the study. Ask your parents to explain it to you if you want. You can email us, speak to us on the telephone or ask us more questions if you come to the hospital to take part.

3. What is the purpose of the study?

We know that most children like you who were born premature seem to grow like their friends, although some grow more slowly. We want to see how your growth and feeding when you were a baby affects how big you are now. We would like to take some blood (about two teaspoons) and do a scan that looks at your tummy, liver and leg (MRI scan). We would like to know what you eat and how much exercise you do. We also want to understand more about how the body 'remembers' the way that you grew when you were smaller.

4. Why have I been chosen?

You have been chosen to take part because you were part of a growth study at the RVI when you were first born. In total we will be inviting about 150 children like you to take part in this study.

5. Do I have to take part?

It is up to you to decide if you want to take part. If you do decide to take part you will be asked to sign a form. Even then, you can still withdraw at any time, without giving a reason.

6. What will taking part involve?

This will involve visiting the hospital with your mum or dad for about 3 to 4 hours. The study involves:

A. Measuring height, weight, skin-folds, head, tummy size and blood pressure. None of these hurt.

B. Measuring how much muscle and fat your body is made up of with a BODPOD. This uses air to measure these, and it does not hurt and is completely safe. There is a picture at the end of this leaflet that shows you what it looks like.

C. A single MRI scan (Magnetic Resonance Image) to look at your tummy, liver and leg that takes about one hour. There is a picture at the end of this leaflet that shows you what it looks like. It does not hurt and is completely safe.

D. 2 small blood samples. We will put some 'magic cream' on your arm before we take the samples so it does not hurt. The doctor who takes it is a very experienced children's doctor.

E. A sample of saliva (spit) to look at DNA

F. Answering some questions about diet using a laptop computer that takes about 20 minutes, and a written questionnaire giving us your opinion about what things children might like to do to improve their health.

G. Wearing a small device on your waist belt called an activity monitor (Actigraph) for a few days. We will give you a print out of the results for you to keep if you want.

7. Are there any risks?

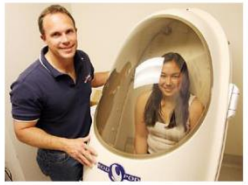
We don't think that there are any risks to you in taking part in our study, but we would like to tell you a bit more about the study.

Blood tests: You do not need to have a blood test if you don't want to. When we do the first blood test, we will make sure that the magic cream has numbed your skin so that it doesn't hurt. We leave a small cannula (small plastic tube) after taking the blood so we can take the second sample, without using another needle.

We would like you not to have eaten any food for a few hours when we do the blood sample (this is called 'fasting'). You may feel hungry, but your body is can manage a few extra hours without food. At the end of the study, we will give you a snack and drink before you go home. We will keep some of your blood and saliva in a freezer so we can do tests at a later stage.

Measurements: To see how much muscle and fat your body is made of, we use tapes and callipers (see the picture below). The researcher gently squeezes some skin and uses the callipers to measure the thickness of the skin. It might feel funny but it doesn't hurt.

We use the BodPod machine (see the picture below). When the door is shut small changes in air movement help us calculate how much fat and muscle you have.



The MRI scanner. You do not need to have this scan if you don't want to. If you do, we will need you to lie in the MRI scanner while it takes pictures of your tummy and liver, then your leg. The scanner uses a magnet to take the pictures. Before you are scanned, you will be asked some questions to make sure it is safe for you (we need to check you do not have any metal in your body). You will lie down in the scanner. It can be quite noisy. If you want, you can bring a CD of music to listen to using our special headphones while you're being scanned.

When you are inside the BodPod and MRI scanner, you may feel a sensation of being stuck in quite a small space. This is normal; most children are not frightened by this sensation and relax and quickly get used to it. If you (or your parents) think that you won't feel comfortable in the scanner, we can discuss whether or not it would be appropriate for you to have a scan. You do not need to do anything you do not want to do. Most children do not have any problems, but if you are having a scan and want to stop that is fine. Your parents can stay in the room next to you so they are close by and can see you. For most children, the hardest thing is lying still for long enough to get good pictures!!

Occasionally we might find something that we were not expecting to find during the study (because the scanner takes pictures of your insides and we will be measuring things in your blood). If this happens, we will talk to you and your parents about it. The chance of this happening is very small indeed.

The whole study process will take a morning or afternoon. There are lots of things to do in that time and you might feel tired afterwards. As there is a lot to do, you might feel a bit nervous about things before deciding whether to join in the study. We have chosen to do the study this way to make sure that we do not inconvenience you and your family more than necessary. If you have any questions or worries about how long the tests will take or about any of the tests themselves, you can contact us (details at the end of this sheet) We will chat to you about it again before we start, if you decide to take part in the study. If you don't want to do the scans and blood tests on the same day we can talk to you about that.

8. What are the possible benefits of taking part?

The information we get from this study may help in the care of premature babies in the future.

9. Will anyone else know if I take part?

Your GP will be notified if you want. All information that is collected about you will be kept strictly confidential and private.

10. What will happen to the information we get?

We will look at the results of all children who take part and tell other doctors what we have found in case it is helpful to them. We will do this at meetings or in medical journals (medical magazines). We will not tell anyone the names of the children who took part.

11. Contacts for further information

If you want to find out more you can ask your parents, or speak to us on the telephone or by email. We would like you to ask your parent's permission before you try and contact us.

1. Dr Nicholas Embleton
Consultant Neonatal Paediatrician
Special Care Babies
Royal Victoria Infirmary
Tel: 0191 282 5156
Email: n.d.embleton@ncl.ac.uk

2. Dr Robert Tinnion
Trust Doctor in Neonatal Paediatrics
Special Care Baby Unit
Royal Victoria Infirmary
Tel 0191 282 5737
Email: robert.tinnion@nuth.nhs.uk

Thank you for your time.

6.2 Participant's consent form

Newcastle Hospitals NHS Foundation Trust headed paper V2

Patient Identification Number:

CHILDREN'S CONSENT FORM

Growth and metabolic outcome in children born preterm (GROW-MORE study)

Name of doctor in charge: Dr. Nicholas Embleton

- I have read about the study and asked any questions I want to
- I am happy to take part in the study
- I know I do not need to have a blood test if I do not want to
- I know I do not need to have an MRI scan or BodPod test if I do not want to (or the study will be stopped if I change my mind during one of the tests)
- I know that if anything unusual is found during the study the doctors will talk to me and my parents and arrange for me to see my own doctor if needed

.....	.....	.....
My Name	Date	My Parent's Name

.....
My Signature

.....	.....	.....
Researcher	Date	Signature

Dr ND Embleton, Consultant Neonatal Paediatrician
Ward 35 Special Care Babies, RVI
Tel 0191 282 5156

PARENT'S INFORMATION SHEET

1. Study title

Growth and metabolic outcome in children born preterm (GROW-MORE study)

2. Invitation paragraph

You and your child are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information, by calling the contact number given at the end of this sheet. Take time to decide whether or not you wish to take part.

3. What is the purpose of the study?

Most children who were born premature seem to grow much like other children, although some grow more slowly. We are interested to see how their early growth and feeding whilst still on the Special Care Baby Unit (SCBU), and over the first few years of life, affects how they grow in later childhood. We want to look at aspects of their body chemistry (for example, sugar/glucose levels in the blood), how much fat they have in their tummy (abdomen) or liver, what sort of diet they have, and how much exercise they do. We also want to understand more about how the body 'remembers' nutritional experiences in our early life.

4. Why have I been chosen?

You have been chosen to take part in this research because your child was part of the growth study at the RVI. We performed an assessment of your child's development when she or he was 18 months old and we saw most of the children again sometime over the last few years. In total we will be inviting about 150 children and their families to take part in this study.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. Even then, you can still withdraw at any time, without giving a reason. Any data already collected about you and your child would then be left out of the study.

6. What will taking part involve?

This will involve visiting the hospital with your child for about 3 hours. The study involves:

A. Measuring height, weight, skin-folds, head, abdominal size and blood pressure.

B. Measuring body composition with 'BODPOD©', which uses air pressure changes (not detectable by the person undergoing the test) to measure fat/muscle levels in the body as a whole.

C. A single MRI scan (Magnetic Resonance Image) to look at the abdomen, liver and muscles that takes about 1 hour to complete.

D. 2 small blood samples to study various markers of body metabolism (e.g. glucose and lipid levels) and liver function, and to look at differences in which genes are switched on or off.

E. A sample of saliva to extract DNA, also to look at which genes are switched on or off

F. Answering some questions about diet using a laptop computer that takes about 20 minutes

G. Wearing a small device on the waist called an activity monitor (Actigraph) for a few days

H. Answering a short paper questionnaire about changes in lifestyle that might make children's lives more healthy

7. What are the possible disadvantages and risks of taking part?

We do not think there are any risks to taking part. The blood test will be done by an experienced paediatrician (Dr Rob Tinnion). We will be measuring blood sugar levels with your child fasting (not having anything to eat or drink besides water since the night before) and then again following a sugar (glucose) drink (this is the oral glucose tolerance test). This will only be done with the full consent from you and your child. Children sometimes feel hungry whilst fasting, but their blood sugar levels are unlikely to go too low or too high unless they have some pre-existing blood-sugar disorder such as diabetes. If your child has a medical condition which relates to their blood sugar control, we would need to discuss with you whether it would be appropriate to do the fasting glucose tolerance test. We will make the blood tests as painless as possible by using anaesthetic cream or spray. We will insert a cannula (small plastic tube) to take the two blood samples so it will feel like having one blood test rather than two. If children do not want a blood test we would still like to see them to measure growth and do the scan if they are happy.

The study also aims to look at differences in how genes work because of differences in growth or nutrition in early life. This will not involve looking at gene mutations. All laboratory work will be conducted on coded samples to keep the identity of study participants confidential. Genetic information will be stored securely and will not be available to anyone other than the study team. We are waiting for further funding to do these tests so we will store the samples in a freezer if you consent.

The body composition measurements involve use of measuring tapes and skin-fold callipers, which do not cause any pain when used.

The BodPod© is an enclosed capsule (see Children's information sheet for picture) which uses small changes in air pressure to measure body composition. The children will have to wear lightweight clothing to allow this to work. They will not be able to detect the changes in air pressure once inside the BodPod.

The risks of an MRI scan are very slight and are mainly due to the fact that the scanner uses a powerful magnet to produce the images. If a child has had any medical procedures that required metal items to be left inside them, such as surgical pins or plates, or if they have had any injuries that may have resulted in metal objects being left inside them we would not include them in the study. The scanner looks like a narrow bed with a cylinder at one end (see Children's information sheet for a photograph of our scanner) and is quite noisy. People who do not like enclosed spaces may not like it (claustrophobia). We will check both of these things with you and your child before taking part. If your child felt uncomfortable during either the BodPod test or the MRI scan the investigation would be stopped.

Incidental findings: There is a very small chance even in healthy people that we may detect something abnormal. This is called an incidental finding and could be harmless. If we do detect an incidental finding in *any* of our scans or tests we will discuss this with you and pass the information on to your GP if appropriate. If needed a consultant paediatrician would see you but we do not think this is likely to happen.

Sudden illness: If any child were to feel unwell during the visit, the doctor organising the tests will always be there (a paediatrician) and able to provide the necessary care. We do not think this is likely, although some teenagers feel a little faint when having blood taken.

We will cover travel and parking costs and provide your child with a snack and a drink during the visit. We will discuss with you the sort of times or days that best suit work and school commitments. We will provide the children with print-outs of their results and scans for them to keep.

The whole study visit is likely to take 3 to 4 hours for a child participating in all of the tests. We appreciate that this is a long time for some children, but it means that we are able to collect all the data in one visit, rather than inconvenience families for multiple visits. If you or your child only want to do bits of it that is fine, or come back at another time. We expect you to stay with your child during the visit. We want to tell you everything that we are doing so we are happy to be contacted by you if you have any questions or anxieties about the study. We will also make sure you are happy before commencing the study, and ask you to sign a consent form to record this. Even if you sign the form, you can still stop any of the tests at any stage.

8. What are the possible benefits of taking part?

The information we get from this study will help us understand how better to feed premature babies when they are first born, and the long term growth of these children. There are no other similar studies like this taking part worldwide so we believe the information we will obtain is unique.

9. Will my taking part in this study be kept confidential?

Your GP will be notified of your participation in this study should you decide to take part and if you so wish. All information that is collected about you, or your child, during the course of the research will be kept strictly confidential.

10. What will happen to the results of the research study?

Following completion of the research, it is expected that the results will be published in a medical journal. You or your child will not be identified in any publication. A copy of the published results can be made available to you if you request, by marking the box on the consent form. We will keep the data from the study on a computer but we will anonymise it so that no one else can work out who the child is.

If we do find that your child has any problems that you did not already know about we will discuss these with you. We will not pass the results to your child's GP or anyone else without

your permission but if you want we can provide a report as appropriate, and arrange any further appointments if needed.

11. What should I tell my child?

It is up to you to decide what to say to your child, but they will need to know that this is a research study that will involve them undergoing some tests. It is also important they know that they do not have to take part if they don't want to. Most of the children will be 11-16 years of age and will be able to read this information leaflet. They can make the choice once all the information is given. You can either show them this sheet or you can give them the attached version designed for children. We do not need to do blood tests or scans on every child who takes part and can discuss which bits of the study (if any) you or your child do not want. We would like to see you even if we only collect information about their growth for example. We can explain things in more detail by telephone, email and when we see you at the hospital.

12. Contacts for further information

1. Dr Nicholas Embleton BSc MBBS MD FRCPCH
Consultant Neonatal Paediatrician
Special Care Babies
Royal Victoria Infirmary
Tel: 0191 282 5156
Email: n.d.embleton@ncl.ac.uk

2. Dr Robert Tinnion BMedSci MBBS MRCPCH
Trust Doctor in Neonatal Paediatrics
Special Care Baby Unit
Royal Victoria Infirmary
Tel 0191 282 5737
Email: robert.tinnion@nuth.nhs.uk

Please feel free to contact us by email if you or your child would like to.

13. Who has reviewed the study?

The study has been reviewed by a Research Ethics Committee who have given their approval for the study. The study is sponsored by Newcastle Hospitals NHS Foundation Trust.

Thank you for your time.

6.4 Parent Consent Form

Patient Name and Identification Number:

CONSENT FORM

Growth and metabolic outcome in children born preterm (GROW-MORE study)

Names of responsible researcher: Dr. Nicholas Embleton

Please initial box

1. I confirm that I read and have understood the information sheet *(version 2, January 2011)* for the GROW-MORE study and have had an opportunity to ask questions. ☐
2. I understand that participation is voluntary and that I am free to withdraw at any time, without giving a reason. ☐
3. I understand that sections of my child's [(name) ……] medical notes may be looked at by responsible individuals involved in the research and give permission for this. ☐
4. I agree to take part and also give permission for my child to take part, and for the results to be recorded on a computer. ☐
6. I would like to know how to get a copy of any published results from this study. ☐
7. I am happy to be contacted about future studies like this. ☐
8. I understand that my child may provide a blood or saliva sample that will be stored and used for the analysis of gene expression. ☐
9. I agree to my GP being informed of any incidental findings (from my child's MRI scans or blood tests) after discussion with me, in the very rare event that this occurs. ☐

……	……	……
Name	Date	Relationship to child

……
Signature

……	……	……
Researcher	Date	Signature

6.5 Young Adult Consent Form

Patient Name and Identification Number:

CONSENT FORM

Growth and metabolic outcome in children born preterm (GROW-MORE study)

Names of responsible researcher: Dr. Nicholas Embleton

Please initial box

1. I confirm that I read and have understood the information sheet *(version 1 August 2012)* for the GROW-MORE study and have had an opportunity to ask questions. ☐
2. I understand that participation is voluntary and that I am free to withdraw at any time, without giving a reason. ☐
3. I understand that sections of my medical notes may be looked at by responsible individuals involved in the research and give permission for this. ☐
4. I agree to take part and for the results to be recorded on a computer. ☐
5. I would like to know how to get a copy of any published results from this study. ☐
6. I am happy to be contacted about future studies like this. ☐
7. I understand that my blood or saliva sample will be stored and used for the analysis of gene expression. ☐
8. I agree to my GP being informed of any incidental findings after discussion with me, in the very rare event that this occurs. ☐

……	……	……
Name	Date	Signature

……
Date of Birth

……	……	……
Researcher	Date	Signature

NUTH Headed paper **NHS logo**

Child's name:

CHILDREN'S ASSESSMENT OF LIFESTYLE INTERVENTIONS

Growth and metabolic outcome in children born preterm (GROW-MORE study)

We would like to know what you think about potential ways we might make children's lifestyles healthier, and form good habits to make them more healthy in adult life. This will help us decide and plan studies in the future that might improve children's health status.

Please make a mark on the scales below to indicate what you think about these suggestions – where 1 is a bad idea and 10 is a really good idea:

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

Please Rate the following suggestions:

1) One to one sessions with a dietician (to learn how to balance healthy foods and tasty treats)

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

2) A Wii fit device & exercise programme which is reviewed with a trainer each month

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

3) Lessons for the *whole family* in cooking healthy meals

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

4) Free access a local swimming pool in the evenings or at weekends

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

5) Weekend activity and lifestyle camps for children

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

6) Activity and lifestyle camps during school holidays

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

7) Gym membership for a year

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

8) Space on an allotment and teaching how to grow your own food (fruit and vegetables)

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

9) Organised walks to and from school

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

10) Regular coaching and training sessions in a team sport (choice of three or four)

1…..…..2……...3……...4…..…..5……...6……...7……...8…..…..9……...10

It would be very helpful if you have any ideas or suggestions as to what you would like to do to improve childrens' health and lifestyles that aren't included in the suggestions above. Please write down your idea for us in the box below:

Thankyou for taking the time to complete this questionnaire!!!!

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