The Influence of Parental Factors on the Growth and Metabolism of Children

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A thesis submitted in partial fulfilment of the requirements for the degree of PhD

The University of Auckland, 2013

Abstract

We are currently undergoing the most remarkable shift in human reproductive behaviour since the post-war baby boom. Couples are having children at more advanced ages, leading to an increase in maternal and paternal ages at childbirth. Couples are also choosing to have fewer children, leading to a dramatic increase in the proportion of first-born children in the population. Uptake of fertility treatment is increasing exponentially worldwide, with much attention given to children conceived by assisted reproductive technology (ART). Despite constituting a much larger proportion of the population conceived with fertility treatment, offspring conceived with ovarian stimulation alone (OS_A) are remarkably under-researched.

The impact of changes in the early fetal environment on offspring phenotype and disease risk is well recognised. Maternal and paternal ages at childbirth, birth order, and OS_A fertility treatments all affect the early environment at gametogenesis and/or embryogenesis. We hypothesised that alteration of these environments by parental factors would lead to changes in growth, adiposity, metabolism, and hormonal profiles in childhood. The possible impacts of these parental factors on childhood phenotype have not been previously examined.

In my studies of almost 400 children, I found that:

- Increasing maternal age at childbirth was associated with taller stature and reduced adiposity in her children, independent of paternal age and birth order.
- Similarly, increasing paternal age at childbirth was associated with taller stature and reduced adiposity in children, but with less favourable lipid profiles.
- First-born children were taller than second-born children, who were in turn taller than third-borns.
- Children conceived with OS_A fertility treatment were shorter than naturally conceived children of fertile and subfertile parents. OS_A children also had reduced adiposity and displayed subtle metabolic changes compared to those conceived naturally.

Thus, my studies demonstrate that a number of parental factors impact on childhood phenotype and disease risk profiles. As a result, the on-going demographic shift in reproductive behaviour is leading to changes in childhood height, adiposity, and metabolism, which may have implications for their future health and disease risk.

Acknowledgements

I would like to sincerely thank my supervisory team of Prof Wayne Cutfield, Assoc Prof Paul Hofman, Dr John Peek, and Dr Mark Green for their advice, support, and training throughout my PhD. I would especially like to thank Wayne, who was a constant source of encouragement and support throughout.

I would also like to thank Eric Thorstensen, Dr Yannan Jiang, Elizabeth Robinson, Dr Harriet Miles, Dr Fran Mouat, Dr Craig Jefferies, and in particular Dr José Derraik for their invaluable help in many aspects of my PhD. This thesis would not have been possible without their help and advice.

I would also like to thank the National Research Centre for Growth and Development (NRCGD) and the Australasian Paediatric Endocrine Group (APEG) for their financial support for the studies in this thesis. I am also grateful to the Paykel Trust for long-term funding of the Maurice & Agnes Paykel Clinical Research Unit at the Liggins Institute, University of Auckland.

I would also like to thank my girlfriend Marianne and my family (in particular my brother John) for their unwavering support and faith in my ability to complete the journey of a PhD.

The greatest debt of gratitude is owed to the many children and their families who voluntarily gave their time by taking part in the studies. None of the work in this thesis would have been possible without their help.

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ALSPAC – Avon Longitudinal Study of Parents and Children

ART – assisted reproductive technology

BMI – body mass index

BMISDS – body mass index standard deviation score

CV – coefficient of variation

DOHaD – developmental origins of health and disease

DXA – dual-energy x-ray absorptiometry

FSH – follicle-stimulating hormone

GH – growth hormone

HDL-C – high density lipoprotein cholesterol

HOMA-IR – homeostasis model assessment-estimated insulin resistance

HtSDS–MPHSDS – height standard deviation score minus mid-parental height standard deviation score

ICSI – intra-cytoplasmic sperm injection

IGF – insulin-like growth factor

IGFBP – insulin-like growth factor binding protein

IUGR – intra-uterine growth restriction

IUI – intra-uterine insemination

IVF – *in vitro* fertilisation

LBW – low birth weight

LDL-C – low density lipoprotein cholesterol

LH – luteinizing hormone

MPBMISDS – mean parental body mass index standard deviation score

MPHSDS – mid-parental height standard deviation scores

mRNA – messenger RNA

OS_A – ovarian stimulation alone

PCOS – polycystic ovarian syndrome

SD – standard deviation

SDS – standard deviation scores

SGA – small for gestational age

TFR – total fertility rate

Chapter 1. Introduction

1.1. The impact of the early environment on phenotype, metabolism & future disease risk

1.1.1. Developmental origins of health and disease

The time around conception and that during *in utero* embryonic development are recognised to have a critical influence on offspring phenotype and long-term health (Godfrey *et al.* 2010). This concept is broadly known as the "developmental origins of health and disease" (DOHaD) hypothesis (Godfrey *et al.* 2007).

The ability to adapt to environmental stimuli during *in utero* development is an important survival tool for some animals, and these adaptations are often positive, as they enable the organism to change to better suit the external environment (Rickard & Lummaa 2007). However such "developmental plasticity" is not always positive, and can lead to fetal 'programming that predisposes to future disease. For example, a fetus that is growth-restricted *in utero* may "anticipate' a nutritionally scarce post-natal environment, and consequently is programmed to conserve energy. This is an example of a "predictive adaptive response" (Gluckman & Hanson 2004a). However, if the post-natal environment is one of abundance, the adaptation will be 'mismatched', and the person will ultimately be at increased risk of obesity, type 2 diabetes mellitus, and cardiovascular disease later in life (Barker 2006).

Barker & Osmond (1986) carried out some of the pivotal studies underpinning the concept of DOHaD, linking geographical areas of increased infant mortality to higher risk of adult cardiovascular disease. This was further strengthened by the observed association between lower birth weight and a higher rate of metabolic and other diseases in adulthood (Hales *et al.* 1991). These observations have since been reproduced in a multitude of studies demonstrating an association between birth weight and long-term disease risk (as reviewed by Mcmillen & Robinson 2005). Crucially, such studies demonstrate that there is no birth weight threshold, but rather a continuous association between birth weight reduction and increased risk of adult disease. While this continuum is not linear, it shows that even

relatively subtle changes within the 'normal' birth weight spectrum may impact disease risk in the long-term (Barker 1995).

The relationship between birth weight and later disease risk has been proposed to be a result of altered fetal nutrition, and consequent adaptation to this altered environment by the fetus (Martin-Gronert & Ozanne 2010). Such adaptation may help the fetus survive in the short-term, by directing limited resources to vital organ development and nutrition (Martin-Gronert & Ozanne 2010). However, as discussed above, if this adaptation is 'mismatched' to the external environment, the altered metabolic programming may pre-dispose the individual to a number of adult diseases (Martin-Gronert & Ozanne 2010) (Figure 1.1).

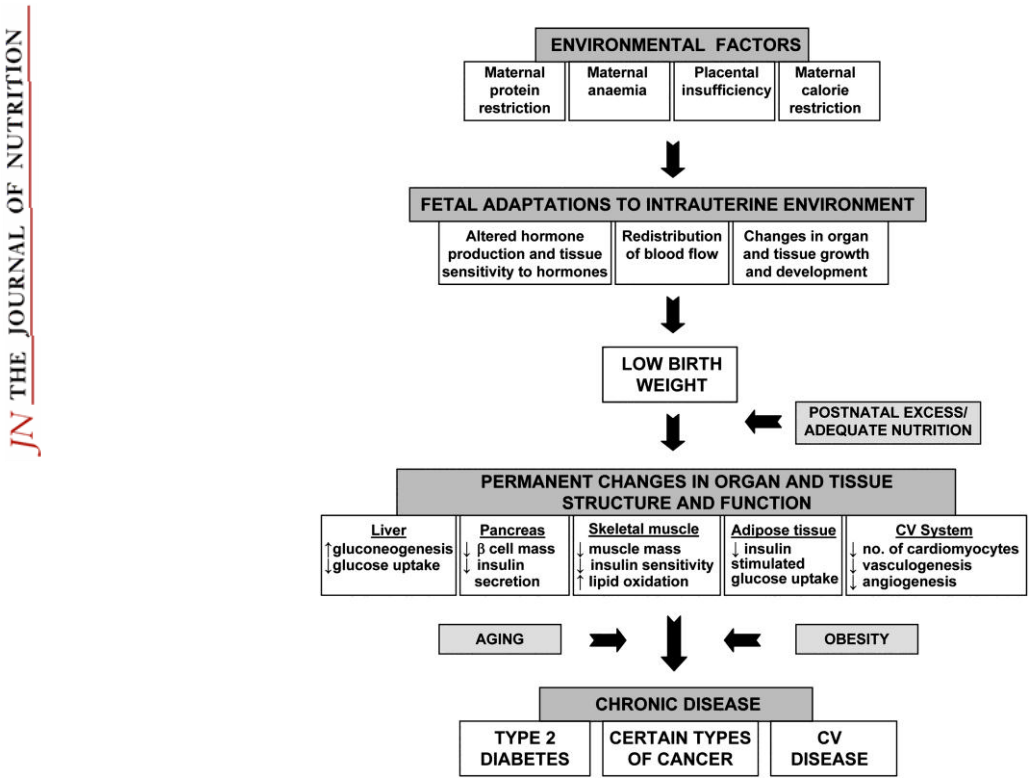

Figure 1.1. Developmental programming of type 2 diabetes and cardiovascular disease.
Adapted with permission from Martin-Gronert & Ozanne (2010).

Assessments of offspring born to mothers who experienced the Dutch Famine represent further landmark work in this field, demonstrating the importance of the timing of fetal environmental alterations to fetal programming. For example, mothers who experienced famine in the first or second trimester of gestation had offspring with increased obesity risk (Ravelli *et al.* 1976); in contrast, maternal famine exposure in late gestation was associated with an increased risk of glucose intolerance in the offspring (Ravelli *et al.* 1998).

Elegant animal studies have provided further evidence of fetal programming and the DOHaD concept (Godfrey *et al.* 2007). Studies in rats showed that alternations in maternal diet during pregnancy can lead to offspring hyperphagia and obesity (Vickers *et al.* 2000) or altered nephron development and increased blood pressure (Langley-Evans *et al.* 1999).

The peri-conceptional period has also been shown to affect the offspring, with changes in maternal nutrition around the time of conception found to be associated with post-natal alterations in phenotype and hypertension in the offspring (Kwong *et al.* 2000). Indeed, even changes in post-natal maternal behaviour (such as grooming and offspring care) may lead to phenotypic alterations in the offspring (Weaver *et al.* 2004). Many animal studies (outlined in an extensive review by Mcmillen & Robinson 2005) have demonstrated that changes in the early environment *in utero* can lead to short- and long-term alterations in offspring outcomes. Short-term changes include alterations in fetal growth, leading to changes in birth weight. Longer term consequences include increased risk of obesity, hypertension, insulin resistance, and perturbations of growth (Mcmillen & Robinson 2005).

Human studies in this area have expanded exponentially, with the effects of the early environment on offspring growth and metabolism generating particular interest. Many studies have focused in particular, on the effects of birth weight on childhood and adulthood outcomes. Birth weight reflects the *in utero* environment, and is associated with alterations in childhood height, body size, and metabolism (Hack *et al.* 2003; Sørensen *et al.* 1999). Children who were born small for gestational age (SGA) have been extensively studied, with evidence that they are at risk of being shorter as children compared to their peers born at full term (Chernausek 2012). Furthermore, SGA children have higher rates of obesity and insulin resistance, tend to experience an earlier onset of puberty, as well as having a tendency towards less favourable lipid profiles (Chernausek 2012; Eriksson *et al.* 2006). Later in life, SGA children are also at a greater risk of developing a number of diseases, such as obesity and the metabolic disturbances (Barker 1998).

Childhood studies examining the effects of adverse early life conditions on childhood outcomes tend to evaluate children who were subjected to more extreme stressors *in utero* (Clayton *et al.* 2007; Hofman *et al.* 2004; Regan *et al.* 2006). Such extreme stressors include being born at very low birth weight or small for gestational age. These children are more likely to display detectable adverse effects in childhood and adolescence, including increased risk of insulin resistance and obesity (Clayton *et al.* 2007; Hofman *et al.* 2004; Regan *et al.* 2006).

However, more subtle stressors may lead to adverse effects that are not apparent in childhood, but which may become evident in adulthood. An example is a reduction in birth weight from 3.5 to 3.0 kg, which is associated with altered disease risk in adulthood (Barker 2001). Thus, the impacts of such changes in the early fetal environmental may only emerge with the passage of time.

The tempo or rate of growth in childhood is another area which has become a recent focus of attention. There is some evidence that some children born at lower birth weight may undergo excessive 'catch up growth' in infancy, leading to a risk of increased body size during childhood, as well as a possible increased risk of diabetes and obesity in the long-term (Leunissen *et al.* 2009; Nobili *et al.* 2008; Ong *et al.* 2000). Low birth weight and more rapid weight gain during childhood has also been linked to earlier onset of puberty (Sandhu *et al.* 2006; Sloboda *et al.* 2011), which is in turn associated with increased metabolic and cardiovascular disease susceptibility in later life (Lakshman *et al.* 2009). Studies assessing the possible consequences of lower birth weight and 'excessive catch-up growth' during childhood on phenotype and disease risk in childhood and adulthood are on-going.

1.1.2. Epigenetics

As a result of these observations, the possible mechanisms responsible for such changes have been the subject of intense and on-going research. It is thought that epigenetic changes form much of the molecular basis underpinning these "early environmental effects" on offspring phenotype and later disease risk. Epigenetics is defined as the study of heritable changes in phenotype or gene expression that occur independently of alterations in DNA sequence (Waterland & Michels 2007). In simpler terms, the original concept of epigenetics suggested that epigenetics may be broadly referred to as the study of how genes interact with the environment, and the possible consequences of such interactions to phenotype (Waddington 1940).

Epigenetic modifications include histone acetylation and/or cytosine methylation, both of which alter chromatin organisation, leading to DNA conformational change and gene silencing. Methylation is the best characterized form of epigenetic modification, and can be further subdivided into hypermethylation (methylation gain) and hypomethylation (loss) occurring on either the maternal or paternal allele (Goldberg *et al.* 2007).

Epigenetic modification is a normal process that is intrinsic to the switching on and off of genes, and is therefore crucial to the regulation of gene expression (Jaenisch & Bird 2003). Epigenetic mechanisms are involved in gene regulation in both somatic and germ cells (Reik 2007). Although epigenetic changes are important from prior to conception until old age (Reik 2007), they are particularly important in germ cells and during fetal development. This is because many epigenetic patterns are re-programmed during gametogenesis and embryogenesis, making these stages key in human epigenetic programming.

The best characterised epigenetic event in early development is genomic imprinting, i.e. the epigenetic silencing of either the maternal or paternal allele that occurs by methylation (Waterland & Michels 2007). Imprinting is crucial for many aspects of pre- and post-natal growth and development, as well as in tumour suppression (Waterland 2009). Importantly, imprinting is a normal process that is required for gene regulation, and up to 1% of all human genes are imprinted (Luedi *et al.* 2007). In humans, there have been over 100 imprinted genes described, most of which are involved in fetal development (Luedi *et al.* 2007).

However, as with any other regulatory process, genomic imprinting is subject to errors, which in extreme cases can lead to imprinting disorders. *IGF2* is the best characterised imprinted gene, in which an imprinting loss is associated with several imprinting disorders, such as Beckwith-Wiedemann Syndrome (Ohlsson *et al.* 1993), Silver-Russell Syndrome (Gicquel *et al.* 2005), and Wilms' Tumor (Ogawa *et al.* 1993).

1.1.2.1. Epigenetics and gametogenesis

During gametogenesis, it is known that more than 50 human imprinted genes undergo modification, and it is likely that a much greater number also undergo changes during this critical time (Luedi *et al.* 2007). The development of germ cells (oocytes and sperm) is genetically and epigenetically regulated (Morgan *et al.* 2005), and these cells undergo extensive epigenetic reprogramming from the earliest stages of development (Kimmins & Sassone-Corsi 2005). Epigenetic changes take place in early primordial germ cells, with sex-

specific de-methylation and re-methylation occurring at crucial time points during their development into mature gametes (Sasaki & Matsui 2008; Seki 2011). This re-setting of the epigenome means that, when these gametes fuse at fertilisation, the zygote is able to give rise to any cell type. This process helps reduce the passing on of epigenetic modifications from parents to offspring (Reik *et al.* 2001b).

Germ cells differ from somatic cells as they have a number of specialized epigenetic regulatory pathways. These pathways have been extensively discussed in detail in reviews examining epigenetic changes on the male gamete (Carrell 2012) and during ovarian folliculogenesis (Pan *et al.* 2012). Briefly, female gametogenesis requires the epigenetic processes of methylation, histone modification, non-coding RNA regulation, and limited chromatin remodelling in order to produce genetically competent and functional gametes. There is a complex interplay between these processes which is strictly regulated. There is emerging evidence that even subtle alteration of the process of gametogenesis or changes to processes which influence the process (such as hormones) may lead to dysregulation of the epigenetic process (Pan *et al.* 2012).

Male gametogenesis also undergoes extensive epigenetic remodelling. However, while it shares many of the above-mentioned processes involved in female gametogenesis, epigenetic regulation of male gametes occurs primarily via chromatin remodelling (Carrell 2012; Kimmins & Sassone-Corsi 2005).

Evidence from animal and human studies suggest that alteration of the latter stages of gametogenesis (e.g. by ovarian stimulation leading to the production of oocytes) is associated with epigenetic changes in the oocyte (Market-Velker *et al.* 2010; Sato *et al.* 2007). There is also evidence (although limited) that similar epigenetic alterations may occur in sperm genes during male gametogenesis (Oakes *et al.* 2003).

It is important to note that there is no definitive proof of the transmissibility of paternal (or maternal) epigenetic changes to the offspring. In fact, several authors have pointed out that the likelihood of transmission of sperm or oocyte epigenetic changes to the offspring is low, because the epigenome undergoes re-programming at embryogenesis, stripping the 'memory' of much of the epigenome (Curley *et al.* 2011). However, not all epigenetic 'memory' is stripped, and epigenetic changes in the germ cell may still be transmitted to the offspring (Kimmins & Sassone-Corsi 2005; Lane *et al.* 2003), but definitive evidence in human studies has yet to be uncovered.

1.1.2.2. Epigenetics and embryogenesis

From the moment of fertilisation, the zygote undergoes important epigenetic changes that are crucial to its development and survival. During the early stages after conception, paternal DNA in the zygote undergoes rapid de-methylation and this occurs more passively than in the case of maternal DNA (Santos *et al.* 2002). Subsequently, embryonic DNA undergoes de novo methylation until the blastocyst stage is reached (Santos *et al.* 2010a). Thereafter, further epigenetic changes likely take place in the developing embryo, involving complex mechanisms that have not been clearly identified (Senner 2011).

Although most data on epigenetics at embryogenesis is derived from animal studies, there is some limited information from human studies suggesting that mechanisms may be broadly similar (Fulka *et al.* 2004). Also, for obvious ethical reasons, these data are derived from studies of embryos conceived via assisted reproductive technology (ART), which may have quite different epigenetic patterns to naturally conceived embryos (Sato *et al.* 2007). Work in the complex area of epigenetic change during embryogenesis is on-going, and will likely shed further light upon this important process.

While not the focus of work in this thesis, it is important to mention that epigenetic modifications also occur in later gestation. There is some evidence that alteration of the fetal environment (such as placental insufficiency during later gestation) is associated with changes in imprinted genes and phenotypic changes in offspring (MacLennan *et al.* 2004; Pham *et al.* 2003; Waterland & Garza 2002). Human studies, particularly in the area of fetal growth and intra uterine growth restriction are shedding further light on the process of epigenetics and imprinting in later gestation (Fowden *et al.* 2006; Nafee *et al.* 2008).

1.1.2.3. Epigenetics and the placenta

The importance of the placenta in the field of epigenetics and fetal programming is becoming increasingly recognised (Maltepe *et al.* 2010; Nelissen *et al.* 2011). The placenta contains all known imprinted genes in abundance, and it is clear that the placenta has a crucial role in the epigenetic process itself. In fact, epigenetics and gene imprinting are critical to embryo implantation and subsequent placental development (Nelissen *et al.* 2011). Animal and human studies showed that epimutations of these genes can lead to abnormal placentation and subsequent complications, such as abnormal fetal growth (Nelissen *et al.* 2011; Steinhoff *et*

al. 2009). The importance of the placenta in the epigenetic process is outlined in detail in a review by Nelissen *et al.* (2011).

1.1.2.4. Linking epigenetics to phenotype

The dynamic and active nature of epigenetics and imprinting at gametogenesis and embryogenesis, as well as their vulnerability to subtle external environmental stimuli, is apparent (Figure 1.2).

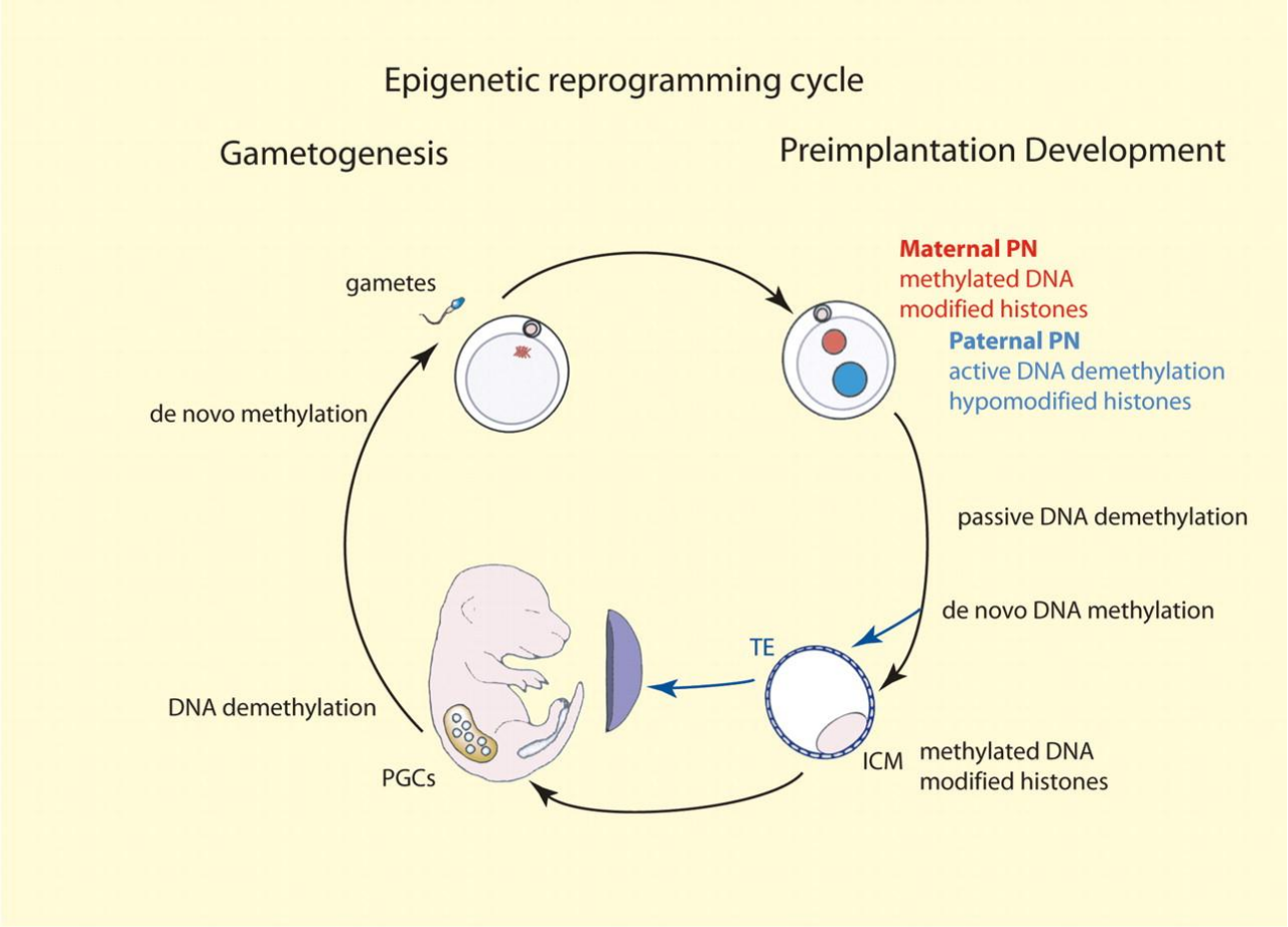

Figure 1.2. Epigenetic reprogramming cycle.
"Epigenetic modifications undergo reprogramming during the life cycle in two phases: during gametogenesis and preimplantation development. PGCs arise from somatic tissue and develop into mature gametes over an extended period of time. Their genome undergoes DNA demethylation in the embryo Following demethylation, the genomes of the gametes are de novo methylated and acquire imprints; Fertilization signals the second round of reprogramming during preimplantation development. The paternal genome is actively demethylated. The embryo's genome is passively DNA demethylated during early cell cycles before blastulation. Despite this methylation loss, imprinted genes maintain their methylation through this preimplantation reprogramming. De novo methylation coincides with the differentiation of the first two lineages of the blastocyst stage, and the inner cell mass (ICM) is hypermethylated in comparison to the trophectoderm (TE). These early lineages set-up the DNA methylation status of their somatic and placental derivatives" (Morgan *et al.* 2005). Reproduced with permission from Oxford University Press.

There are two seminal studies that are most often cited linking environmental change, phenotypic alterations, and differences in imprinted genes (Heijmans *et al.* 2008; Wolff *et al.* 1998). Wolff *et al.*'s (1998) Agouti mouse model is the classic example of alterations in fetal environment (by dietary change) leading to epigenetic modifications (increase in DNA methylation), and consequent phenotype changes (coat colour). The second study by Heijmans *et al.* (2008) is one of many assessing adults conceived during the Dutch Famine, and revealed lower DNA methylation of the *IGF2* genes compared to their siblings. Such changes have been subsequently linked to higher risk of obesity, type 2 diabetes, and cardio-vascular disease (Ravelli *et al.* 1976; Roseboom *et al.* 2001).

Numerous other animal studies have found epigenetic changes or differences in imprinted genes in association with alterations in the early fetal environment. However, these have been discussed in detail by Mcmillen & Robinson (2005) and Gluckman *et al.* (2008), and will not be discussed here. Nonetheless, such studies have led to further research, particularly of *IGF2* and its role in phenotypic changes and disease risk in offspring (Juul *et al.* 1995). *IGF2* has been extensively linked with fetal growth and subsequent post-natal growth (Baker *et al.* 1993; Kent *et al.* 2012), and changes in *IGF2* imprinted gene have been implicated in some of these observations (as detailed by Piedrahita (2011) in a recent review on this specific topic).

Other studies have also attempted to elucidate associations between physical changes observed in offspring and alterations in imprinted genes or epigenetic changes (Gaunt *et al.* 2001; Kaku *et al.* 2007; Petry *et al.* 2005). These studies have found some differences in serum concentrations of IGF-II and alterations in expression of H19 (a methylated gene associated with IGF-II) in association with birth weight, childhood body mass index (BMI), and adult obesity (Gaunt *et al.* 2001; Kaku *et al.* 2007; Petry *et al.* 2005). However, these studies have yielded conflicting results, due at least in part to the highly variable populations studied.

Overall, it is clear that the early fetal environment is vulnerable to environmental changes and that such changes may be associated with alterations in offspring phenotype and altered disease risk. Epigenetics is the most likely the mechanism underpinning such changes, but evidence defining the role of epigenetics as the mechanism underpinning these changes in humans has yet to be established.

1.1.3. Parental factors

With an outline of the importance of the early environment and the possible consequences to the phenotype of even subtle alterations to this crucial stage in human development, it is possible to proceed onto the background of the topics addressed in the studies covered by this PhD project. The introduction to this thesis will therefore discuss a number of important parental factors that will be investigated, which are known to be associated with changes in the early environment.

1.2. The impact of increasing maternal age at childbirth on the growth & metabolism of children

1.2.1. Demographic patterns and causes of increasing maternal age at childbirth

Over the past several decades, there has been a major change in human reproductive behaviour, with parents choosing to have children at increasing ages. While there is overlap between the demographics and reasons associated with the increase in maternal and paternal ages at childbirth, I will examine these separately.

Postponement of parenthood is a phenomenon occurring in almost every country in the developed world Between 1970 and 2010 the average maternal age at first childbirth has increased by almost 4 years (OECD 2011b) (Figure 1.3). As a result, the mean maternal age at first childbirth has increased from an average of 25 to 29 years of age (Collins *et al.* 2010; OECD 2011b). Similarly, in the US the mean maternal age at first childbirth has risen from 23 years in the 1970s to 26 years by 2005 (Collins *et al.* 2010). As a consequence of such trends, the birth rate among women aged less than 30 years is decreasing steadily, while that for women over 35 years of age is experiencing the most persistent increase (Martin *et al.* 2009). The reasons for this shift in reproductive behaviour are multi-factorial. They include greater availability of contraception, increased educational and career opportunities for women, economic pressures, and personal choice (Beets 2011).

The impact of the widening availability of contraception since the 1970s on postponement of childbirth should not be underestimated. Not only does contraception allow women to plan pregnancy and to postpone parenthood, it also means that many women can continue to study and/or work (Goldin & Katz 2002). This 'knock on effect' of contraception remains

important, as motherhood may lead many women to decide against further education or employment. Thus, contraception has had a major influence on women's decision-making process regarding motherhood.

Increased opportunities for women in the workplace and in education have also led to a behavioural and societal shift, whereby women seek to obtain higher education, remain in the workplace, and postpone motherhood to a later period in their lives. Higher levels of education are closely linked to older maternal age of childbirth, with tertiary educated women often postponing parenthood into their thirties, with highly educated and high-earning women often having their first child beyond 35 years of age (Andersson *et al.* 2008; Berrington 2004; O'Donoghue *et al.* 2011). Thus, in tandem with education, higher socio-economic status and personal economic independence are also linked to postponement of motherhood (Blossfeld & Huinink 1991).

Maternity leave is increasingly available in developed countries, and has helped somewhat to stem the tide of postponement of childbirth. However, there is some evidence that women who choose to have children while in a career and obtain maternity leave may be at risk of poorer career progression than those who postpone motherhood (O'Donoghue *et al.* 2011). This is another factor encouraging highly educated women to have children later on. It is uncertain if increasing unemployment rates as a result of the current economic downturn will lead women to have children at a younger age, especially since it is also evident that economic uncertainty leads to postponement of parenthood (Mills 2005). In addition, the decline in marriage rates and increase in the practise of co-habitation before marriage is associated with "relationship uncertainty", a factor which has led many couples to postpone the timing of their first childbirth (Manning & Smock 2002).

Overall, many of these factors have influenced women's decision-making regarding their timing of pregnancy. The change in belief and attitude as to when is a suitable time to become a mother has permeated throughout society, where it is now 'normal and acceptable' for most women to attempt motherhood for the first time over the age of 30 years, or even over 35 years.

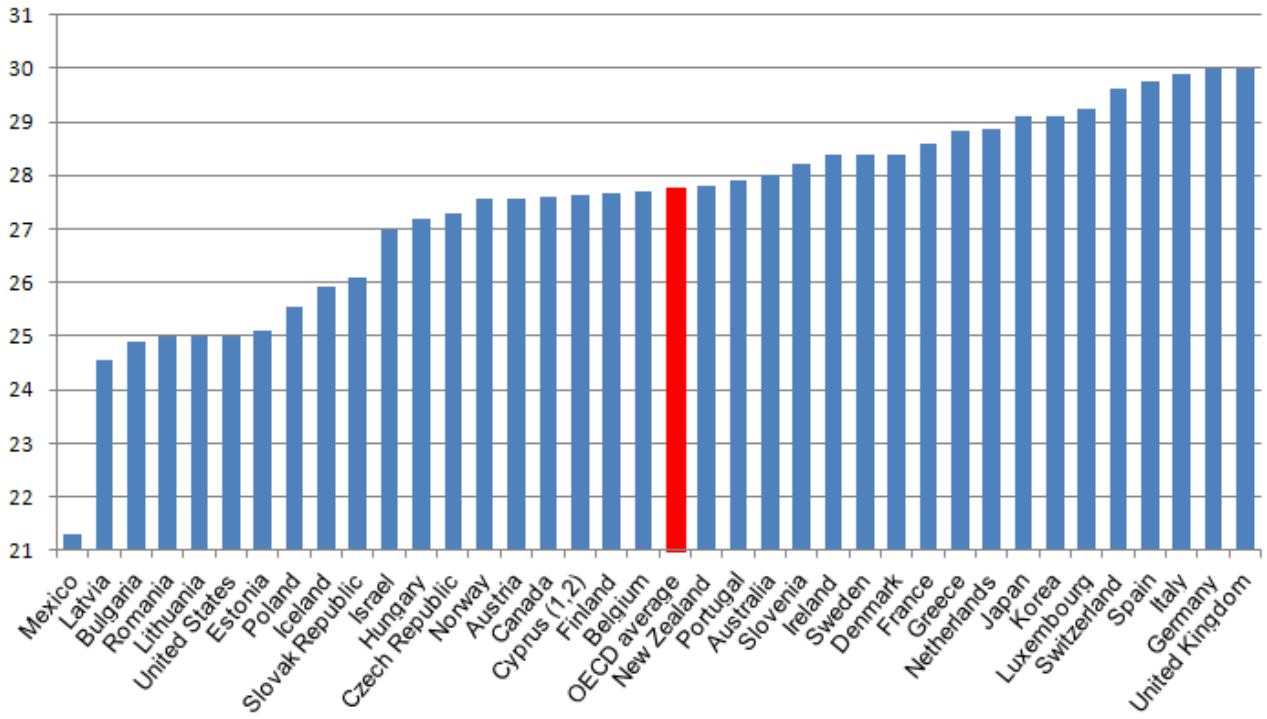

Figure 1.3. Mean age of women at the birth of the first child in 2009.
Countries are ranked in ascending order of the mean age of mothers at first birth in 2009. Source: Eurostat (2011).

While these changes in societal attitude and values are difficult to quantify, they should not be underestimated as a continuing driving force behind the increasing maternal age at childbirth (Benzies *et al.* 2006). This shift in maternity to older ages shows no sign of abating, and it is likely that the majority of children will continue to be born to mothers who are over 30 years of age. Importantly, there is emerging evidence of poor knowledge amongst the general public of the associated risks of attempting pregnancy at older ages; particularly the risk of infertility, feto-maternal morbidity, and risks to offspring (Benzies 2008; Bretherick *et al.* 2010).

1.2.2. Effects on mother and pregnancy of advancing maternal age

Increasing maternal age at childbirth is associated with many changes in maternal physiology, as well as risks to offspring. This complex process will not be discussed in detail, but it is worthwhile very briefly outlining the process of female gametogenesis, and examine how increasing age adversely affects this process. In addition, it is important to discuss how increasing age impacts on maternal physiological aspects that are critical to conception and the birth of a healthy infant.

Female gametogenesis begins early in life, during fetal development when 6–7 million follicles (oocytes surrounded by granulosa cells) are formed by 20 weeks gestation. Thereafter, the process of atresia begins, with a decline in follicle number to 1–2 million by birth, 300,000 by menarche, and a steady decline to less than a few thousand follicles at the end of fertility, with possibly a few hundred follicles remaining in the peri-menopausal period (Egli & Akutsu 2011) (Figure 1.4).

Ovulation begins after menarche and is strictly regulated by a number of processes, including systemic hormones and local factors. Prior to ovulation of a mature oocyte, a primordial follicle is initiated or 'selected' from the pool of follicles through a process that appears to be initially gonadotrophin (FSH, LH) independent (Eppig *et al.* 1997). Subsequent follicle progression and maturation is dependent on FSH and LH, respectively, as well as growth factors, steroids (e.g. oestrogen), amongst others (Buffet & Bouchard 2001; Hillier 1994).

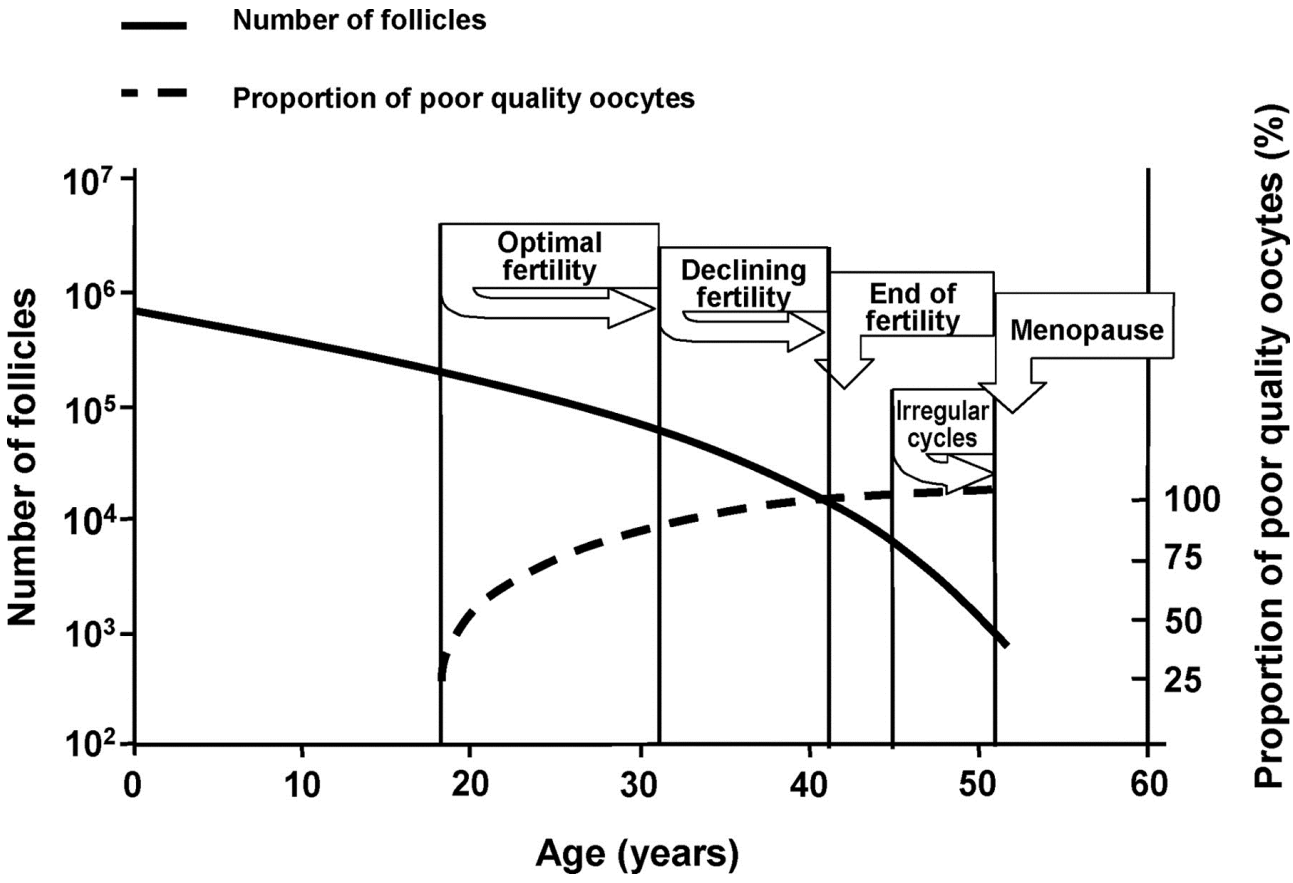

Figure 1.4. Reduction in oocyte pool and declining quality.
Adapted from Broekmans *et al.* (2009).

These processes undergo many changes with increasing age, which impact on oocyte quantity and quality, with consequent risks to offspring.

1.2.2.1. Maternal fertility

One of the most important effects of increasing maternal age at childbirth is reduced fertility. In strict terms, fertility is used to define the actual reproductive rate in population demography (Broekmans *et al.* 2004), and fecundity means the potential ability to reproduce (van Noord-Zaadstra *et al.* 1991). However, here, the term fertility will be used to describe the ability to reproduce or conceive, as it is broadly used in this manner in the medical literature on human reproduction.

It is generally believed that monthly fertility declines from approximately 30 years of age (van Noord-Zaadstra *et al.* 1991). As a result, postponement of parenthood is leading many couples to attempt conception during a time where there is a lower chance of achieving pregnancy.

Studies of fertility are logistically difficult to carry out, but some reliable data have been obtained from populations where contraception is not practised for religious, cultural, or other reasons. For example, Larsen & Yan (2000) calculated monthly conception rate as a function of a woman's age by studying a Christian sect opposed to fertility control. They found that the ability to achieve pregnancy diminished as a woman aged, so that monthly conception rates were 24% at 25 years of age, 17% at 30, 12% at 35, and 5% at 40 years of age. This decline is further illustrated by the reduction in pregnancy and live birth rates in ART with increasing maternal age (Malizia *et al.* 2009). The improvement in ART success rates in older women when donor oocytes from younger women are used (rather than the women's own oocytes) again demonstrates the effect of ovarian aging on pregnancy success (Andersen *et al.* 2009; Sauer & Klein 2011; Wright *et al.* 2008). Figure 1.4 also illustrates the progressive increase in the proportion of poor quality oocytes with increasing maternal age (Broekmans *et al.* 2009).

1.2.2.2. Ovarian aging

There is an inevitable decline in natural fertility with time, which is primarily a consequence of ovarian aging secondary to a fall in oocytes quantity and quality (te Velde & Pearson

2002). There is on-going debate on the rate of decline of ovarian reserve with age. Earlier studies suggested that the decline is bi-exponential, with a slower decline before the age of 35 to 37 years and a more rapid decline thereafter (Faddy *et al.* 1992). However, more recent evidence suggests that there is a steady decline in ovarian reserve from birth until peri-menopause (Broekmans *et al.* 2009; Hansen *et al.* 2008).

Hormonal changes in LH, FSH, inhibin B, and anti-Mullerian hormone also accompany the decline in ovarian reserve (Broekmans *et al.* 2009). While LH levels are recognised to increase with reduction in ovarian reserve, this increase occurs relatively late, and much closer to menopause than any change in FSH (Broekmans *et al.* 2009). FSH levels increase with ovarian aging, with subtle increases from approximately 30 to 35 years old, and a more marked increase in the peri-menopausal phase (Hansen *et al.* 2005). This rise in FSH levels has been linked to declining oocyte numbers, and is thought to be caused by reduced negative feedback of ovarian hormones, particularly inhibin B (Hale *et al.* 2007).

1.2.2.3. Other maternal pregnancy changes with increasing age

As well as the many changes that occur at a gene and hormonal level in mothers with advancing age, women also undergo age-related changes in phenotype and disease risk. As part of increasing age, pregnant mothers are subject to the same increase in morbidity as the general population. In particular, increasing maternal age is associated with greater risk of pregnancy complications, such as diabetes and hypertension, which increase almost linearly with age (van Katwijk & Peeters 1998). Maternal gestational diabetes can lead to offspring risks in the short-term, but is also associated with effects in the offspring, including alterations in insulin sensitivity (Plagemann *et al.* 1997) and increased risk of obesity (Boney *et al.* 2005) in childhood. Also, gestational hypertension (more common in older mothers) is also associated with an increased risk of elevated blood pressure in their offspring during childhood (Himmelmann *et al.* 1994).

Further, women tend to have higher BMI with increasing age (Vahratian 2009). As well as contributing further to pregnancy and peri-natal morbidity, this may impact offspring phenotype and disease risk, as childhood obesity is associated with maternal obesity (Fogelholm *et al.* 1999). Therefore, when assessing childhood outcomes in association with maternal age, all of these factors need to be considered.

1.2.2.4. Twinning

Despite a decline in fertility, increasing maternal age is associated with an increased rate of spontaneous twin pregnancies (Abel & Kruger 2012; Hoekstra *et al.* 2008). Increasing maternal age is associated with elevated levels of FSH, which may drive more than one single follicle into dominance, resulting in 'double ovulation' (Beemsterboer *et al.* 2006; de Koning *et al.* 2008). The increased ovarian stimulation by a higher level of FSH leading to production of more than one follicle has been aptly referred to as "endogenous ovarian hyperstimulation" (Prior 2005). This physiological phenomenon is of interest, such 'endogenous ovarian hyperstimulation' may lead to subtle alterations in oocyte genes or changes in oocyte quality (Prior 2005).

1.2.2.5. Epigenetics

Increasing age and epigenetic changes in humans is attracting much research interest, and there is emerging evidence that epigenetics may play an important role in the aging process itself (Fraga & Esteller 2007). While such research focuses on somatic cells, there is some evidence that germ cells (in particular maternal germ cells) may also be subject to age-related epigenetic changes (Grøndahl *et al.* 2010; Hamatani *et al.* 2004).

Epigenetics is intrinsic to the regulatory process of gametogenesis and the multitude of changes that take place in maternal physiology, including hormonal and other changes in the oocytes themselves may lead to epigenetic changes in oocyte genes. While, there is no current evidence that such maternal age related changes in gametes confer phenotypic differences or age-related changes in offspring, such alterations in phenotype are possible.

1.2.3. Effects on the fetus

1.2.3.1. Aneuploidy and other chromosomal disorders

Increasing maternal age is linked to higher rates of oocyte/embryo aneuploidy, and the increased risk of trisomies (particularly trisomy 21) is well recognised (Pellestor *et al.* 2006). Most aneuploidy is caused by errors in maternal meiosis, and advancing maternal age is a risk factor for most (if not all) human trisomies (Chiang *et al.* 2012). There is evidence that the size of the ovarian reserve or the 'oocyte pool' is related to aneuploidy risk (Kline & Levin 1992): as this 'pool' reduces, aneuploidy risk increases (Figure 1.4) It is also possible that FSH itself may contribute to aneuploidy risk (Oliver *et al.* 2008; Ottolenghi *et al.* 2004;

Warburton 2005), and higher FSH levels with increasing age have been linked to trisomic births (Kline *et al.* 2011). However, there is no definitive proof of a direct causative effect (Kline *et al.* 2011).

1.2.3.2. Congenital abnormalities, imprinting disorders and other genetic changes

Advancing maternal age is linked to a slight increase in the risk of congenital malformations in the offspring (e.g. cardiac defects, diaphragmatic hernia) (Cleary-Goldman *et al.* 2005; Hollier *et al.* 2000).

While there is some limited evidence that advancing maternal age confers a marginally increased risk of imprinting disorders (such as Prader-Willi syndrome, Beckwith Wiedemann Syndrome or Silver Russell Syndrome) in offspring, this risk remains to be established (Hannula *et al.* 2001; Maher *et al.* 2003; Matsubara *et al.* 2011).

1.2.3.3. Obstetric and perinatal complications

It is well recognised that there are higher rates of miscarriage, stillbirth, low birth weight (LBW), SGA, and pre-term delivery with increasing maternal age (Cleary-Goldman *et al.* 2005; Jacobsson *et al.* 2004; van Katwijk & Peeters 1998). The higher miscarriage rate with increasing maternal age can be largely attributed to the increase in aneuploidy rates in older mothers. However, the aetiology of other maternal age-related complications can be mostly attributed to the age related increase in pregnancy complications such as pre-eclampsia, gestational diabetes, caesarean section, obesity, and hypertension (Balasch & Gratacós 2011; Luke & Brown 2007). For example, the increasing proportion of premature (and often LBW babies) deliveries in older mothers are often as a result of medical decisions based on maternal well-being, rather than spontaneous pre-term births (Aliyu *et al.* 2010).

In addition, the higher rate of SGA infants in older mothers (even in those others with no co-morbidity) may be due to subtle placental insufficiency associated with increasing maternal age (Balasch & Gratacós 2011). Indeed, there is evidence of subtle reduction in uterine elasticity and perfusion with increasing age (Cano *et al.* 1995; Zenke & Chetkowski 2004), as well as more subtle alterations in implantation efficiency (Yamada *et al.* 2001).

All of these factors serve to increase the complication rate for mother and baby during pregnancy and the peri-natal period with increasing maternal age.

1.2.4. Post-natal effects in offspring childhood and adulthood

1.2.4.1. Adiposity, height, and pubertal onset

Despite the fact that childhood obesity is the subject of enormous research (Monasta *et al.* 2010), very few studies have examined the possible effects of maternal age on childhood BMI or body composition, and none have examined this issue with maternal age as the primary factor (Blair *et al.* 2007; Reilly *et al.* 2005). Reilly *et al.* (2005) found that children of mothers aged less than 25 years had a higher obesity risk than those born to mothers aged over 25. Blair *et al.* (2007) found a weak relationship between increasing maternal age and childhood adiposity, but 40% of the children were born SGA (which also impacts on childhood adiposity (Chernausek 2012; Norris *et al.* 2012).

One recent study in Japan assessed data from "well-baby checks" of over 50,000 subjects, and found no association between maternal age and height at 1 and 12 months of age (Iwayama *et al.* 2011). However, their method of height measurement was not described, and genetic height was not accounted for (Iwayama *et al.* 2011). No other studies assessing the impact of maternal age on childhood height appear to have been carried out.

Increasing maternal age has also been associated with later age at menarche in girls (Blell *et al.* 2008; Comings *et al.* 2001), but such findings have not been replicated in larger studies. Nevertheless, these are potentially important findings, as later age of menarche is associated with relatively shorter stature in childhood (Johansson & Ritzén 2005). Thus, studies assessing the effects of maternal age at childbirth on childhood height need to consider the possibility of differences in childhood biological maturity with advancing maternal age.

1.2.4.2. Diabetes mellitus

A recent meta-analysis of 30 studies found that increasing maternal age at childbirth is associated with a small linear increase in type 1 diabetes risk in the offspring. While possible childhood immune differences or changes in birth weight were offered as possible explanations, no convincing mechanism was described (Cardwell *et al.* 2010). An association between advancing maternal age with type 2 diabetes risk in adulthood (Lammi *et al.* 2007) and insulin resistance in twins (Loos *et al.* 2002) has also been described. These authors proposed that the finding may be related an increase in fetal restriction in the later part of pregnancy, leading to altered fetal programming of childhood and adult disease (Loos *et al.* 2002).

1.2.4.3. Blood pressure

Increasing maternal age at childbirth has also been found to be associated with increased offspring blood pressure in the neonatal period (Gillman *et al.* 2004) and in childhood (Lawlor *et al.* 2004; Whincup *et al.* 1989). Gillman *et al.* (2004) found a blood pressure increase of 0.8 mmHg in newborns per 5 year increase in maternal age, with a similar observation amongst 5-year-olds (Lawlor *et al.* 2004; Whincup *et al.* 1989). The possible mechanism proposed for this increase in BP by the authors is similar to that proposed for insulin resistance (i.e. possible fetal nutrient restriction in older mothers leading to altered fetal programming and subsequent increase in BP) (Edwards *et al.* 1993).

1.2.4.4. Cancer and lifespan

Several studies (some of which were conducted in large populations) have shown a slight increase in cancer risk amongst children in association with increasing maternal age at childbirth (Hemminki & Kyyrönen 1999; Hemminki *et al.* 1999; Johnson *et al.* 2009; Yip *et al.* 2006). Cancers implicated include leukaemia, retinoblastoma, and brain tumours, amongst others. While mechanisms remain elusive, it is proposed that the increased risk may be associated with epimutations in maternal oocyte genes (Johnson *et al.* 2009) or chromosomal aberrations in oocytes (Yip *et al.* 2006). Advancing maternal age at childbirth has also been linked to an increased risk of cancer in the offspring in adulthood, such as breast cancer (Innes *et al.* 2000; Xue & Michels 2007). Proposed triggers of this risk include higher pregnancy oestrogen levels observed with increasing maternal age (Trichopoulos 1990).

It has also been suggested that increasing maternal age at childbirth is associated with changes in offspring lifespan (Gavrilov & Gavrilova 1997). However, such inferences were based largely on historical and no reliable conclusions can be drawn.

1.2.5. Can increasing maternal age at childbirth benefit the offspring?

We have outlined above many complications and risks associated with increasing age at motherhood. However, it is important to consider that those mothers who give birth in their 30s or 40s may actually provide an advantageous pre- and post-natal environment to their offspring.

For example, there is a suggestion that older mothers may have a healthier lifestyle during pregnancy (Mohsin *et al.* 2011). However, it must be emphasised that increasing maternal

age at child-birth is strongly linked to higher socio-economic status and better education and these may be the reasons for healthier pregnancy behaviours with increasing maternal age (Mills *et al.* 2011).

Similarly, increasing maternal age was found to be associated with higher rates and a longer duration of breastfeeding (Scott *et al.* 2001). Again, higher socio-economic status and better education may be the driving force behind breastfeeding behaviours (Thulier & Mercer 2009). Increasing maternal age may confer other possible advantages upon their children, such as better maternal knowledge on nutrition and greater financial security, amongst others (Mills *et al.* 2011).

Possible disadvantages of older ages at motherhood include findings that older mothers tend to have less energy than their younger counterparts (Sayer *et al.* 2004), and are therefore likely to engage in less active playtime with their children (MacDonald & Parke 1986). On the whole, there is a paucity of reliable data on the possible effects of the post-natal rearing environment in association with maternal age.

Overall, increasing maternal age is associated with many physiological changes in the mother, as well as an increased risk of chromosomal disorders and other complications to offspring. There is some evidence that increasing maternal age is also associated with a higher risk of some childhood diseases. Clearly, there is a paucity of data on the possible effects of maternal age at childbirth on offspring outcomes.

1.3. The impact of increasing paternal age at childbirth on the growth & metabolism of children

1.3.1. Patterns and causes

While advancing maternal age at childbirth has received much attention, a simultaneous and possibly larger and more rapid upward shift in the age of fatherhood is taking place. The mean paternal age at first childbirth in the United Kingdom increased from 29 years of age in 1980 to 32 years in 2000 (Martin *et al.* 2009; Office for National Statistics 2002). In tandem with this increase in paternal age, by 2003, over 40% of children in the UK were born to fathers over 35 years of age, while a further 10% of children were born to fathers aged over 40 (Office for National Statistics). In Germany, the mean age of fathers increased from 31.3

to 33.1 years between 1991 and 1999 (Kühnert & Nieschlag 2004). While information on paternal age at childbirth is lacking for many countries, a similar trend is most likely present in many other countries in the Western world (Bray *et al.* 2006).

The ages of men and women in couples are relatively closely correlated in the majority of cases. As a result, factors influencing maternal age at childbirth also impact on paternal age at childbirth, and vice-versa. However, it is thought that maternal choice as to when she wishes to have children has one of the strongest influences in the upward trend of paternal age at first fatherhood (Mills 2005).

Other factors leading to the increase in paternal age at childbirth are somewhat similar to those of advancing maternal age of first childbirth. The tendency amongst men to wait for personal economic stability before deciding to have children is described as another important factor (Roberts *et al.* 2011). Further, there seems to be a widespread belief amongst men that they can perpetually postpone fatherhood, due to a reduced sense of a 'biological clock' (Langdridge *et al.* 2005; Roberts *et al.* 2011). While the pattern of increasing paternal age at childbirth is common to all societal groups, a more advanced age at fatherhood is more prominent amongst men of higher socio-economic status (Mills *et al.* 2011). This is particularly important when assessing possible effects of paternal age on childhood outcomes.

1.3.2. Effects on paternal fertility

Charlie Chaplin, William Shatner, Anthony Quinn, and many other famous men fathered children in their 70s or 80s. Such stories contribute to the myth of eternal fertility for all men.

Unlike female fertility (which ceases relatively abruptly) male fertility usually undergoes a steady decline over the years. While subject to marked individual variability, changes in male fertility are associated with increasing paternal age (Hassan & Killick 2003; Kidd *et al.* 2001; Sartorius & Nieschlag 2010). These include a reduction in sperm motility, sperm counts, semen quality, and androgen levels (Belloc *et al.* 2008; de La Rochebrochard & Thonneau 2002; Handelsman 2002; Pasqualotto *et al.* 2005). There is also little doubt that with increasing age, male sexual dysfunction increases (Nicolosi *et al.* 2006) and frequency of intercourse decreases (Weinstein & Stark 1994). This is notwithstanding other factors associated with increasing age such as the increased risk of obesity (Carone *et al.* 2010), which also impacts on fertility (Hammoud *et al.* 2008).

Environmental toxicants may also impact on male fertility, but so far studies have yielded conflicting data (Sartorius & Nieschlag 2010; Sharpe 2012). This will be explored in more detail in the section on Fertility. However, it is obvious that increasing male age inevitably leads to a more prolonged period of exposure to such toxicants and thus they may be a contributory factor to age related changes in male fertility (Delbès *et al.* 2010).

1.3.3. Effects on the fetus

1.3.3.1. DNA damage, genetic disorders, and epigenetics

There is debate as to whether advancing paternal age confers an independent slight increased risk of offspring aneuploidy, but maternal age clearly has a far stronger effect (Fonseka & Griffin 2011; Sloter *et al.* 2004; Wyrobek *et al.* 2006).

However, increasing paternal age has been linked to an increase in the risk of gene mutations in offspring (Crow 2000). It is increasingly recognised that the quality of male gametes deteriorates with increasing age, leading to higher rates of DNA damage and gene mutations in the sperm (Barratt *et al.* 2010; Singh *et al.* 2003; Slama *et al.* 2005; Wyrobek *et al.* 2006). Male germ cells undergo mitotic division every 16 days and there is potential for error with each replication. The potential for de novo mutations in male germ cells increases exponentially with age (Crow 2000). This is thought to be at least partially responsible for the observed increase in the frequency of single-gene defects in offspring of fathers of advancing age (Chandley 1991; Crow 2000).

DNA repair is a crucial process in the genetic competence of male sperm DNA, and there is accumulating evidence that this process is vulnerable to age-related changes in males. Consequently, increasing paternal age is likely to be associated with greater likelihood of defective DNA repair (Leduc *et al.* 2008 1290), increased DNA fragmentation (Singh *et al.* 2003 1291), and reduced chromatin integrity (Wyrobek *et al.* 2006). Such paternal age-related DNA "copy errors" (Penrose 1955) may be responsible for the greater frequency of congenital disorders in the offspring, such as Apert syndromes (Crow 2000; Wyrobek *et al.* 2006), situs inversus (Lian *et al.* 1986), heart defects, tracheo-oesophageal fistula, oesophageal atresia, and musculoskeletal anomalies (Yang *et al.* 2007).

Increasing paternal age is also associated with greater risk of other disorders in their offspring, such as autism (Reichenberg *et al.* 2006), childhood behavioural problems, and

adult-onset diseases including schizophrenia (Malaspina 2001) and bipolar disorder (Frans *et al.* 2008). These disorders may be caused by an age-related increase in the frequency of DNA point mutations in sperm (Flatscher-Bader *et al.* 2011). Alternatively, the underlying mechanism may be age-related epigenetic changes in sperm DNA (Farrer *et al.* 1991; Peedicayil 2010; Perrin *et al.* 2007).

There is increasing evidence that epigenetic changes may contribute to the normal "aging process" in humans. Thus, epigenetic change in somatic cells is a focus of intense interest, particularly in cancer research (Fraga & Esteller 2007). However, the possible association between increasing paternal age and epigenetic changes in germ cells is also of some interest (Probst *et al.* 2009). This is because there are suggestions that epigenetic changes taking place in sperm DNA with increasing paternal age may be transmitted to offspring (Curley *et al.* 2011; Oakes *et al.* 2003). The possible extent of the transmission of such changes and any impact they may have of offspring phenotype remains uncertain (Curley *et al.* 2011).

1.3.3.2. Obstetric and perinatal complications

Independently of maternal age, increasing paternal age has been reported to be associated with complications during pregnancy and in the peri-natal period (Chen *et al.* 2008). These include increased rates of miscarriage (Kleinhaus *et al.* 2006; Lian *et al.* 1986), maternal pre-eclampsia (Harlap *et al.* 2002), as well as an increased risk of caesarean section (Tang *et al.* 2006), low birth weight, pre-term delivery (Astolfi *et al.* 2006; Zhu *et al.* 2005), and twinning (Abel & Kruger 2012). Post-natally, advancing paternal age has been associated with reduced APGAR scores (i.e. poorer physical condition at birth) in one study (Sun *et al.* 2006).

1.3.4. Outcomes in offspring childhood and adulthood

1.3.4.1. Neurological disorders

There are limited data on the impact of paternal age on health outcomes in their offspring, and most have focused on neurological outcomes (Liu *et al.* 2011b). Several studies have shown an increased risk of autism in children of older fathers, and the risk appears to be greatest when fathers are aged over 35 years, and especially over 40 years old (Croen *et al.* 2007; Durkin *et al.* 2008; Reichenberg *et al.* 2006). While there is suggestion of a possible maternal age effect on offspring autism risk (Croen *et al.* 2007; Durkin *et al.* 2008), the

association with paternal age is far more consistent, particularly in more robust studies (Reichenberg *et al.* 2006).

Other neurological manifestations in childhood linked to increasing paternal age (either >35 or >40 years) are behavioural problems (Saha *et al.* 2009), lower IQ (Auroux *et al.* 1989; Malaspina *et al.* 2005), poorer neurocognitive outcomes (Saha *et al.* 2009), poorer social functioning (Weiser *et al.* 2008), and epilepsy (Vestergaard *et al.* 2005). Psychiatric illness such as schizophrenia (Peedicayil 2010) and bipolar disorder (Frans *et al.* 2008) are also more frequently observed amongst adult offspring born to fathers aged more than 50 or 55 years old.

It seems likely that the increased rates of *de novo* mutations in germ cells of older fathers are responsible for the higher rates of neurocognitive and psychiatric disorders in their offspring (Flatscher-Bader *et al.* 2011). However, several authors have cautioned that the personality of older fathers, (particularly those aged more than 50 years old) may be quite different to those of younger fathers, with an increased rate of psychiatric illness and anti-social personalities amongst men who father children at older ages (Hemminki & Kyyrönen 1999; Zammit *et al.* 2003). Therefore, it is possible that this factor contributes, at least in part, to the offspring risk of psychiatric disorders. Nonetheless, it is likely that the contribution of paternal age related *de novo* mutations are responsible for the majority of the offspring risk.

1.3.4.2. Adiposity, height, and pubertal onset

While causative factors of childhood obesity continue to be intensively investigated (Monasta *et al.* 2010), a possible independent impact of paternal age has been examined in surprisingly few studies to date. Advanced paternal age is associated with higher socio-economic status (Mills *et al.* 2011), which in itself is protective against childhood obesity (Parsons *et al.* 1999; Wang & Beydoun 2007) and consequently a major confounder for studies examining such outcomes. Only one study has assessed the potential effects of paternal age on childhood BMI, finding no difference between children of fathers aged over 37 years compared to those under 37 (Huus *et al.* 2007). However, it is unclear if the analysis was adjusted for socio-economic factors.

While many influences on childhood height have been examined (Batty *et al.* 2009), the possible impact of paternal age has not been investigated. One study has assessed the possible impact of paternal age on the timing of their daughters' menarchal onset, finding no effect

(Shrestha *et al.* 2010). The latter is important when we consider that the timing of menarche influences childhood growth and that earlier menarche may lead to shorter final height.

1.3.4.3. Diabetes mellitus and cancer

While advanced maternal age has been associated with a slightly increased risk of type 1 diabetes in childhood, there is limited evidence of an independent paternal age effect (Cardwell *et al.* 2010). Three studies found that a paternal age of over 30 or 35 years was associated with a minor increase in the risk of type 1 diabetes in children (Bingley *et al.* 2000; Cardwell *et al.* 2005; Memon *et al.* 2011), whereas one study found no effect (Stene *et al.* 2001).

A slight increase in the incidence of leukaemia and certain types of brain tumours in children has been linked with older paternal age (Chen *et al.* 2008; Hemminki & Kyyrönen 1999; Hemminki *et al.* 1999; Huus *et al.* 2007; Lu *et al.* 2010; Yip *et al.* 2006). The increasing age at which parents are having children may be linked to the observed increase in the incidence of certain childhood cancers over the past 30 years, possibly a result of age-related mutations in male germ cells (Yip *et al.* 2006). Similarly, adult onset cancers have also been linked to advancing paternal age, including breast cancer (Choi *et al.* 2005), prostate cancer (Zhang *et al.* 1999), and non-Hodgkin's lymphoma (Gavrilov *et al.* 1997).

1.3.4.4. Mortality

Two Scandinavian studies have explored possible associations between paternal age and mortality in the offspring, from age 1–18 years in Denmark (Zhang *et al.* 1999) and 1–39 years in Finland (Zhu *et al.* 2008). The Danish study found that fathers aged over 45 years had children with a higher mortality rate due to congenital malformations, injury, or poisoning (Zhang *et al.* 1999). Similarly, the Finnish study also found that a paternal age over 45 years was an important threshold of increased mortality in the offspring, but causes of death were not outlined in detail (Zhu *et al.* 2008).

Several studies, which took place in developing countries, also examined for a possible association between increasing paternal age and childhood mortality (Becher *et al.* 2004; Brittain 1992; Miller *et al.* 2010). Two studies found no effect (Becher *et al.* 2004; Miller *et al.* 2010), and another observed a higher mortality rate amongst children of older fathers (Brittain 1992). However, any relationship between paternal age and childhood mortality in developing countries is likely confounded by important socio-economic factors, which would

strongly influence the availability of food as well as childhood disease risk. Thus, a real effect of paternal age itself is difficult to elucidate.

Increasing paternal age has also been described to be associated with reduced longevity in the offspring, albeit in historical cohorts (Gavrilova *et al.* 2003; Larfors *et al.* 2012). Interestingly, the effect of paternal age was much greater amongst daughters of older fathers, losing an average of 4.4 years of their life span. While these limited studies have several methodological weaknesses, they further stimulate interest in the possible impacts of paternal age on offspring phenotype and disease risk.

Overall, there is emerging evidence that increasing paternal age is associated with gene and other changes in male gametes, with associated risks to offspring. There is evidence of an association between increasing paternal age and disease risk in their offspring; particularly neurological and behavioural disorders. Any association between increasing paternal age and changes in childhood phenotype and metabolism is unknown.

1.4. Birth order

1.4.1. The increasing proportion of first-borns

There is an established trend towards couples having fewer children, leading to a continuing increase in the proportion of first-born relative to later-born children in the population (Sobotka & Toulemon 2008). The trend towards two-child families began early in the 20th century and has continued unabated. However, over the past two decades an increasing proportion of couples are deciding to have just one child (Sobotka & Toulemon 2008).

There is a relative paucity of accurate population statistics which directly examine the number of children borne by each couple. However, information from Eurostat and the OECD point towards an on-going reduction in the number of couples having more than two children (Eurostat 2011; OECD 2011a).

The total fertility rate (TFR) and data on household size (the number of persons in the household) also help provide a crude estimate of family size. TFR is the average number of children that would be born to a woman over her lifetime. TFR has fallen dramatically in OECD countries over the past thirty years, from 2.7 children per woman in 1970 to just 1.7 in

2009 (OECD 2011a). Figures in Europe from 2008 show a TFR of just 1.56 live births per woman (Eurostat 2011). However, it must be noted that these figures are not a direct reflection of a reduction in family size *per se*, as there are an increasing proportion of women choosing not to have any children. Nevertheless, these figures do help demonstrate the trend of a reduction in the number of children being borne by couples.

While some data is available on household size (OECD 2011a), this figure reflects all occupants in the same household, which may not comprise exclusively of parents and children. Therefore the household occupants may comprise of relatives or adult children. This may vary widely between countries and socio-economic groups depending on cultural practises as well as economic circumstances. Nevertheless, this data demonstrates a fall in household size over the past thirty years (OECD 2011a), and provides some useful information on the number of children being borne by couples.

Thus, it is evident that the proportion of first born children in the population in the developed world is increasing and it is likely that first borns represent at least 50% of children born today.

There is some optimism that these trends will change over the coming decades, based on the continuing desire of most couples for two or more children (Bacci 2001). However, for a variety of reasons, many couples do not achieve their desired number of children.

Reasons for the reduction in family size are increasing maternal age at childbirth, personal choice and socio-economic factors, amongst other reasons (Joffe *et al.* 2009).

There is overlap between the reasons for the reduction in family size and those associated with the increase in maternal and paternal ages at first childbirth (Mills *et al.* 2011). The trend towards older maternal and paternal ages at first childbirth is pushing an increasing proportion of couples into an age range where their fertility may be in decline. This is risk is particularly great among women who are highly educated and pursuing a career, who will often postpone childbirth beyond 35 years of age (Joffe *et al.* 2009). There is increasing evidence that decline in biological fertility may be a more important reason for smaller family size than previously thought (Jensen *et al.* 2002; Joffe *et al.* 2009; Leridon & Slama 2008). Furthermore, it has been demonstrated that couple who take more than 12 months to conceive their first child are less likely to have subsequent children (Joffe *et al.* 2009). Therefore, by virtue of postponing starting a family, many couples do not realise their desired family size.

Personal choice of having fewer children is an on-going trend, whereby the "normal" or socially acceptable family size is one or two children. Indeed, personal choice on family size evolves with increasing age, whereby a person's desired number of children reduces as they themselves get older (Liefbroer 2009). This was well demonstrated in a recent study which demonstrated that the majority of adults aim for smaller families with increasing age (Liefbroer 2009). Thus, the increase in mean parental age at first childbirth is not only leading to a biological impact on fertility, but also has an impact on future parent's plans regarding family size.

Governments often have financial incentives in place in an effort to increase total fertility rates. Examples include tax refunds, 'children's allowance', and extended paid maternity leave, amongst other benefits. Despite these incentives, couples may refrain from having more than one or two children for financial reasons. Recent figures from the New Zealand Inland Revenue Department put the cost of raising a child at NZ$ 300–500 per week (Claus *et al.* 2009). This increases by approximately NZ$ 150–200 dollars per week for each additional child. Such costs inevitably exert an influence the number of children a couple decide to have (Gauthier 2007).

The global reduction in marriage rate also has had an impact on family size. It is evident that many no longer perceive marriage as a pre-condition for childbirth, which is demonstrated by the fact that 37% of children in Europe were born outside of marriage in 2009 (Eurostat 2011) (Figure 1.5). Couples who are co-habiting rather than officially married are more likely to have fewer children, which may be related to a perceived or real reduction in family stability (Beets *et al.* 1998; Liefbroer 2009).

It is clear that the causes of the observed reduction in family size are inter-dependent and multi-factorial. One exception is the "one-child" policy in China that has been in effect for more than 30 years, which has also favoured the birth of boys over girls. While current thinking is that this policy may change, it is clear that at least 50% of the Chinese population were heavily influenced towards having only one child since its implementation (Hvistendahl 2010). This has had an enormous impact on the current population in China, where men outnumber women by 1.2:1 (Branigan 2011). It also has obvious major implications in terms of the proportion of first- compared to later-borns, when we consider the total births per annum in China exceeds 16 million (UNICEF 2010). Nonetheless, it is clear that overall the

proportion of first-born children continues to grow on a global scale with no signs of changing.

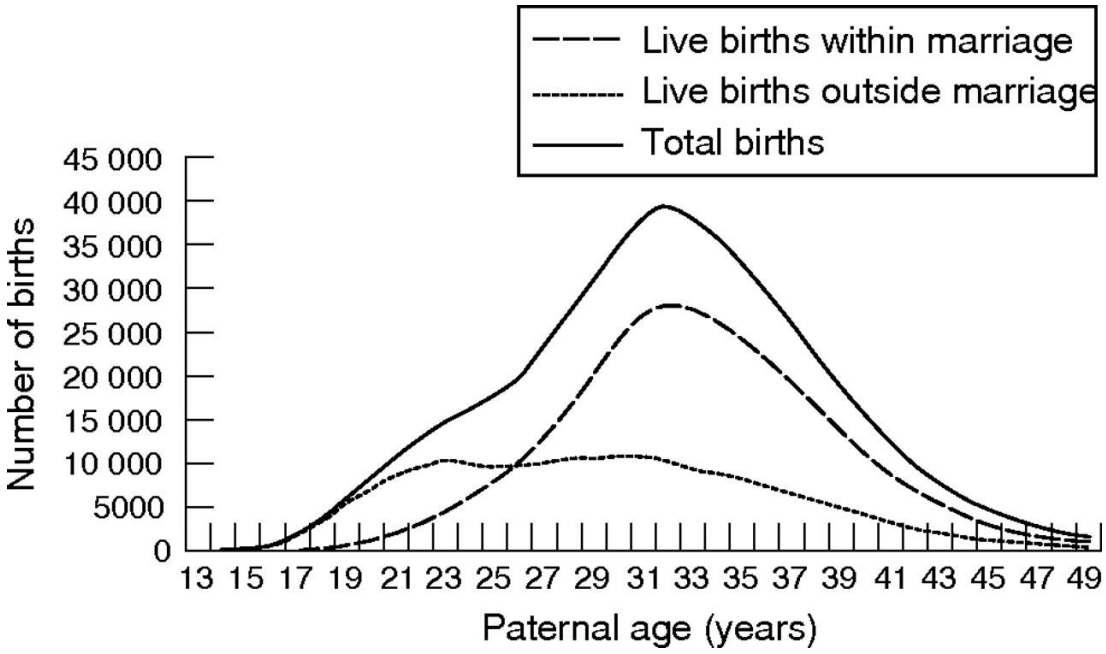

Figure 1.5. Distribution of paternal age for births in England and Wales in 2003.
Total births and live births within and outside marriage to fathers aged 13–49. Reproduced and adapted with permission from Bray *et al.* (2006).

1.4.2. Impact of birth order on offspring phenotype and disease risk

1.4.2.1. Birth weight

It is established that first-borns are approximately 200 grams lighter at birth compared to later-borns among offspring born at term (>37 weeks gestation) (Oken *et al.* 2003; Shah 2010). While there is no clearly defined mechanism for this weight difference, it is most likely associated with differences in implantation and placentation between first and subsequent pregnancies.

Normal implantation is an intrinsically inefficient process. Approximately 50% of pregnancies do not continuing beyond 20 weeks, and 75% of failed pregnancies are due to ineffective implantation (Wilcox *et al.* 1988). This inefficiency may be partially explained by the need for a well synchronized and efficient interaction of many physiological factors for successful implantation, including local paracrine and autocrine factors, as well as molecular and cellular interactions (Norwitz 2006; Norwitz *et al.* 2001). Furthermore, other factors (such as maternal hormonal levels) are also important, and altered maternal oestrogen levels have been shown to effect implantation rates (Ma *et al.* 2003).

Trophoblast invasion into the uterine endometrium and myometrium are critical steps, which when altered may affect pregnancy outcomes (Norwitz 2006). Uterine receptivity to the invading trophoblast is also critical to successful implantation (Aplin & Kimber 2004). Endometrial angiogenesis and vascular permeability are just two examples of the many changes that take place in the uterus at the time of early implantation. Such changes are dependent on over thirty known cytokines and growth factors (including IGF-I and IGF-II) that are required for effective endometrial receptivity (Norwitz 2006). Ineffective or inadequate trophoblastic invasion into the uterine wall may establish a poor flow of nutrients to the fetus from the outset, and this is one of the possible mechanisms leading to intra-uterine growth restriction (IUGR) (Bjercke 1999; Goldman-Wohl & Yagel 2002).

It has been proposed that implantation during first pregnancy leads to permanent changes in the uterine wall, which remain for subsequent pregnancies (Khong *et al.* 2003). Khong *et al.* (2003) carried out histological examination on hysterectomy specimens from women of varying parity, finding that first parity permanently alters the uterine anatomy. Anatomical changes in spiral arteries after first pregnancy may modify vascular remodelling in the next pregnancy, likely leading to improved nutrient flow to the fetus in subsequent pregnancies (Khong *et al.* 2003). They suggested that this process may explain the difference in birth weight (Khong *et al.* 2003). Their findings were supported by later findings of more enhanced trophoblast invasion in non-primiparous pregnancies (Prefumo *et al.* 2006), and increased materno-placental perfusion in later compared to first pregnancies (Hafner *et al.* 2000). According to Hafner *et al.* (2000) this increased flow demonstrated improved trophoblastic invasion, and consequently better dilatation of spiral arteries in the second and subsequent pregnancies. These is some evidence from animal models to support these assertions at a molecular level, as the expression of vascular endothelial growth factors was shown to be greater in non-primiparous pregnancies (Litwin *et al.* 2010).

Immunological changes are another proposed mechanism behind the difference in birth weight between first and later pregnancies (Vernier *et al.* 2010). There is evidence suggesting that a mother's immune system perceives non-primiparous pregnancies to be less antigenic, allowing more efficient invasion of the uterine wall by the trophoblast (Vernier *et al.* 2010). However, the immunological differences between first and subsequent pregnancies are far more complex, and are explored in detail elsewhere (Moffett & Loke 2006; Sacks *et al.* 1999).

Nevertheless, while alternative mechanisms are possible, the proposed mechanism that first pregnancies confer permanent anatomical changes to the uterus leading to improved fetal nutrition in subsequent pregnancies remains the most plausible explanation for the higher birth weight in later-born offspring.

This difference between first and later pregnancies is of importance when we consider the observed differences in the outcomes between first born and later born offspring.

1.4.2.2. Height

Several studies have found an association between first birth order and taller stature, in both children (Tanner 1989; Wells *et al.* 2011; Wingerd & Schoen 1974) and adults (Ghosh & Bandyopadhyay 2006; Siervo *et al.* 2010). However, these findings are not always consistent, and two studies found no height differences in childhood across birth order groups (Hermanussen *et al.* 1988; Whitley *et al.* 2008). Importantly, none of the childhood studies corrected for parental height, which is the most important determinant of childhood stature (Tanner *et al.* 1975). Furthermore, all studies included populations across the socio-economic spectrum. Families of lower socio-economic status tend to have larger families compared to those of higher socio-economic status (Newman 2009). Importantly, socio-economic status has a strong impact on height (Silventoinen 2003). As a result, the outcomes from all these studies were likely affected by confounding factors. The authors generally did not put forward a convincing mechanism for the taller stature of first-borns.

There is some evidence that onset of puberty is earlier in first-borns (Apraiz 1999; Morris *et al.* 2010), and this means that childhood biological maturity needs to be considered when assessing any association between birth order and childhood height.

1.4.2.3. Body mass index, body composition, and obesity

There is an association between being first-born and an increased risk of a higher body mass index (BMI) and/or a less favourable body composition in adults (Dahly & Adair 2010; Reynolds *et al.* 2010; Siervo *et al.* 2010; Stettler *et al.* 2000).

Wells and Siervo have published several studies on findings from assessment of the 1993 Pelotas Birth Cohort (Victora *et al.* 2008). They found that adult first-borns had higher BMI and fat mass than later-borns in one study (Siervo *et al.* 2010); and in the other, found that first-born 4-year-olds had increased BMI, but no difference in BMI or weight was found at adolescence (Wells *et al.* 2011). Recently, Reynolds *et al.* (2010) assessed 267 adult finding higher BMI and fat mass in first-borns. A large study by Dahly & Adair (2010) assessing 970 Filipino adults found that being first-born conferred a greater risk of central adiposity, irrespective of socio-economic status. Stettler *et al.* (2000) assessed 447 African American adults and described a strong association with first-born status and increased BMI. The authors proposed that first-borns had increased adiposity by virtue of their relative restriction *in utero*.

The association between first-born status and higher BMI and fat percentage has been assessed as one of many outcomes in studies by Ong & Dunger in assessments of the ALSPAC cohort (Ong *et al.* 2009; Ong *et al.* 2000; Ong *et al.* 2002b). However, these authors argue that the relationship between first-born status and later increased risk of obesity is primarily associated with lower birth weight and more rapid catch-up growth, rather than first-born status itself. As already outlined, first-borns tend to be lighter at birth, and therefore tend to have a higher risk of excessive catch up growth which may be responsible for increased adiposity. While this infers an association between first-born status and higher BMI; the association is not predominantly dependent on first birth per-se. It appears to be dependent the associated lower birth weight of first-borns, their consequent more rapid post natal weight gain and risk of obesity. This is an important differentiation when one considers possible mechanisms and causation for these associations.

1.4.2.4. Blood pressure, metabolism, and glucose homeostasis

Higher blood pressure in first- compared to later-borns has been described in children (Lawlor *et al.* 2004; Whincup *et al.* 1989) and adolescents (Siervo *et al.* 2010; Wells *et al.* 2011). For example, Wells *et al.* (2011) found that adolescents who were first-borns had a BP

which was 4 mmHg higher than their later born counterparts. In contrast, Johnson *et al.* (1965) observed no effects of birth order on blood pressure in adulthood.

A slightly less favourable lipid profile among first-born adults has been described in one study (Siervo *et al.* 2010). However, these first-born adults also had a higher BMI, which is well recognised as a risk factor for poorer lipid profiles (Must *et al.* 1999). A recent study found that first-borns have a slightly increased risk of type 1 diabetes (Cardwell *et al.* 2011), with relatively lower exposure to early childhood infection compared to later-born children proposed as a possible mechanism. Dunger *et al.* (2007) have described reduced insulin sensitivity in a proportion of first-born children with higher BMI and greater adiposity. However, this reduced insulin sensitivity was related to their smaller size at birth and greater adiposity in childhood, rather than a direct association with birth order (Dunger *et al.* 2007).

Overall, there is some evidence that the first-born fetus is subject to a degree of relative *in utero* restriction compared to later-borns. First- and later-borns also appear to have phenotypic differences and differing disease risks. The possible differences in phenotype and metabolism between first- and later-borns require further exploration.

1.5. Fertility treatment

1.5.1. Background and demographics

Fertility treatment dates back to the first century, when Shang Han Lun (the oldest published medical textbook) suggested the use of herbal remedies and acupuncture for treatment of infertility (Dharmananda 1989). The emergence of fertility treatment in 'conventional' medicine occurred in the mid-20th century, with the use of ovulation induction (Anonymous 1946). Over the past 60 years, there have been huge advances in fertility treatment, and work in this area continues to expand.

The demand for fertility treatment and the number of children conceived with the help of fertility medications and ART has increased exponentially, particularly in the past two decades. Epidemiological data suggest that 8–20% of couples experience difficulty conceiving after 12 months of unprotected intercourse, and that 1 in 10 women of reproductive age have had a healthcare visit associated with infertility (Chandra *et al.* 2005; Sunderam *et al.* 2009).

In industrialised countries, it is estimated that 1 to 4% of all children are conceived with the help of ART (De Mouzon *et al.* 2010; Schieve *et al.* 2009), but rates vary widely between countries, largely due to differences in state funding for fertility treatment (De Mouzon *et al.* 2010; Sunderam *et al.* 2009). In Europe, the proportion of ART conceived infants ranges from 1% in Italy, 1.5–1.7% in Germany, France and UK, and as a high as 4.1% in Denmark (De Mouzon *et al.* 2010). However, the rate of ART births continues to increase steadily. In the USA for example, the number of children conceived by ART rose from 20,000 in 1996 to 55,000 in 2006 (1.2% of all live births) (Sunderam *et al.* 2009).

Fertility medication treatment (without ART) is widely used in clinical practise, but the number of children conceived with their help is unknown, as there is a lack of reliable data for most countries (De Mouzon *et al.* 2010). Nonetheless, it is estimated that 2.5 to 7% of children in Western countries are conceived with the help of fertility medications or ovarian stimulation alone (De Mouzon *et al.* 2010; Schieve *et al.* 2009). Practice varies widely between countries regarding the prescription of fertility drugs. For example, in some countries such as New Zealand the use of drugs such as clomiphene citrate can only be prescribed by specialists (The Pharmaceutical Management Agency of New Zealand 2011). However, in some European countries and the USA more liberal policies allow these drugs to be prescribed by general practitioners (EMA 2011; FDA 2011). More recently, a large number of fertility medications have become available for purchase on the internet without prescription. These factors make it extremely difficult to accurately ascertain the number of children conceived with the assistance of fertility drugs.

However, population studies in the USA have attempted to evaluate the use of fertility drugs among the general population. According to data on approximately 6,000 births from 1997–2002, 4.2% of women reported the use of some type of maternal fertility treatment, 1.0% reported ART use, 1.6% reported clomiphene citrate use without ART, while 1.7% reported the use of other fertility treatments (Duwe *et al.* 2010). These figures of non-ART fertility treatment is lower than that obtained by Schieve *et al.* (2009), who calculated that 4.6% of children born in the USA in 2005 were conceived via non-ART fertility treatments. In Europe, estimates suggest that percentage of infants were born after non-ART fertility treatments was 2.5% in Finland (Gissler 2003) and 2.3% in Denmark (Nyboe Andersen & Erb 2006). Overall, it is clear that children conceived with the help of fertility drugs comprise a significant proportion of the population.

1.5.2. Factors responsible for the increased uptake of fertility treatment

The reasons for the exponential increase in the use of fertility treatment are multifactorial and complex. These include the increasing maternal and paternal ages when couples are attempting parenthood, the availability of fertility treatment itself and its funding by government agencies. Other more complex reasons include changing societal beliefs whereby subfertility or infertility have become less acceptable, leading to couples demanding fertility treatment rather than accepting their difficulty or inability to conceive as their 'fate'.

As outlined previously, fertility declines with increasing maternal and paternal ages, and current societal trends mean that an increasing proportion of couples are attempting parenthood at a time when their fertility is in decline (Ledger 2009). Therefore, an increasing proportion of couples fail to conceive naturally, and then go on to seek fertility treatment, with varying degrees of success.

The availability of fertility treatment has increased worldwide, and this vast industry continues to grow (Beckman & Harvey 2005). While uptake of fertility treatment remains dominated by the relatively wealthy (Jain 2006), some countries are providing limited state funding for treatment, thus increasing availability to a wider section of the population. As discussed by Ledger (2009), the motivations of governments to fund fertility treatment vary. Apart from the more obvious public demand for assistance, there are socio-demographic interests as well. As a long-term investment, it may be a financially sound investment for the national treasury to fund fertility treatment, as these children (who may not otherwise have been born) will generate tax revenue throughout their productive lives, by virtue of the treatment itself (Chambers *et al.* 2009; Connolly *et al.* 2010; Connolly *et al.* 2008). Obviously, funding of fertility treatment as a financial investment is optimised if a healthy singleton infant is born, as demonstrated by the "Belgian project" (Ombelet *et al.* 2005). The Belgian Project demonstrated that single embryo transfer, and consequent singleton births, led to a dramatic cost reduction in ante natal and neonatal care (Ombelet *et al.* 2005).

Another important factor accounting for the increasing rates of fertility treatment uptake is the wider eligibility criteria for treatment, with many centres deeming a couple eligible after only 6 months of attempting natural conception. While this is clearly important for woman over 35 years of age, it has been suggested that it may be more pertinent to adopt a 'wait and see' approach in younger women (Hughes *et al.* 2004). However, in line with an increasing

societal trend towards ‘instant success’, a ‘wait and see’ approach is likely to be unacceptable for many couples.

The increasing social acceptability of fertility treatment in modern society is another important factor leading to increasing fertility treatment uptake, whereby couples are becoming more open about fertility problems (Thorn *et al.* 2009; van den Akker 2001). This contrasts with a sense of shame or failure which was quite pervasive in previous decades (Darsney 1996; Thomas 1995).

1.5.3. Infertility, and types of fertility treatment

Prior to discussing the specifics of fertility treatment, it is necessary to define some commonly used terms:

- Fertility – although ‘fertility’ and ‘fecundity’ are often used interchangeably in the literature, ‘fertility’ is the ability to conceive whereas fecundity is the potential ability to conceive. While these two terms are similar, it is important to differentiate that a fertile woman is able to conceive whereas a fecund woman is potentially able to conceive, but may never go on to conceive (Gnoth *et al.* 2005).
- Subfertility – generally accepted as the failure to conceive after more than 12 months of regular, unprotected intercourse (Fauser *et al.* 2005; Gnoth *et al.* 2005).
- Infertility – although inconsistently defined in the literature, the World Health Organization defines infertility as the inability to conceive within two years of exposure to the risk of pregnancy (Larsen 2005; World Health Organization 2001).

It is not necessary to outline all of the causes of subfertility or infertility for the purpose of this thesis. In broad terms the causes and frequency of subfertility and infertility can be classified as: unexplained (28%), male factor (24%), ovulatory failure (21%), tubal factors (14%), endometriosis (6%), and other causes (7%) (Ganeshan & Malik 2012).

Investigations of infertility are directed by clinical history, and may comprise of serum hormone levels, semen analysis, abdominal ultrasound, as well as more invasive tests such as hysteroscopy and laparoscopy. A basic work-up of a couple with difficulty conceiving varies among clinics and countries, but tends to be a clinical assessment followed by basic semen analysis, female serum hormone levels, and ultrasound (Balasch 2000). In recent years, time consuming and invasive tests tend to be avoided unless strongly indicated, as the diagnostic

yield is low. Furthermore, time and money is often better spent on empiric treatment for infertility, rather than on exhaustive investigations (Brosens *et al.* 2004).

Fertility treatment can be divided into two main areas: namely, ART and others (non-ART). The definition of ART varies, but the CDC (US Centre for Disease Control and Prevention) defines it as all fertility treatment in which both eggs and sperm are handled (Centers for Disease Control and Prevention 2006). The CDC does not classify ovarian stimulation (via medication) or sperm manipulation alone (e.g. intra-uterine insemination) as ART. In the following sections, I will discuss the various aspects of fertility treatment, the departure of this treatment from natural conception, and the changes it causes in gametes and to the early fetal environment.

1.5.4. Assisted reproductive technology (ART)

Our research focuses on the outcomes of children conceived with ovarian stimulation fertility treatment (without ART), but there is a marked paucity of information on its possible effects on maternal physiology, genes, epigenetics, and offspring outcomes. Therefore, it is necessary to explore the available information on ART, which has been the target of far more research. In addition, a number of aspects of ART are central to our hypotheses, as they provide an insight into the possible impact of ovarian stimulation alone on gametes, maternal factors, and childhood outcomes. As a result, while assessment of ART offspring is not the subject of this research, it is important to outline its various aspects.

ART consists largely of either *in vitro* fertilisation (IVF) or intra-cytoplasmic sperm injection (ICSI). Recent data show that ICSI comprises 50 to 90% of ART procedures, without a clear reason for the greater uptake of this procedure over IVF (De Mouzon *et al.* 2010; Nyboe Andersen *et al.* 2008). ART involves several steps that are substantially different from natural conception and to ovarian stimulation alone:

1. Ovarian stimulation – discussed in detail is section 1.5.6.
2. Oocyte retrieval and preparation – oocytes are removed by needle aspiration, and are then denuded of their surrounding cumulus cells prior to micromanipulation.
3. Sperm preparation – removal of semen and extraction of spermatozoa.
4. External fertilisation or ICSI – in IVF, sperm and oocytes are externally fertilised; in contrast, in ICSI a sperm is injected into the oocyte, thus introducing sperm acrosome and digestive enzymes into the ooplasm (Allen & Reardon 2005).

5. Embryo/blastocyst culture
6. Embryo freezing and thawing – it is estimated that over 25% of ART children are now born after cryopreservation of embryos, blastocysts, or oocytes.
7. Pre-implantation genetic diagnosis (PGD) – only performed in <1% of ART procedures.
8. Embryo selection and intra-uterine transfer

1.5.5. Non-ART fertility treatments

Non-ART fertility treatments fall outside the aforementioned definition of ART, and thus encompass a wide range of drug and non-drug treatments of infertility. It is not practical to outline all of the various non-ART fertility treatments on offer, as they range from acupuncture and herbal remedies to a variety of drug treatments and dietary interventions. For the purpose of this thesis, only ovulation induction and ovarian stimulation are discussed.

Ovulation induction and ovarian stimulation are often confused in the literature, and there is considerable overlap between the drugs utilised for both treatments. However, the two treatments have different aims and also differ according to the type of patient treated. Ovulation induction is a pharmacological intervention in anovulatory women to induce mono-ovulatory menstrual cycles. Patients are generally women with polycystic ovarian syndrome or hypogonadotrophic hypogonadism (Hughes 1997; Macklon *et al.* 2006). In contrast, ovarian stimulation is a pharmacological intervention in women with normal menstrual cycles, with the aim to induce development of more than one dominant follicle (Fauser & Macklon 2004).

There is again some confusion in the literature regarding terminology as ovarian stimulation may mean the stimulation of multiple follicles production (6 to 10) for the purpose of ART, or the stimulation of one to three follicles for the purpose of fertility drug treatment without ART. Therefore, for clarity I will refer to ovarian stimulation for the purposes of non-ART fertility treatment as "ovarian stimulation alone" and abbreviate it as "OS_A". When referring to any other type of ovarian stimulation, the context will be made clear.

1.5.5.1. Ovarian stimulation

As mentioned previously, ovarian stimulation is a process common to OS_A and ovarian stimulation for ART, and not surprisingly, there is some overlap in the drugs used in these processes. OS_A drug regimens tend to be more straight-forward and usually involve just one

or two medications. Two drugs in particular are frequently used either alone or in combination: clomiphene citrate and FSH. However, OS_A may also involve human chorionic gonadotrophin and intra-uterine insemination as adjunctive treatments.

ART drug regimens may also include FSH (but at comparatively higher doses) or human menopausal gonadotrophin (HMG, a combination of FSH and LH). In addition, ART often includes adjunctive treatment with GnRH agonists and antagonists as part of ovarian stimulation.

Clomiphene citrate

Clomiphene citrate (or just 'clomiphene') is a non-steroidal oestrogen antagonist, which was originally developed in the 1950s for the treatment of breast cancer and endometrial hyperplasia (Kistner & Smith 1960). In 1961, Greenblatt *et al.* first reported its use as an ovulation induction agent in anovulatory women. Since then, clomiphene remains the most prescribed drug therapy for anovulatory infertility worldwide (Macklon *et al.* 2006). Clomiphene is also used for unexplained infertility as it increases the likelihood of pregnancy by increasing the number of pre-ovulatory follicles or possibly by correcting subtle ovulatory dysfunction (Guzick *et al.* 1998). Clomiphene was used for ovarian stimulation for ART in many centres in the 1980s and 1990s, but has been largely replaced by FSH and other drugs (Gibreel *et al.* 2010).

Clomiphene contains an unequal mixture of two isomers, enclomiphene and zuclomiphene. Enclomiphene has a relatively short half-life. In contrast, zuclomiphene not only has a much longer half-life (detectable in plasma one more than one month after administration), but it may also accumulate over consecutive cycles (Macklon *et al.* 2006). Clomiphene primarily works by blocking hypothalamic oestrogen receptors. This signals a lack of circulating oestrogen to the hypothalamus, inducing a change in the pattern of release of GnRH, and thus increasing endogenous FSH secretion (Macklon *et al.* 2006). Release of even a small amount of FSH from the pituitary will often induce ovulation (Hillier 1994).

Clomiphene is usually given orally for 5 days from the beginning of a cycle, generally commencing at a lower dose of 50 or 100 mg per day. Dose requirements for the desired end-point of ovarian stimulation vary widely amongst recipients of clomiphene, ranging from exquisite sensitivity and excess follicle production at a low dose, to clomiphene resistance and failure to ovulate even at higher doses (Fauser & Macklon 2004; Homburg 2005). Therefore, in order to monitor the number of follicles produced by ovarian stimulation, some

centres measure serum oestradiol and/or LH levels as well as perform an ovarian ultrasound. In this way, timed intercourse or intra-uterine insemination can be avoided if excess follicle production has occurred. This helps to avoid multiple pregnancies, which is one of the most serious complications of OS_A. Unfortunately, many centres do not monitor follicle production, and the risk of twins and higher order multiple pregnancies after OS_A treatment still runs at 5 to 40% (Fauser *et al.* 2005; Källén *et al.* 2002; Wright *et al.* 2008).

There are conflicting views regarding the efficacy of clomiphene in the treatment of unexplained infertility (Hughes *et al.* 2010). While clomiphene leads to ovulation in almost 70% of treated women, the relatively low pregnancy rate of 4 to 5% is thought to be due to the anti-oestrogenic effects of clomiphene on the endometrium (Dickey 1993), cervical mucous (Acharya *et al.* 1993), and increased levels of LH (Homburg *et al.* 1988), all of which causing luteal phase dysfunction. There are several combinations of therapies with clomiphene that attempt to overcome these unwanted effects. These include measures such as intra-uterine-insemination (IUI) so that sperm can by-pass hostile cervical mucous (ESHRE *et al.* 2009), and additions of drugs such as human menopausal gonadotrophin, recombinant FSH, and other fertility drugs (Macklon *et al.* 2006).

Clomiphene alone achieves pregnancy rates of approximately 5.3% per cycle, but this rate increases to approximately 8% when combined with IUI (Guzick *et al.* 1998; Homburg 2005). Clomiphene combined with IUI and/or other drugs is recommended as first line treatment for unexplained infertility in many countries (National Institute for Clinical Excellence 2004; Pandian *et al.* 2009; Quaas & Dokras 2008; Royal College of Obstetrics and Gynaecology 1998). However, large scale studies have yielded conflicting findings on its effectiveness, with some studies finding an increase in pregnancy rates, but others finding poor evidence for its use (Bhattacharya *et al.* 2008; Chambers *et al.* 2010; Hughes *et al.* 2010; Morris *et al.* 2011; Wordsworth *et al.* 2011). Nonetheless, OS_A with clomiphene citrate and/or FSH (with or without IUI) is still widely used as a fertility treatment, possibly because it is less invasive and less expensive than ART (Chambers *et al.* 2010; Quaas & Dokras 2008).

It is important to note that clomiphene is associated with actions other than stimulation of ovulation. These include changes in the endometrial thickness (Nakamura *et al.* 1997; Sereepapong *et al.* 2000) and adhesion (Valbuena *et al.* 2001), alterations in oestrogen receptor numbers (Nakamura *et al.* 1997), as well as changes in intra-uterine chemokines,

cytokines, and growth factors (Boomsma *et al.* 2010). Further, clomiphene has been associated with changes in meiotic spindles in oocytes in animal studies (London *et al.* 2000), and it may affect oocyte quality and embryo development (Oktay *et al.* 2000). Although there is no direct evidence linking clomiphene to methylation changes, such changes can be associated with tamoxifen (Badia *et al.* 2007), which is a structurally similar compound to clomiphene (Homburg 2005).

Human chorionic gonadotrophin (HCG)

HCG is often used as an adjuvant in OS_A treatment, as there is evidence that, given at the appropriate time, HCG triggers ovulation when there is a delayed or absent LH surge (Homburg 2005). Although it is unclear if it leads to an improvement in pregnancy rates, the utilisation of HCG triggering as part of OS_A treatment is relatively common (Homburg 2005).

Intra-uterine insemination (IUI)

IUI is widely used as a combination treatment with ovarian stimulation using clomiphene citrate or gonadotrophins. This is despite mixed results on its effectiveness at improving pregnancy rates in couples with unexplained infertility (Crosignani 2009; Wordsworth *et al.* 2011). Regardless of its effectiveness, the procedure of sperm preparation for IUI deviates from natural conception. Although subject to various procedural differences, IUI usually involves selection of the most motile sperm. IUI often involves also separation of sperm from semen ejaculate (Crosignani 2009), which may remove male seminal cytokines and other important semen constituents (Boomsma *et al.* 2007; Robertson *et al.* 2009; Sjoblom *et al.* 2005).

Follicle stimulating hormone (FSH)

FSH has been used for ovarian stimulation in fertility treatment since the 1960s and has largely replaced clomiphene as the main agent for ovarian stimulation for ART (Macklon *et al.* 2006; Sterrenburg *et al.* 2011). FSH is also used for OS_A, either as mono-therapy or in combination with clomiphene (Berker *et al.* 2011).

During the early follicular phase of the normal menstrual cycle, endogenous FSH levels rise and then gradually fall in the mid to late follicular phase. This leads to the selection of a single dominant follicle. Ovarian stimulation with exogenous FSH (or with agents such as clomiphene that indirectly increase endogenous FSH) maintains FSH concentrations above the so-called “threshold” level for a longer period of time. This threshold level is required to

maintain follicle development and maturation, and prolongation of this phase leads to production of increased follicle numbers (Zeleznik 2004).

Not unlike clomiphene, the individual dose response to exogenous FSH is not always predictable, with failure to ovulate and ovarian hyperstimulation syndrome at the two ends of the dose-response spectrum. However, FSH is usually well tolerated and highly effective at increasing follicle numbers. Furthermore, monitoring of dose-response with serum LH or oestradiol levels (and/or ultrasound) guides the physician in subsequent treatment steps. Required doses of FSH vary considerably, depending on whether the treatment aim is 2 or 3 follicles for OS_A or 6 to 10 follicles for ovarian stimulation for ART. FSH doses as mono-therapy for OS_A are generally 75-150 IU per day, and similar doses are given on alternative days when used as adjunctive treatment with clomiphene (Willis *et al.* 2011). Doses of FSH for ovarian stimulation for ART are appreciably higher, starting from 100–300 IU per day, which are often increased in subsequent cycles if the response is poor (Macklon *et al.* 2006). FSH is much more rapidly cleared from the circulation than clomiphene, with a terminal half-life of approximately one and one-half days (Balasch *et al.* 2003).

While exogenous FSH administration does not lead to the same marked anti-oestrogenic effects as clomiphene, it has been shown to have several effects other than ovarian stimulation. FSH may lead to alterations in endometrial receptivity, as well as changes in endometrial growth factor concentrations and cell adhesion molecule profiles (Macklon & Fauser 2000).

FSH and 'genetic competence'

Elevations in FSH (both from exogenous and endogenous sources) have been implicated in alterations in 'genetic competence' of the human oocyte and embryo, with suggestions that FSH may have a role in oocyte aneuploidy (Broekmans *et al.* 2009). The exact mechanism leading to aneuploidy in human oocytes remains poorly understood. While increasing maternal age is definitely linked to higher rates of aneuploidy (Hassold & Hunt 2001), there is some debate as to whether endogenous FSH has a direct causative effect on human aneuploidy (Dursun *et al.* 2006; Kline *et al.* 2011). However, animal studies suggest that ovarian stimulation leads to DNA changes and an increase in chromosomal abnormalities when compared to unstimulated oocytes and embryos (Roberts *et al.* 2005; van Blerkom & Davis 2001). Furthermore, several human studies have linked exogenous FSH used in ovarian stimulation for ART with increased aneuploidy rates (Kaleli *et al.* 2005; Katz-Jaffe *et al.*

2005; Munne *et al.* 1997). It is proposed that FSH may alter oocyte maturation and the completion of meiosis, leading to oocyte and/or embryo chromosome dysfunction and aneuploidy (Hodges *et al.* 2002; Massie *et al.* 2011; Verpoest *et al.* 2008).

Another possible reason for the observed higher rate of aneuploidy in stimulated oocytes is that ovarian stimulation (for ART or OS_A) bypasses the natural process of selecting the most competent oocyte for ovulation, which may lead to recruitment of poorer quality immature oocytes (Verberg *et al.* 2009). Baart *et al.* (2007) demonstrated a relationship between higher FSH doses in ovarian stimulation for ART and elevated aneuploidy rates, suggesting that milder ovarian stimulation may lead to fewer aneuploid oocytes or embryos.

Thus, although limited, there is some evidence that FSH may have a role in DNA competence, and may be associated with an increased risk of generation of aneuploid oocytes. However, it is clear that there are many other factors involved in aneuploidy risk. These include maternal age, background subfertility of parents, amongst others (Broekmans *et al.* 2009; Massie *et al.* 2011; Oliver *et al.* 2008; Verpoest *et al.* 2008).

Ovarian stimulation, exogenous FSH, imprinting, and epigenetics

The purpose of OS_A is to ensure ovulation of one (and often more than one) oocyte to increase the chances of conception. Therefore, the very nature of this intervention means that immature oocytes are directed to ovulate. Gametogenesis is a complex process whereby oocytes gain 'genetic competence' in preparation for ovulation, and epigenetic regulation is intrinsic to this process (Allegrucci *et al.* 2005).

As outlined, OS_A treatment leads to a surge of endogenous FSH through the actions of clomiphene and/or exogenous FSH. Several animal studies (reviewed by van Montfoort *et al.* 2012), but only one human study (Sato *et al.* 2007), have been conducted to date on the impact of exogenous FSH administration for ovarian stimulation on epigenetics and imprinted genes. These studies have been almost exclusively carried out on oocytes that have undergone ovarian stimulation for ART, and some have found that ovarian stimulation (for ART) is associated with epigenetic changes in oocyte genes (Liu *et al.* 2011a; Market-Velker *et al.* 2010; Sato *et al.* 2007). Such changes include alteration in DNA methylation and gene expression, as well as changes in the methylation of *H19, an imprinted gene involved in* post-natal growth (Market-Velker *et al.* 2010). A recent study suggests that the degree of ovarian stimulation in ART may influence the extent of methylation change in the stimulated oocyte (Market-Velker *et al.* 2010).

Several studies have also examined the effects of ART on DNA methylation of imprinted and non-imprinted genes on embryos (Santos *et al.* 2010a) and offspring (Gomes *et al.* 2009; Horsthemke & Ludwig 2005; Katari *et al.* 2009; Oliver *et al.* 2011; Puumala *et al.* 2012a; Santos *et al.* 2010b; Sato *et al.* 2007; Tierling *et al.* 2010), yielding conflicting results. However, these studies assessed genes of ART offspring, and it is unclear whether ovarian stimulation or the subsequent steps in ART were responsible for any observed differences.

Ovarian stimulation has also been implicated in changes in the homocysteine pathway (Ebisch *et al.* 2007) and folate metabolism (Boxmeer *et al.* 2008), which are important in gametogenesis and folliculogenesis respectively. Research in this area is on-going, and is likely to yield further insight into the possible contribution of OS_A and ovarian stimulation for ART and other mechanisms to the generation of epigenetic changes in stimulated oocytes.

1.5.6. Complications and consequences of ART

As already stated, there is a marked paucity of research on outcomes of children conceived with OS_A. Therefore, the relative wealth of information on outcomes and risks to ART children provides an insight into the complications and consequences of fertility treatment to offspring.

The following is the unaltered version of a review published in Human Reproduction outlining the complications and consequences to offspring of ART fertility treatment.

- *Authors:* Savage T, Peek J, Hofman PL, and Cutfield WS
- *Title:* Childhood outcomes of assisted reproductive technology
- *Journal:* Human Reproduction
- *Year of publication:* 2011
- *Volume:* 26
- *Issue:* 9
- *Pages:* 2392-2400
- *Impact factor:* 4.48
- *Journal's aims and scope:* "Human Reproduction features full-length, peer-reviewed papers reporting original research, clinical case histories, as well as opinions and debates on topical issues. Papers published cover the scientific and medical aspects of reproductive physiology and pathology, endocrinology, andrology, gonad function, gametogenesis, fertilization, embryo development, implantation, pregnancy, genetics,

genetic diagnosis, oncology, infectious disease, surgery, contraception, infertility treatment, psychology, ethics and social issues. The highest scientific and editorial standard is maintained throughout the journal along with a rapid rate of publication."

1.5.6.1. Introduction

The definition of assisted reproductive technology (ART) varies widely, but the CDC (US Centre for Disease Control and Prevention) defines it as all fertility treatment in which both eggs and sperm are handled [Sunderam et al., 2009]. Until relatively recently, IVF made up the majority of ART procedures, but the utilization of ICSI has steadily increased, and it now represents 40–70% of ART procedures [de Mouzon et al., 2010]. Further, approximately three-quarters of ART pregnancies are achieved through fresh embryo transfer, and the remainder through frozen embryos that are thawed and then transferred [Sunderam et al., 2009].

From its inception in 1978, there has been a worldwide increase in uptake and demand for ART with recent data suggesting that children conceived by ART comprise anywhere between 1 and 4% of the newborn population in industrialized countries [de Mouzon et al., 2010]. There is a continued worldwide increase in the number of ART conceived children due to several possible reasons. These include a shift in reproductive behaviour towards the postponement of childbirth leading to a consequent increase in the proportion of women at risk of infertility; increased availability of treatment and state funding for ART in some countries [Leridon and Slama, 2008; Sunderam et al., 2009]. The indications for ART treatment of infertility have also broadened significantly over the past decade, leading to many couples utilizing ART instead of expectant management or other treatment options [Reindollar et al., 2010].

Outcomes of ART-conceived pregnancies are an area of intense scrutiny and research; with vast publications assessing ART treatment success, perinatal outcomes and longer-term outcomes of ART children. The overwhelming majority of studies to date have assessed ART childhood outcomes in comparison with those of the general population. There is emerging evidence that in order to properly determine any actual impact of the ART process itself, ART outcomes may be better compared with those of children born to subfertile parents. Subfertility is generally accepted as the failure to conceive naturally after 12 months or more of unprotected intercourse [Gnoth et al., 2005] and affects between 8 and 20% of couples [Oakley et al., 2008]. As the majority of ART parents are subfertile [Zhu et al., 2006; Källén

et al., 2010d], it is becoming increasingly evident that underlying maternal or paternal subfertility may be an important factor in obstetric, neonatal and childhood outcomes in the ART population [Williams and Sutcliffe, 2009].

The possible impact of parental subfertility on childhood outcomes is largely accounted for by utilizing naturally conceived children of subfertile parents as a comparison group for ART children. However, this effect is not completely eliminated, as children of subfertile parents were eventually conceived naturally, whereas the ART children were not.

In this review, we examine the evidence to date assessing if outcomes of ART-conceived children are different from those of naturally conceived children, and whether mechanisms such as epigenetics influence these changes.

1.5.6.2. Multiple births and perinatal outcomes

Multiples births occur more commonly in ART pregnancies. In most developed countries, 30–50% of all twin pregnancies result from ART [Ombelet et al., 2005; Pandian et al., 2009; Sunderam et al., 2009; Gelbaya et al., 2010]. Multiple pregnancies occur in 25–50% of ART [Martin et al., 2009; Sunderam et al., 2009; de Mouzon et al., 2010] and 5–40% of ovarian stimulation pregnancies [Källén et al., 2002; Fauser et al., 2005; Wright et al., 2008]. The wide variation in these figures is due to the differing practices between centres and countries on the number of embryos transferred and ovarian stimulation practices [Maheshwari et al., 2011]. While doubleembryo transfer remains common practice, single embryo transfer (SET) is increasing in popularity; particularly, when involving extended culture to blastocyst to increase the likelihood of successful implantation [Papanikolaou et al., 2008]. SET in ART significantly reduces the rate of twins and higher order multiples, yet this practice remains sporadic [Bergh, 2005; Ombelet et al., 2005; Gerris, 2009]. The Belgian project encouraging SET led to a reduction in the rate of twins from 19 to 3% and of higher order multiples to almost 0% [Ombelet et al., 2005]. A long-term Swedish population study showed that the increasing utilization of SET led to a reduction in twins from 20 to 5%, as well as a corresponding reduction in rates of premature births and low birthweight infants in the ART population [Källén et al., 2010d].

While some reviews suggest that cumulative live birth rates from SET is superior to that of multiple embryo transfer [Pandian et al., 2009; Gelbaya et al., 2010], a recent meta-analysis concluded that SET yields a lower live birth rate than a double-embryo transfer (27 versus

42%); but that this difference is almost completely overcome by an additional frozen SET cycle in the SET patients [McLernon et al., 2010]. Increase in the rate of SET has led to greater availability of frozen embryos for future cycles, and it is estimated that over 25% of ART children are now born after cryopreservation of embryos, blastocysts, or oocytes [Källén et al., 2010d]. Recent evidence suggests that obstetric and perinatal outcomes are comparable irrespective of whether pregnancy is a result of fresh or frozen embryo transfer [Wennerholm et al., 2009].

Perinatal outcomes in ART are most significantly influenced by multiple pregnancies, which have more than a 60% risk of low birthweight or premature delivery in the ART population [Sunderam et al., 2009]. Helmerhorst et al. found that ART twins fared better in the peri-natal period in comparison with all naturally conceived twins (monozygotic and dizygotic) [Helmerhorst et al., 2004]. However, perinatal outcomes of dizygotic are better than those of monozygotic twins, and the proportion of dizygotic twins in the ART population is greater than among naturally conceived twins [Källén et al., 2010e]. Recent studies examining the outcomes of dizygotic twins in isolation showed that ART twins fare worse than naturally conceived twins in the perinatal period [Hansen et al., 2009; Källén et al., 2010e].

The contribution of the ART population to the premature and low birthweight population varies between countries, but remains high. Over 41% of all ART infants born in the USA in 2006 were delivered preterm, accounting for 4% of all preterm infants that year, costing more than US$1 billion in neonatal care alone [Martin et al., 2009; Sunderam et al., 2009]. Even in ART singleton pregnancies, there is an almost 2-fold increase in the risk of premature birth, as well as an associated higher rate of small for gestational age (SGA) and low birthweight infants [Helmerhorst et al., 2004; Sunderam et al., 2009; Källén et al., 2010d].

However, the role of ART as a pivotal factor in the increased risk of premature delivery and low birthweight has been called into question; with several studies [Williams et al., 1991; Joffe and Li, 1994; Henriksen et al., 1997; Draper et al., 1999; Basso and Baird, 2003; Ludwig, 2009; Williams and Sutcliffe, 2009] suggesting that background subfertility and time to pregnancy may be more important. For example, a study of singleton ART offspring compared with naturally conceived sibling controls found no differences in rates of low birthweight, premature or SGA births [Romundstad et al., 2008]. Overall, there is growing evidence that the contribution of parental subfertility and other factors to adverse perinatal outcomes may be equally, if not more important, than the contribution of ART itself.

1.5.6.3. Neurological and neuro-developmental outcomes

Neurological abnormalities appear to occur more commonly in ART-conceived children. However, there is increasing evidence that factors other than the ART procedure itself, such as perinatal events, may be largely responsible for these findings.

Several studies have found an increased risk of cerebral palsy (CP) or neurological abnormalities among the ART population [Ericson et al., 2002; Strömberg et al., 2002; Pinborg et al., 2004; Källén et al., 2005; Lidegaard et al., 2005; Hvidtjørn et al., 2006, 2010; Klemetti et al., 2006; Romundstad et al., 2008]. However, the authors concluded that these outcomes were generally explained by higher rates of multiple pregnancies, prematurity and low birthweight among ART offspring. The wider use of SET has reduced the number of premature and low birthweight ART infants [Källén et al., 2010d]. Consequently, it would be expected that a reduction in CP rates would follow, and there is some indication that this is the case [Källén et al., 2010a].

Recent studies have included children of subfertile parents as a comparison group to ART children, finding similar neurological outcomes [Middelburg et al., 2009; Middelburg et al., 2010]. However, a recent large population study suggested that ART may be a more important factor than parental subfertility in CP risk [Zhu et al., 2010]. Further studies are needed to clarify the possible contribution of background subfertility to neurological outcomes in ART children.

The vast majority of studies assessing neuro-cognitive outcomes (i.e. locomotor, cognitive, speech and language and behaviour) in ART children showed no differences between ART-conceived and naturally conceived singletons; when adjusted for recognized confounding factors such as low birthweight and prematurity [Koivurova et al., 2003; Place and Englert, 2003; Ponjaert-Kristoffersen et al., 2005; Leunens et al., 2008; Hvidtjørn et al., 2009]. Similarly, there is no evidence of an increased risk of autism among ART children [Maimburg and Væth, 2007; Hvidtjørn et al., 2009, 2011]. Current evidence suggests that the ART process itself does not lead to an increased risk of adverse neuro-cognitive outcomes or autism.

A weak association between ART and drug-treated attention deficit hyperactivity disorder has been reported, but the association is no longer significant with adjustment for subfertility [Källén et al., 2011]. Further, there are no concerns about psycho-social issues or family

relationships in the ART population, with a suggestion that ART children may fare a little better than naturally conceived children in this area [Barnes et al., 2004; Basatemur and Sutcliffe, 2008; Colpin and Bossaert, 2008; Wagenaar et al., 2008; Wagenaar et al., 2011].

1.5.6.4. Growth

The large offspring syndrome is a well-recognized adverse consequence of cultured embryos in cattle and sheep [Young et al., 1998]. This is proposed to be due to altered expression of the insulinlike growth factor (IGF)-II receptor, which is imprinted in animals but not humans [Young et al., 2001]. These observations have triggered interest in the growth patterns of ART-conceived children.

There are several studies that have examined the growth and weight patterns of ART-conceived children, and these have revealed conflicting results. Most of these studies included children that were born prematurely, SGA or as a result of multiple pregnancies. This is not surprising considering the higher rate of multiple and premature births associated with ART [Sunderam et al., 2009; de Mouzon et al., 2010]. At one extreme, earlier studies reported lower weight and length percentiles in ART-conceived children at 2 and 3 years of age [Brandes et al., 1992; Koivurova et al., 2003]. However, a large uncontrolled study of 400 IVF-conceived children aged 6–13 years found no difference in height or weight compared with population-based growth data [Olivennes et al., 1997]. In addition, inclusion of ICSI in the IVF process did not influence children's height or weight compared with controls [Belva et al., 2007]. Further, a large European multi-centre study found no difference in height or weight among IVF, ICSI and naturally conceived children at 5 years of age [Bonduelle et al., 2005]. Kai et al. assessed two cohorts of ART children (longitudinally from 0–36 months and a cross-sectional cohort at 5 years of age) finding that ICSI children were shorter than IVF and naturally conceived controls at 3 years, but no different at 5 years [Kai et al., 2006]. They also found a lower serum IGF-I level in ICSI boys and IVF-conceived girls at 3 months, but this difference did not persist into childhood [Kai et al., 2006]. In contrast, others found that ART children were taller than naturally conceived controls or the general population [Saunders et al., 1996; Pruksananonda, 2001; Miles et al., 2007; Makhoul et al., 2009].

Few studies have provided longitudinal assessment of growth in childhood. Celeen et al. analysed height and weight data (from 3 months to 4 years) of IVF-conceived children compared with naturally conceived controls of subfertile parents [Ceelen et al., 2009]. They

found that ART infants had a lower weight, height and BMI standard deviation score (SDS) at 3 months, and a lower weight SDS at 6 months of age [Ceelen et al., 2009]. ART children then showed greater growth velocity than controls in late infancy, but with no difference in height at 3 years of age [Ceelen et al., 2009]. However, this study did acknowledge the potential effect of parental subfertility on growth outcome. We have identified only one other study that utilized this important comparison group [Makhoul et al., 2009].

In a recent study of growth in ART children, Basatemur et al. found no difference in height or weight in IVF or ICSI subjects between 5 and 12 years [Basatemur et al., 2010]. However, the study population did include children who were low birthweight and born as early as 32 weeks gestation.

As previously stated, most studies included children born prematurely, SGA or from multiple pregnancies, factors that are known to be associated with impaired childhood growth [Leger et al., 1997; Fewtrell et al., 2000; Peralta-Carcelen et al., 2000]. Thus, ART populations including these subjects would likely be shorter than naturally conceived populations that have far fewer low birthweight or prematurely born children. Therefore, it is surprising that these studies largely show no difference in height between IVF- and naturally conceived offspring.

There have been only a handful of studies that have corrected for prematurity and birthweight to determine any actual effects of the ART process itself on childhood growth [Miles et al., 2007; Makhoul et al., 2009; Green et al., 2010]. In a matched control study, Miles et al. analysed the growth and metabolic parameters of 69 ART-conceived children from fresh embryo transfers [Miles et al., 2007]. Compared with controls, ART-conceived girls were taller, had higher IGF binding protein 3 levels and displayed a trend towards higher IGF-I levels. ART children also had higher high-density lipoprotein (HDL) and lower triglyceride levels than controls. The findings were particularly relevant as it was the first study to correct for parental heights and exclude children born premature or with low birthweight. The same group also assessed ART-conceived children from frozen embryo transfers, again showing that ART-conceived girls were taller than matched controls [Green et al., 2010].

Recently, Makhoul et al. published findings on the follow-up of children with very low birthweight aged 6 to 10 years conceived by ART or fertility medications [Makhoul et al., 2009]. This group accounted for the genetic height and the low birthweight status in their analyses, and found that the ART and fertility medication-conceived groups were

significantly taller than controls [Makhoul et al., 2009]. Currently, there is no clear explanation for the taller stature or the gender bias observed.

It is clear that many studies on the growth of children conceived by ART had confounding factors and methodological weaknesses. Larger studies including appropriately matched groups are needed to confirm these latter findings. Further, given that ICSI and/or frozen embryo transfer are common major additions to ART, the effects of these processes on offspring outcome also need to be clarified.

1.5.6.5. Metabolism and gonadal function

There are limited and conflicting data on the metabolic and hormonal profiles of ART children, as summarized by a recent review [Kanaka- Gantenbein et al., 2010]. Examples of observed differences in ART children compared with controls include higher fasting glucose [Ceelen et al., 2008b], higher HDL and lower or higher triglycerides [Miles et al., 2007; Sakka et al., 2010]. Such differences were minor and remained well within the normal range.

Marginal increases in systolic and diastolic blood pressure have also been observed in ART children [Ceelen et al., 2008b]. Although not clinically significant in childhood, subtle blood pressure increases may be amplified in adulthood [Law et al., 1993].

There is evidence of an increased rate of early adrenarche and polycystic ovarian syndrome in women who were born prematurely or SGA [Pandolfi et al., 2008; Melo et al., 2010]. However, only one study has assessed puberty in ART children, finding no difference in pubertal staging in comparison with children of subfertile couples [Ceelen et al., 2008a]. Interestingly, ART-conceived girls had a significantly higher dehydroepiandrosterone sulfate and luteinizing hormone levels than naturally conceived controls. Although the cause for this was unclear, there were no apparent clinical effects [Ceelen et al., 2008a]. The group was not evaluated further for disorders such as polycystic ovarian syndrome.

There are potential concerns for the future fertility of ART offspring, but these are yet to be clarified as the majority of this population is still relatively young. Current concern relates mostly to the future fertility of ICSI-conceived boys. Limited evidence suggest that ICSI boys have an increased rate of genital abnormalities [Ludwig et al., 2009], lower serum testosterone levels at 3 months [Mau Kai et al., 2007], but have salivary testosterone levels in puberty comparable with controls [Belva et al., 2011]. A proportion of adult males requiring ICSI have very low sperm counts, which is associated with a greater risk of carrying

chromosomal abnormalities [Aittomäki et al., 2004; Marchina et al., 2007].There is limited evidence that paternal sex chromosomal disorders, including micro-deletions, are rarely transmitted to male ART offspring [Feng et al., 2008; Mau Kai et al., 2008]. Their potential effects on the gonadal function of ART boys is yet to be determined. While current evidence is inconclusive; the future fertility of ART offspring, particularly ICSI-conceived males, warrants further research.

1.5.6.6. Congenital abnormalities

There is a higher incidence of congenital abnormalities following ART [Rimm et al., 2004; Hansen et al., 2005; Lie et al., 2005], with considerable speculation as to the causes. Meta-analyses indicate a 30% increased risk of major malformations in children conceived by ART compared with spontaneous conception [Rimm et al., 2004; Hansen et al., 2005; Lie et al., 2005]. Although much of the focus has been on the process of ART itself, mounting evidence suggests that parental subfertility may be an important factor [Rimm et al., 2004; McDonald et al., 2005; Rimm et al., 2011]. A large population study in Denmark compared congenital abnormality rates among naturally conceived and fertility treatment offspring of subfertile couples, finding no differences in overall prevalence of congenital malformations [Zhu et al., 2006]. In addition, this study indicates that parental factors are important, as increasing 'time to pregnancy' was associated with a greater risk of congenital abnormalities [Zhu et al., 2006].

A recent Swedish study provided further evidence on the important contribution of parental factors to the higher rates of congenital abnormalities in the ART population [Källén et al., 2010b]. While the overall rate of congenital abnormalities in ART children remained elevated, the incidence of certain types of abnormalities such as neural tube defects, cardiac defects and oesophageal atresia were reduced [Källén et al., 2010b]. The authors speculated that this reduction might be due to a greater proportion of ART-treated couples with a shorter period of unwanted childlessness [Källén et al., 2010b].

1.5.6.7. Epigenetics and imprinting disorders

There is growing evidence of an increased risk of imprinting disorders in ART children. Epigenetics is the study of heritable changes in phenotype or gene expression that occur independently of alterations in the DNA sequence [Waterland and Michels, 2007]. DNA methylation and histone acetylation lead to DNA conformational change and gene silencing.

Methylation is the best characterized epigenetic modification of DNA with cytosine guanosine (CpG) islands being particularly vulnerable [Waterland and Michels, 2007]. A reduction in gene expression generally occurs when these islands are methylated within unmethylated gene promotor regions. Imprinting involves the silencing of either the maternal or paternal allele and is crucial for many aspects of pre- and post-natal growth and development [Waterland and Michels, 2007]. Imprinting occurs at gametogenesis and embryogenesis and imprinted genes undergo de-methylation followed by re-methylation during early embryonic development.

Although environmental influences on gene regulation are increasingly studied in ART offspring, parental factors may lead to epigenetic modification [Horsthemke and Ludwig, 2005]. Studies support the assertion that a subfertile couple may have a greater risk of preexisting methylation defects and consequent imprinting disorders in their offspring [Horsthemke and Ludwig, 2005; Ludwig et al., 2005; Hartmann et al., 2006; Doornbos et al., 2007].

Nonetheless, it is proposed that the process of ART may lead to epigenetic and consequent imprinting changes. While animal studies support the role of DNA methylation changes in ART [Reik et al., 2001], there are very few human studies in this area. Fertility medications that cause ovarian stimulation are used in isolation for fertility treatment or as a component of the ART process. Ovarian stimulation is associated with an increased risk of aneuploidy in artificially matured oocytes [Kaleli et al., 2005], and may alter the methylation process [Sato et al., 2007; Market-Velker et al., 2010]. However, the risk of alteration of the normal process of methylation is much greater in ART, as it departs more substantially from natural conception. The timing of ART also coincides with critical early embryonic DNA methylation and re-methylation.

Imprinted and epigenetically controlled genes play a key role in implantation and subsequent placental development [Nelissen et al., 2011]. It is evident from animal and human studies that epimutations of these genes can lead to abnormal placentation and subsequent complications such as abnormal foetal growth [Steinhoff et al., 2009; Nelissen et al., 2011].

The effect of the culture medium on the growth of ART offspring is well established in animal studies [Young et al., 2001]. Recent evidence has emerged that different culture media lead to a small but significant change in birthweight in humans [Dumoulin et al., 2010]. It was suggested that the observed difference (245 g) may partially explain the greater

incidence of low birthweight among ART-term singletons [Dumoulin et al., 2010]. The authors speculated that the weight difference associated with different culture media may be due to distinct epigenetic changes, leading to changes in placental function [Dumoulin et al., 2010].

It is recognized that male seminal cytokines play an important role in implantation regulation [Robertson, 2007; Robertson et al., 2009], and these cytokines may influence foetal programming [Sjöblom et al., 2005]. It was found that removal of seminal fluid prior to needle sperm injection into the mouse oocyte led to a reduction in embryo and offspring size [Sjöblom et al., 2005]. When the culture medium of these smaller embryos were treated with a male seminal cytokine (granulocyte-macrophage colony-stimulating factor), embryo and offspring size were corrected to normal [Sjöblom et al., 2005]. Work in this area of periconceptual immunology is ongoing, and development of periconceptional immune modulators in human ART is a distinct possibility in the future [Salmassi et al., 2005; Guerin et al., 2009].

Evidence of subtle changes has emerged from a small cohort of ART subjects who were comprehensively screened for changes in DNA methylation and gene transcription [Katari et al., 2009]. A very small study showed hypomethylation of a growth regulating gene (KvDMR1) in 3 of 18 ART children [Gomes et al., 2009]. In contrast, work from our centre examined DNA methylation of four likely candidate genes and found no differences between ART and naturally conceived children [Cutfield et al., unpublished data]. Further, a more recent study found similar rates of DNA methylation imprints in ART children and controls [Tierling et al., 2010]. Overall, however, there are limited data to suggest that ART leads to changes in DNA methylation. Importantly, none of these studies have aligned these changes with alterations in gene expression producing an altered phenotype. Therefore, the impact of ART on phenotype or biochemical profile through DNA methylation has yet to be determined.

Nonetheless, there is increasing evidence of a link between ART and dramatic changes in methylation that lead to rare imprinting disorders, namely Beckwith–Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) [DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003; Halliday et al., 2004; Chang et al., 2005; Bowdin et al., 2007; Doornbos et al., 2007; Paoloni-Giacobino, 2007; Lim et al., 2009; Manipalviratn et al., 2009; Choufani et al., 2010]. BWS has an estimated incidence of 1 in 13 700 live births in the general population

[Choufani et al., 2010], but the risk is estimated to be 6–9 times higher among ART offspring [Manipalviratn et al., 2009]. While this would represent a dramatic increase in relative risk, the actual incidence of BWS in the ART population remains low (1 in 4000–5500) [Manipalviratn et al., 2009]. It is significant that the rate of methylation defects as the cause of BWS in the general population is 60%, whereas this figure approaches 100% among the ART population [Manipalviratn et al., 2009]. AS has an estimated incidence of 1 in 12 000 with imprinting abnormalities the aetiological factor in only 5% of cases [Steffenburg et al., 1996]. Thus, the rate of imprinting disorder as a cause of AS in the general population is 1 in 240 000. In contrast, 5 of the 7 reported cases of AS born after ART had an imprinting defect as the cause [Manipalviratn et al., 2009]. However, it is worth noting that subfertility appears to be associated with an increased risk of AS [Doornbos et al., 2007].

There is emerging evidence for an increased relative risk of BWS and AS in the ART population. However, it is possible the case ascertainment of BWS and AS among ART offspring may be higher due to under reporting in the general population, and more intense scrutiny and follow-up of ART-conceived children [Bowdin et al., 2007]. It is nonetheless clear that comprehensive, prospective, multi-centre studies are necessary to ascertain if this association is definitive.

1.5.6.8. Cancer risk

It has been proposed that ART children are at a greater risk of later malignancy, and a possible reason may be a reduction in imprinted gene activity leading to dysregulation in tumour suppression [Lim and Maher, 2010]. Although an initial study suggesting an increased incidence of retinoblastoma in ART offspring caused concern [Moll et al., 2003], expansion of this study found this not to be the case [Marees et al., 2009]. Other studies indicated that cancer risk among children and adolescents is not increased in ART offspring [Bergh et al., 1999; Bruinsma et al., 2000; Klip et al., 2001; Ericson et al., 2002; Marees et al., 2009]. One study detected an increased rate of histiocytosis in ART children [Källén et al., 2005], but there is debate as to whether histiocytosis can be defined as true malignancy [Fadeel and Henter, 2003]. Conversely, a very large recent study of over 26 000 ART-conceived children by the same group showed an increased risk of cancer and histiocytosis (odds ratio 1.48); with the cancer risk remaining elevated (odds ratio 1.35) even when children with histiocytosis were removed from the analysis [Källén et al., 2010c]. This marginal increase in cancer risk was re-affirmed in a recent long-term review of the same

population [Finnström et al., 2011]. While both studies suggest an increased risk of cancer in ART children, the authors cautioned that the risk observed is probably not attributable to the ART procedure itself, but rather a result of many other factors such as the recognized increased risk of cancer among children with a history of prematurity [McLaughlin et al., 2006] or asphyxia [Spector et al., 2005].

Further studies on the growing ART population are necessary to determine any associated cancer risks. As the vast majority of the ART offspring population are under 30 years of age, long-term follow-up studies are warranted to determine if an increased cancer risk emerges with age.

1.5.6.9. Conclusions

There is growing evidence that ART-conceived children are phenotypically and biochemically different from naturally conceived children. However, the mechanism(s) leading to these changes have not been elucidated, and may include parental factors, maternal drug treatment, culture media, as well as egg and embryo manipulation. While there is a possible increase in the risk of imprinted gene disorders in ART offspring, these remain rare. Nonetheless, it is not clear if more subtle changes in DNA methylation can lead to the subtle changes in phenotype observed in some ART offspring. An increased cancer risk among ART children is yet to be conclusively demonstrated, but it is clear that ongoing surveillance is required on this population as it ages.

The population of ART children continues to increase worldwide without a clear understanding of associated long-term outcomes. Further knowledge on ART outcomes will provide health-care professionals, prospective parents and ART offspring with much needed accurate information on any actual risks.

1.5.6.10. References

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1.5.7. Complications and consequences of OS_A fertility treatment

OS_A fertility treatment has many associated risks and complications, many of which overlap with those associated with ART. A brief outline of the known risks associated with OS_A is provided here. Those which overlap with ART and which have been described in the review will not be repeated.

1.5.7.1. Obstetric and perinatal

Not unlike ART, the majority of obstetric and perinatal complications of OS_A can be attributed to the higher rate of twin and higher order multiple births associated with this treatment (Maman *et al.* 1998). Mothers with twin pregnancies have higher rates of pregnancy-induced hypertension, pre-eclampsia, gestational diabetes, hyperemesis, and other obstetric complications (Campbell & Templeton 2004). Women undergoing OS_A also tend to be older, and higher age during pregnancy is another risk factor for these complications (Maman *et al.* 1998). As previously mentioned, multiple birth rates vary widely with OS_A treatment, depending on whether or not follicle numbers are monitored (Macklon 2005).

Perinatal complications in twin and higher order multiple pregnancies are well described, and include being born prematurely or of low birth weight (Corsello & Piro 2010). In turn, both are associated with a multitude of perinatal complications (Santema *et al.* 1995), such as perinatal mortality and morbidity, including neurological and other acute and chronic sequelae (Santema *et al.* 1995; Sherer 2001).

It appears that, even in singleton OS_A pregnancies, the rates of maternal and perinatal complications are higher than in those resulting from natural conception, although lower than in ART pregnancies (Adler-Levy *et al.* 2007; Källén *et al.* 2002). This increased rate of complications in OS_A in the perinatal period (compared to naturally conceived pregnancies) is likely attributable to maternal characteristics and background subfertility (Evers 2002).

1.5.7.2. Aneuploidy and other congenital malformations

Aneuploidy rates in OS_A pregnancies are difficult to quantify, as aneuploid pregnancies tend to result in early miscarriages. Nonetheless, limited data suggest that OS_A offspring have a similar rate of chromosomal disorders to those of subfertile parents and the general population (Tulandi *et al.* 2006). There are however, concerns that OS_A offspring have an increased risk of congenital abnormalities such as neural tube defects (Schardein 1993; Tulandi *et al.* 2006). It is of interest therefore, that repeated doses of clomiphene may be associated with a slightly increased risk of congenital malformations, including neural tube defects and hypospadias (Elizur & Tulandi 2008). Overall, the data are conflicting, and further studies are required. Currently, evidence suggests that OS_A offspring have a similar rate of congenital abnormalities compared to those who were conceived naturally, after maternal factors including age, parity, and subfertility are taken into account (Klemetti *et al.* 2005; 2010; Zhu *et al.* 2006).

1.5.7.3. Growth and adiposity

Only one study has assessed height outcomes in OS_A conceived children, and no studies have assessed BMI or body composition in these children. The one study assessing height compared 45 OS_A children to 203 naturally conceived controls and to 83 ART-conceived children (Makhoul *et al.* 2009). This study found that height was significantly different among the groups (Figure 1.6).

However, the height of OS_A children was not significantly different to that of naturally conceived children. It is worth noting that the cohort consisted entirely of children born of very low birth weight and almost half of participants were twins (Makhoul *et al.* 2009), both factors known to affect childhood height (Hack *et al.* 2002; Hack *et al.* 2003; IJzerman *et al.* 2001). In addition, the socio-economic status of the cohort is unclear, which is another factor known to influence height in childhood (Silventoinen 2003).

1.5.7.4. General health, cancer, and other risks

There is a marked paucity of studies assessing the general health of children conceived with OS_A (Klemetti *et al.* 2010). OS_A children seem to have poorer health (as assessed by hospital admissions) than naturally conceived counterparts (Klemetti *et al.* 2010). However, the poorer health of OS_A children was mostly attributable to perinatal problems, which were associated with complications of prematurity and low birth weight. The risk of childhood cancer in OS_A children compared to the general population is unknown as the data are extremely limited (Lightfoot *et al.* 2005; Puumala *et al.* 2012b).

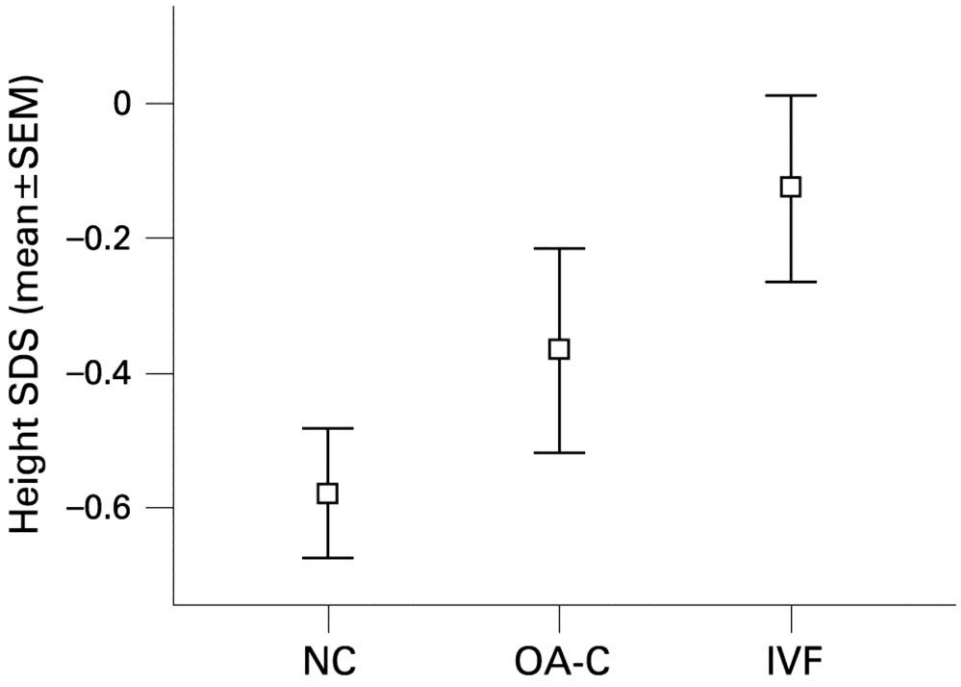

Figure 1.6. Height standard deviation score (SDS) at 6–10 years of age in IVF-conceived (IVF-C), ovulating agents-conceived (OA-C) and naturally conceived (NC) groups.
Reproduced with permission form BMJ Publishing Group Ltd and the Royal College of Paediatrics and Child Health (Makhoul *et al.* 2009).

Overall, it is clear that OS_A children are an under-researched group, with a marked paucity of robust studies assessing physical outcomes in childhood. The limited number of studies on certain childhood outcomes included participants who were born prematurely and/or of low birth weight. Furthermore, background parental subfertility is often suggested as a possible contributing factor. Thus, the true impact of OS_A on childhood outcomes has not been assessed to date.

1.5.8. Subfertility

As previously outlined (Savage *et al.* 2011), parental subfertility may be an important factor in obstetric and perinatal outcomes, as well as in many childhood outcomes in OS_A (and ART) offspring. As a result, several studies have attempted to assess the potential effects of subfertility. Many of these studies have been already outlined in relation to outcomes on ART and OS_A offspring, so that they will only be briefly discussed here.

Parental subfertility is associated with poorer obstetric and perinatal outcomes, such that subfertile mothers are more likely to suffer miscarriage and have children born prematurely, of low birth weight, and SGA (Ludwig 2009). Rates of congenital abnormalities (Zhu *et al.* 2006) and rare imprinting disorders (Doornbos *et al.* 2007) are also higher among the offspring of subfertile parents. While associations between parental subfertility and childhood cancer risk have been explored, there is currently no proven link (Puumala 2010; Puumala *et al.* 2012b).

It is clear that OS_A fertility treatment represents a departure from natural conception and that this departure leads to many changes in the oocyte and the early fetal environment. Nonetheless, there is a remarkable paucity of research assessing the various outcomes of this large population in childhood and adulthood. The vast majority of research to date has assessed outcomes on ART offspring. As a result, currently, the effects of ovarian stimulation on offspring outcomes can only be inferred from these studies. Importantly however, as outlined OS_A and ART only share ovarian stimulation in common, with ART going on to a far more invasive and complex process. Therefore, it is necessary to specifically investigate the possible impact of OS_A on offspring outcomes. Further, it is also important to separate the effects of OS_A from those associated with background parental subfertility.

1.6. Hypotheses

As outlined, changes in the early environment, particularly at the times of gametogenesis and embryogenesis, are associated with alterations in offspring phenotype and metabolism. There is evidence that the process of ART is associated with such alterations in the offspring. Since ovarian stimulation comprises part of the ART process, we hypothesised that children conceived with ovarian stimulation alone (without ART) would differ to those conceived naturally. In addition, advancing maternal age is associated with an increase in endogenous FSH production in the mother, which is a form of 'endogenous ovarian hyperstimulation'. Therefore, we also hypothesised that increasing maternal age would be associated with phenotypic and metabolic changes in the offspring.

However, following an extensive literature review and ample discussions on study design to address the above hypotheses, it became clear that there are other important parental factors that may impact on offspring phenotype and metabolism. We have identified three additional factors deemed to be important: background parental subfertility, paternal age, and birth order. As a result, we have subsequently expanded on our original hypotheses.

Background parental subfertility (i.e. duration to conception) have been shown to affect outcomes in naturally conceived children compared to ART conceived children. Therefore, we hypothesised that children of subfertile children would differ to those who were naturally conceived.

Paternal age is also associated with changes in offspring outcomes, including disease risk. Since average paternal age at childbirth is also increasing and it is closely correlated with maternal ages, the potential effects of paternal age on offspring outcomes require investigation. Birth order is another inherently important 'parental factor' that should be assessed in our cohorts. Thus, we hypothesises that both increasing paternal age and birth order would be separately associated with changes in the naturally conceived offspring in childhood.

As a result, the overarching hypothesis of this thesis is that a number of parental factors would be associated with alterations in offspring phenotype and metabolism.

1.7. Summary of issues and aims

A number of important factors have been discussed so far:

- The importance of the early fetal environment and its vulnerability to even minor changes.
- The possible role of epigenetics in the early fetal environment, as well as its potential role in the developmental origins of health and disease.
- The changing demography of parental factors over the past several decades, with remarkable shifts in human behaviour:
- The increase in maternal and paternal ages at childbirth by almost four years
- The decreasing number of children being born per couple, with first-borns now representing more than 50% of all births
- The large increase in the number of couples undergoing fertility treatment, which is perhaps the greatest change in reproductive behaviour observed so far

Considering the above changes, some important conclusions can be drawn at this stage:

1. As parental factors change (such as increasing age at childbirth), there are many associated physiological changes in the respective parent. These alterations extend to their gametes, which go on to fertilize. Even minor changes in gametes or the fetal environment (including the uterus or placenta) can lead to marked changes in the offspring.
2. Only in relatively recent years have a limited number of studies directly assessed the possible effects of maternal and paternal ages at childbirth on offspring outcomes, with some evidence of resulting changes in phenotype and disease risk.
3. While studies of the impact of birth order on offspring outcomes are more numerous, few have directly assessed the effects of birth order *per se*. Further, none have eliminated all important confounding factors, such as socio-economic status.
4. The outcomes on ART offspring have received comparatively more attention, but in contrast, very few studies have examined OS_A children. In addition, parental subfertility is an important factor in offspring outcomes, but no studies have assessed its potential effect on offspring phenotype or metabolism.

With these issues in mind, this thesis will examine the potential impacts of each parental factor on childhood outcomes:

- conception with OS_A fertility treatment (Chapter 2)
- parental subfertility (Chapter 2)
- birth order (Chapter 3)
- maternal age at childbirth (Chapter 4)
- paternal age at childbirth (Chapter 5)

Chapter 2. Ovarian stimulation alone

Preface

This chapter contains the unaltered text of an already published article.

- *Authors:* Savage T, Peek JC, Robinson EM, Green MP, Miles HL, Mouat F, Hofman PL, Cutfield WS.
- *Title:* Ovarian stimulation leads to shorter stature in childhood
- *Journal:* Human Reproduction
- *Year of publication:* 2012
- *Volume:* 27
- *Issue:* 10
- *Pages:* 3092-3099
- *Impact factor:* 4.48
- *Journal's aims and scope:* "Human Reproduction features full-length, peer-reviewed papers reporting original research, clinical case histories, as well as opinions and debates on topical issues. Papers published cover the scientific and medical aspects of reproductive physiology and pathology, endocrinology, andrology, gonad function, gametogenesis, fertilization, embryo development, implantation, pregnancy, genetics, genetic diagnosis, oncology, infectious disease, surgery, contraception, infertility treatment, psychology, ethics and social issues. The highest scientific and editorial standard is maintained throughout the journal along with a rapid rate of publication."

2.1. Abstract

Background: We aimed to determine whether children conceived with ovarian stimulation alone (OS_A) would differ phenotypically and biochemically from naturally conceived children of fertile and subfertile parents.

Methods: Healthy pre-pubertal children aged 3–10 years, born at term, after singleton pregnancies were recruited in Auckland (New Zealand), and were allocated into three groups: i) children conceived following OS_A, and naturally conceived children of ii) subfertile and iii) fertile parents. Anthropometric, endocrine, and metabolic parameters were recorded. Children's heights and BMI were expressed as standard deviation score (SDS) and corrected for genetic potential (i.e. parental height or BMI).

Results: 352 children were studied: 84 OS_A subjects and 268 naturally conceived controls consisting of 54 children of subfertile parents and 214 children of fertile parents. Children of subfertile and fertile parents did not differ in measured outcomes. Overall, OS_A children were shorter than children of both subfertile (SDS score -0.08 ± 0.09 *vs* 0.32 ± 0.07; *P*=0.001) and fertile (SDS score -0.08 ± 0.09 *vs* 0.45 ± 0.10; *P*=0.004) parents when corrected for genetic height potential. OS_A boys were shorter than boys of subfertile (SDS score -0.18 ± 0.14 *vs* 0.42 ± 0.16; *P*=0.03) and fertile (SDS score -0.18±0.14 *vs* 0.35 ± 0.08; *P*=0.01) parents. There was also a trend towards OS_A girls being shorter than girls of subfertile parents (*P*=0.06), but not significantly shorter than those of fertile parents (*P*=0.17). OS_A children also had lower corrected BMISDS than children of subfertile (SDS score -0.90 ± 0.15 vs -0.37 ± 0.17; *P*=0.06) and fertile (-0.90 ± 0.15 *vs* -0.34 ± 0.10; *P*=0.008) parents. Among metabolic parameters, fasting glucose was lower in OS_A children than in children of fertile parents (4.62 ± 0.07 *vs* 4.81 ± 0.04; *P*=0.006).

Conclusions: Conception after OS_A was associated with shorter stature, particularly in boys, compared to naturally conceived children of fertile and subfertile parents.

2.2. Introduction

Almost 1 in 7 couples have difficulty conceiving, with many going on to seek fertility treatment [1]. It is estimated that 2.5 to 7% of children in Western countries are conceived with the help of ovarian stimulation alone (OS_A) [2,3] compared to 1 to 4% conceived through *in vitro* fertilisation (IVF) [3]. Most fertility treatment utilises ovarian stimulation; either alone or

as part of the process of IVF [4]. Ovarian stimulation is widely used in the treatment of infertility and the majority of women who undergo OS_A receive either clomiphene citrate and/or follicle stimulating hormone (FSH), often in conjunction with timed intercourse or intra-uterine insemination [4].

There is evidence that IVF children are phenotypically different to naturally conceived children, having for example, higher rates of congenital abnormalities [5] and rare imprinting disorders [6]. When childhood stature has been examined, several studies have found that IVF children were taller than naturally conceived children [7-12].

There is a growing recognition that parental subfertility may play an important role in some of the differences seen in children conceived after IVF, suggesting that parental subfertility should be considered in studies assessing outcomes on children conceived via IVF or with the help of other fertility treatments [7,13-15].

While several studies (reviewed elsewhere [16,17]) have assessed childhood outcomes after IVF conception, there is a marked paucity of studies assessing similar outcomes following OS_A conception. Since IVF is a complex treatment combining several steps, including ovarian stimulation, embryo culture, and embryo selection; it is unclear whether ovarian stimulation plays a role in the observed height differences between IVF children and those conceived naturally. Thus, we have studied phenotypic and metabolic measures of children conceived through OS_A in an attempt to answer this question. We aimed to determine whether children conceived with the help of OS_A would be phenotypically and biochemically different to naturally conceived children of fertile and subfertile parents.

2.3. Methods

2.3.1. Subjects

Only healthy pre-pubertal children aged 3 to 11 years, of European ethnicity, born at term (37–41 weeks gestation) after singleton pregnancies, and of birth weight appropriate for gestational age were included in the study. OS_A subjects were recruited from a patient database of couples who attended for infertility treatment at Fertility Associates in Auckland between 2000 and 2006. Couples included in the study were those treated for unexplained infertility or mild male infertility. Children of women with a diagnosis of polycystic ovarian syndrome (PCOS) (based on pelvic ultrasound) were excluded from the study, because of the

possible association between maternal PCOS and phenotypic differences in their children [18]. Mothers of naturally conceived children did not undergo a pelvic ultrasound to rule out PCOS, but the children with a maternal history of PCOS were excluded from the study. The children of mothers who smoked, had a chronic illness, pre-existing or gestational diabetes, or glucose intolerance during pregnancy were also excluded from the study.

OS_A mothers received one of three fertility treatment regimens: 1) clomiphene citrate (Serophene) (Merck Serono, NSW, Australia) for 5 days (dose: 25–100 mg day^{-1}, mean dose 50 mg day^{-1}); 2) clomiphene for 5 days (dose: 25–150 mg day^{-1}, mean dose 75 mg day^{-1}), and FSH (Puregon, Merck Sharp & Dohme, Tipperary, Ireland) or Gonal F (Merck Serono, NSW, Australia) alternative days (75-150 IU $dose^{-1}$, mean total dose: 360 IU); or 3) FSH daily (75-150 IU $dose^{-1}$, mean total dose 375 IU). Ovarian response was monitored by serum oestradiol, LH or ultrasound as appropriate; with hCG triggering (250 µg Ovidrel; Merck Serono, NSW, Australia) if an LH surge had not occurred prior to intra-uterine insemination (82%) or timed intercourse (18%). All OS_A children were conceived using sperm from the mother's partner, so that the offspring of donor sperm were not included. Exclusion criteria for OS_A children included those who had a known medical syndrome, a chronic illness, on treatment with regular medications, or those identified on early scan as multiple pregnancy ('vanishing twin' syndrome).

All OS_A children enrolled were also asked to invite family friends and school friends to participate in the study as controls. Control mothers and children were subject to the same inclusion and exclusion criteria as OS_A children. All participants (OS_A subjects and controls) were of higher socio-economic status according to their residential address and the "decile score" of the school they attended. Every census year, the Ministry of Education compiles a decile score of every school in New Zealand [19]. A decile score indicates the extent to which a school draws students from low or high socioeconomic communities, so that decile 1 indicates lowest and 10 highest socio-economic status. All participants in the study were of decile 9 or 10 socio-economic status. We effectively ensured that control children were of similar age, ethnicity, and socio-economic status as OS_A children.

Study participants were allocated into three groups: children conceived with the help of ovarian stimulation (OS_A), and two control groups of naturally conceived children (of fertile and subfertile parents). In order to identify any potential contribution of parental subfertility to measured outcomes, control children were categorised based on the time taken for their

parents to conceive. Subfertility is generally accepted as failure to conceive after more than 12 months of unprotected intercourse [20], so control children were divided into children of fertile parents (duration to conception <12 months) and those of subfertile parents (duration to conception >12 months). We did not specifically seek out children of subfertile parents when recruiting naturally conceived control children. Instead, we recruited naturally conceived children in large numbers in order to opportunistically capture more children of subfertile parents in the study. At assessment, along with routine medical and obstetric history, we clarified that all control children were naturally conceived. We informed parents in writing that information regarding mode and duration to conception would be sought on the day of their child's assessment. We determined the duration to conception (in months) by means of open-ended interview questions, which were conducted by the same medical doctor. This optimized the accuracy of recall of "time to conception" as described by Joffe [21].

2.3.2. Study design

Standing and sitting height were measured using a Harpenden stadiometer. Children's weight and body composition were assessed using dual-energy x-ray absorptiometry (DXA Lunar Prodigy 2000; General Electric, Madison, WI, USA). Each child had a bone age x-ray to assess biological maturity, which was assessed by a single blinded paediatric endocrinologist using pre-established standards [22]. Parental height, weight, and body mass index (BMI) were recorded. Children's birth weight was transformed into standard deviation scores (SDS), as per standards based on birth weight data for approximately 500,000 "healthy newborns" in Sweden [23], which are also applicable to our study population.

Children's and parental heights were transformed into SDS [24]. Mid-parental height SDS (MPHSDS) was calculated for each child, taking into consideration the sex of the child [25]. Children's heights SDS were then individually corrected for their genetic potential (parental contribution), using the formula: child's height SDS minus MPHSDS. Children's and parent's BMI's were transformed into SDS and mean parental BMISDS (MPBMISDS) was calculated for each child [26]. Each child's corrected BMISDS was calculated individually using the formula: child's BMI SDS minus MPBMISDS. The SDS system expresses the extent of the deviation of a given value from the standard population mean. Each SDS is calculated as the individual value minus the mean value for the reference population (given gender and age), divided by the standard deviation (SD) of the reference population. Thus, the mean reference point is zero [27].

A fasting morning blood sample was obtained from each child for assessment of metabolic and growth factors. Plasma insulin was measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL, USA) by microparticle enzyme immunoassay (Abbott Diagnostics, Wiesbaden, Germany) with an inter-assay coefficient of variation (CV) of <5%. Glucose, triglyceride, cholesterol, HDL-C, and LDL-C concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany) with an inter-assay CV of 1.2% for glucose, and <5% for cholesterol, triglycerides, HDL-C, and LDL-C. Insulin resistance was calculated using the homeostasis model (HOMA-IR) [28]. Commercially available ELISAs (R&D Systems, Minneapolis, MN, USA) were used to evaluate plasma IGF-I (DSL-100, intra-assay CV 2.8%, inter-assay CV 9.2%), and IGF binding protein 3 (IGFBP-3) (DSL-10-6600, intra-assay CV 3.1%, inter-assay CV 9.9%). Commercially available ELISA kits (Meddiagnost,_Reutlingen Germany) were used to evaluate IGF-2 (E-30, intra-assay CV 1.9%, inter-assay CV 6.3%).

2.3.3. Statistical analysis

ANOVA was used to compare baseline characteristics of parents and children in each group (OS_A, subfertile, and fertile). Data were analysed with both sexes combined, as well as separately for boys and girls. Linear mixed models followed by Tukey's *post hoc* comparisons were used to investigate differences in anthropometric measures. Models included family as a random effect to control for the presence of siblings. Metabolic and endocrine data (plasma glucose, insulin, HOMA-IR, triglycerides, cholesterol, HDL-C, LDL-C, cholesterol: HDL ratio, IGF-I, IGF-II, and IGFBP-3) were analyzed in a similar manner with child's age and sex included in the model where appropriate. Multiple analysis of variance (MANOVA) was used to compare the combined effects on corrected height SDS, IGF-I and IGFBP-3 among groups (OS_A, subfertile, and fertile). Means ± SEM are reported for baseline data; means ± SEM adjusted for other variables in the model are reported for other measures. Analyses were carried out in SAS v.9.1 (SAS Institute, Cary, NC, USA) and Minitab v.16 (Pennsylvania State University, PA, USA). Significance was determined if $P<0.05$.

2.3.4. Ethics approval

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee, and written informed consent was obtained from parents of all participants. Depending on the child's age, written or verbal consent was obtained from all children.

2.4. Results

2.4.1. Study population

206 OS_A mothers were eligible to be contacted for participation in the study when inclusion and exclusion criteria were applied. 92 mothers (45%) were un-contactable due to out-of-date contact details and 30 mothers (15%) declined to participate. In all, 84 of 114 (74%) contactable candidates participated in the study. There were no discernible differences in known baseline characteristics between participants and non-participants.

A total of 352 children aged 7.33 ± 0.12 years were studied: 84 OS_A subjects and 268 naturally conceived controls, comprising of 54 children of subfertile parents and 214 children of fertile parents. The mothers of OS_A children received drug treatment for ovarian stimulation with clomiphene alone (n=26; 31%), a combination of FSH and clomiphene (n=53; 63%), or FSH alone (n=5; 6%), and most (n=69; 82%) also underwent intra-uterine insemination as part of treatment. 80% of OS_A children (35 girls and 32 boys) had parents with unexplained infertility, while the remaining 20% (10 girls and 7 boys) had a father with mild male factor infertility (21%). Among the naturally conceived controls, 54 of 268 (20%) were children of subfertile parents, as they were conceived after 12 months or more of unprotected intercourse.

Parental and birth characteristics of each group are summarised in Table 2.1. Boys and girls from each group (OS_A, children of subfertile and fertile parents) were of similar age (Table 2.2 & Table 2.3), and their age and sex distribution was also similar. Comparison of children of subfertile parents to children of fertile parents yielded no differences in measured anthropometric, endocrine, or metabolic parameters. OS_A children were shorter than children of subfertile parents (SDS score -0.075 ± 0.09 *vs* 0.32 ± 0.07, P=0.001) and were also shorter than children of fertile parents (-0.075 ± 0.09 *vs* 0.45 ± 0.10, P=0.004) when corrected for MPHSDS. OS_A children also had a lower BMI SDS than children of subfertile parents (SDS

score -0.90 ± 0.15 vs -0.37 ± 0.17; *P*=0.06) and children of fertile parents (-0.90 ± 0.15 vs -0.34 ± 0.10; *P*=0.008) when corrected for MPBMISDS.

When the sexes were compared separately, OS_A boys were shorter than boys of subfertile (*P*=0.03) and fertile (*P*=0.01) parents after correction for MPHSDS (Table 2.2). Similarly, OS_A girls tended to be shorter than girls of subfertile (*P*=0.06) but not shorter than girls of fertile parents (*P*=0.17) when corrected for MPHSDS (Table 3). OS_A boys had lower IGFBP-3 levels than boys of subfertile (*P*=0.03) and fertile parents (*P*=0.03; Table 2.2). Comparison of the combined effects on corrected height SDS, IGF-I, and IGFBP-3 among groups demonstrated distinct differences between OS_A boys compared to boys of subfertile (*P*=0.002) and fertile parents (*P*=0.003). It should also be noted that boys and girls in all groups displayed similar biological maturity based on individual bone age assessments (Table 2.2 & Table 2.3).

Table 2.1. Parental and birth characteristics of children in ovarian stimulation alone (OS_A), subfertile, and fertile groups.
*P<0.05 for comparison with OS_A, †P<0.05 for comparison with SF. Where appropriate, data are means ± SEM.

Parameter	OS_A	Subfertile	Fertile
n	84	54	214
Mid-parental height SDS	0.95 ± 0.08	0.64 ± 0.08 *	0.86 ± 0.05
Maternal BMISDS	0.30 ± 0.12	0.66 ± 0.15	0.34 ± 0.07
Mean parental BMISDS	0.63 ± 0.09	0.93 ± 0.12	0.70 ± 0.05
Maternal age at delivery (yrs)	36.9 ± 0.4	35.5 ± 0.33 *	32.8 ± 0.3 *†
Time to pregnancy (mths)	24 ± 2.3	22 ± 1.5	2.9 ± 0.15 *†
Gestation (wks)	39.1 ± 0.15	39.3 ± 0.17	39.5 ± 0.08
Birth weight SDS	0.05 ± 0.1	0.14 ± 0.12	0.22 ± 0.06
Firstborns (%)	56	61	50
Sex ratio (% males)	47	46	47
Breast-feeding rates at 6 months (%)	62	59	63

Table 2.2. Anthropometric, and fasting serum endocrine and metabolic parameters in boys from ovarian stimulation alone (OS_A), subfertile, and fertile groups.

*P<0.05 for comparison with OSA. Data are means ± SEM.

BOYS	OS_A	Subfertile	Fertile
n	39	26	123
Anthropometric			
Age (yr)	7.49 ± 0.37	7.56 ± 0.47	7.34 ± 0.19
Bone age–age (yr)	-0.01 ± 0.13	-0.51 ± 0.22	-0.16 ± 0.10
Height SDS	0.74 ± 0.16	0.94 ± 0.19	0.95 ± 0.09
HtSDS–MPHSDS	-0.18 ± 0.14	0.42 ± 0.16 *	0.35 ± 0.08 *
Sitting HtSDS	0.02 ± 0.24	0.36 ± 0.30	0.53 ± 0.15
BMISDS	0.06 ± 0.17	-0.07 ± 0.21	-0.05 ± 0.10
BMISDS–MPBMISDS	-0.6 ± 0.22	-0.32 ± 0.27	-0.13 ± 0.14
Body fat %	16.1 ± 0.9	16.5 ± 1.5	14.8 ± 0.5
Endocrine & metabolic			
Insulin (mU L^{-1})	4.66 ± 0.37	5.34 ± 0.43	4.97 ± 0.22
Glucose (mmol L^{-1})	4.64 ± 0.07	4.86 ± 0.08 *	4.83 ± 0.04
HOMA-IR	0.97 ± 0.09	1.19 ± 0.10	1.10 ± 0.05
Cholesterol (mmol L^{-1})	4.10 ± 0.13	4.15 ± 0.16	4.26 ± 0.08
HDL-C (mmol L^{-1})	1.36 ± 0.06	1.33 ± 0.07	1.35 ± 0.03
Chol: HDL-C ratio	3.26 ± 1.90	3.65 ± 1.00	4.95 ± 1.40
LDL-C (mmol L^{-1})	2.40 ± 0.10	2.45 ± 0.13	2.44 ± 0.06
Triglycerides (mmol L^{-1})	0.68 ± 0.05	0.79 ± 0.06	0.68 ± 0.03
IGF-I (µg L^{-1})	93 ± 6	111 ± 7	102 ± 3
IGF-II (µg L^{-1})	763 ± 19	756 ± 19	733 ± 8
IGFBP-3 (µg L^{-1})	2309 ± 126	2829 ± 161 *	2683 ± 76 *

Table 2.3. Anthropometric, and fasting serum endocrine and metabolic parameters in boys from ovarian stimulation alone (OS_A), subfertile, and fertile groups.

*P<0.05 for comparison with OSA. Data are means ± SEM.

GIRLS	OS_A	Subfertile	Fertile
n	45	28	91
Anthropometric			
Age (yr)	7.10 ± 0.32	7.43 ± 0.47	7.24 ± 0.23
Bone age–age (yr)	-0.26 ± 0.14	-0.09 ± 0.12	-0.11 ± 0.10
Height SDS	0.96 ± 0.14	0.68 ± 0.18	0.87 ± 0.10
HtSDS–MPHSDS	-0.002 ± 0.13	0.48 ± 0.15	0.29 ± 0.09
Sitting HtSDS	0.22 ± 0.20	-0.07 ± 0.24	0.02 ± 0.14
BMISDS	-0.48 ± 0.15	-0.27 ± 0.18	-0.30 ± 0.10
BMISDS–MPBMISDS	-1.10 ± 0.18	-0.49 ± 0.22	-0.40 ± 0.13 *
Body fat %	18.9 ± 0.0	21.6 ± 1.2	19.1 ± 0.6
Endocrine & metabolic			
Insulin (mU L^{-1})	4.66 ± 0.37	5.34 ± 0.43	4.97 ± 0.22
Glucose (mmol L^{-1})	4.64 ± 0.07	4.86 ± 0.08 *	4.83 ± 0.04
HOMA-IR	0.97 ± 0.09	1.19 ± 0.10	1.10 ± 0.05
Cholesterol (mmol L^{-1})	4.10 ± 0.13	4.15 ± 0.16	4.26 ± 0.08
HDL-C (mmol L^{-1})	1.36 ± 0.06	1.33 ± 0.07	1.35 ± 0.03
Chol: HDL-C ratio	3.26 ± 1.90	3.65 ± 1.00	4.95 ± 1.40
LDL-C (mmol L^{-1})	2.40 ± 0.10	2.45 ± 0.13	2.44 ± 0.06
Triglycerides (mmol L^{-1})	0.68 ± 0.05	0.79 ± 0.06	0.68 ± 0.03
IGF-I (µg L^{-1})	93 ± 6	111 ± 7	102 ± 3
IGF-II (µg L^{-1})	763 ± 19	756 ± 19	733 ± 8
IGFBP-3 (µg L^{-1})	2309 ± 126	2829 ± 161 *	2683 ± 76 *

Overall, OS_A children had lower fasting glucose than control children (children of subfertile and fertile parents combined) (4.62 ± 0.07 *vs* 4.81 ± 0.04 mmol/l; *P*=0.006). In addition, HDL concentrations in OS_A girls were higher than in girls of fertile parents (*P*=0.01) and tended to be higher than in girls of subfertile parents (*P*=0.09; Table 2.3). As a result, OS_A girls had lower total cholesterol to HDL ratios than girls of fertile parents (*P*=0.01), and also tended this way compared to girls of subfertile parents (*P*=0.09; Table 2.3).

2.5. Discussion

This study found that, when corrected for their genetic height potential, children conceived following OS_A were shorter than children of fertile and subfertile parents. On average OS_A boys were 3 centimetres shorter than boys of subfertile and fertile parents, with a trend towards shorter stature in OS_A girls compared to girls of subfertile parents. Importantly, the children studied did not display evidence of delayed biological maturation, as children's bone ages matched their chronological age [25]. Thus, the observed height reduction among OS_A children is likely to persist into adulthood. Furthermore, when the combined effects on corrected height SDS, IGF-I, and IGFBP-3 were evaluated, there were clear differences between OS_A boys and boys of fertile and subfertile parents.

To our knowledge, this is the first study to assess auxological, metabolic, and hormonal outcomes in OS_A children born at term and of normal birth weight. It is also the first study of prepubertal outcomes in fertility treatment children (OS_A or IVF) to have two separate control groups, consisting of children of subfertile and fertile parents. The finding of shorter stature in OS_A children was unexpected, and contrasts with a previous study that found similar height to controls in OS_A children born at very low birth weight [9]. Our findings also contrast with the taller stature of IVF children found in several studies [7-11].

IVF differs substantially to OS_A, as IVF involves several further steps, including external fertilisation and embryo culture and selection. In addition, ovarian stimulation for IVF differs to OS_A, as it utilises different drug regimens at higher doses, aiming to produce 6 to 10 follicles for IVF versus 2 to 3 follicles for OS_A [29]. Furthermore, clomiphene is frequently utilised in OS_A, but rarely used in ovarian stimulation for IVF [4]. Hence, the contribution of ovarian stimulation to the height of either OS_A or IVF children is likely to be complex, and may depend on several factors including the type and degree of ovarian stimulation.

One potential reason for the differences observed between OS_A and naturally conceived children may be that ovarian stimulation leads to alterations in genomic imprinting in the oocyte or embryo, with consequent programming of endocrine changes. As the timing of imprinting overlaps with the timing of ovarian stimulation, interventions in oocyte maturation could have an impact on imprinting and methylation [15]. In fact, several studies have found that ovarian stimulation for IVF alters DNA methylation of imprinted and non-imprinted genes [30-33], and a recent investigation suggested that the degree of ovarian stimulation may influence the extent of methylation change in the oocyte [34]. Further, imprinted genes involved

in growth regulation can also be altered by ovarian stimulation for IVF [31]. Although there is no direct evidence linking clomiphene to methylation changes, such alterations can be associated with tamoxifen [35,36], which is a structurally similar compound to clomiphene [37]. Thus, it is possible that ovarian stimulation may cause imprinting changes at critical points in early embryonic development, leading to the phenotypic differences seen in our study population. It is also conceivable that sexually dimorphic imprinting could explain the more pronounced height difference observed in OS_A boys, but this remains speculative at this stage. Future work should examine the DNA of OS_A children for differences in methylation of imprinted and non-imprinted genes when compared to naturally conceived children.

Another possible explanation for the observed differences in OS_A children may be indirect effects of clomiphene citrate or FSH on the oocyte or developing embryo. FSH is used as part of ovarian stimulation for IVF, and this process has been shown to alter intra-uterine chemokines, cytokines, and growth factors; but it is unknown whether these changes affect phenotype [38]. Nonetheless, clomiphene has many well recognised effects on the intra-uterine environment, including altered embryonic adhesion [39] and endometrial thickness [40], which may impact on embryonic development. Clomiphene is also associated with reduced serum levels of IGF-I and increased levels of IGFBP-3 in some women after standard treatment for OS_A, but it is unknown if these alterations affect the developing oocyte or embryo [41,42]. With metabolites of clomiphene detectable in maternal serum more than one month after administration [37], it is possible that clomiphene could have subtle effects in the developing oocyte or embryo, leading to subsequent phenotypic changes.

This study compared outcomes in OS_A children to those born of subfertile and fertile parents in order to differentiate the contribution of parental subfertility separately from that of the fertility treatment itself. It is therefore of interest that children of fertile and subfertile parents were similar in all anthropometric, endocrine, and metabolic parameters measured in our study, even though the subfertile control group was comparatively smaller (n=54). The inclusion of children of subfertile parents in this study was important, as they are the most logical comparison group for children conceived by OS_A or IVF [43]. It seems that parental subfertility is an important factor determining peri-natal and childhood outcomes in IVF offspring [13-15], and may account in part for the higher rate of congenital abnormalities and imprinting disorders amongst the IVF population [5,6,44-46]. However, as children of fertile and subfertile parents had similar heights, the observed shorter stature among OS_A children in our study is unlikely to be explained by parental subfertility. Nonetheless, it is important to

emphasise that OS_A and IVF children are in reality a distinct population to children of subfertile parents, as the latter were eventually conceived naturally, whereas OS_A and IVF children were not.

We also observed metabolic differences (including lower fasting glucose and higher HDL) in OS_A children. To our knowledge, no previous studies have examined metabolism in OS_A children, and those on IVF children yielded conflicting results (reviewed by [47]. It is unclear whether these relatively subtle metabolic changes could have any long-term significance, but these are further evidence that OS_A children are different to those conceived naturally.

The strengths of this study include restriction of the study population to a higher socio-economic group and single ethnicity in an effort to reduce confounders. Further, the height of each child in our study was individually corrected for parental stature (MPHSDS), which is the most important determinant of childhood height [25]. We encountered only three previous studies that corrected for this fundamental factor of genetic height when assessing childhood height in OS_A or IVF children [9-11]. In contrast, a possible limitation of our study is that we cannot rule out with certainty the existence of PCOS cases among the mothers of OS_A or control groups. However, any OS_A mothers with a positive ultrasound or history of PCOS were excluded from the study, and all control mothers had a negative history of PCOS [48]. Furthermore, it is worth noting that no mothers (OS_A and controls) had a history of glucose intolerance, and the mean maternal BMISDS was well within the normal BMISDS range in all groups. Another possible weakness of the study is that the duration to conception reported by parents of children in the subfertile group may be subject to recall bias, but we optimized our methods to maximize recall accuracy. A further possible weakness was the relatively small number of children of subfertile parents (n=54) compared to the number of children of fertile parents (n=214). Therefore, a larger study with a greater number of children in the subfertile group may be required to clarify possible differences in anthropometric, metabolic, or hormonal outcomes between children of fertile and subfertile parents.

2.6. Conclusion

OS_A children, particularly boys, have shorter stature than children of fertile and subfertile parents. There is also evidence that OS_A children have an altered metabolic phenotype. Since OS_A children comprise up to 7% of all births in Western countries, it is important to determine whether these differences persist or accentuate during puberty and into adulthood.

Further studies are necessary to fully understand any potential long-term consequences to children and adults conceived with this widely used fertility treatment.

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Chapter 3. Birth order progressively affects childhood height

Preface

This chapter contains the unaltered text of an already published article online.

- *Authors:* Savage T, Derraik JGB, Miles HL, Mouat F, Hofman PL, Cutfield WS
- *Title:* Birth order progressively affects childhood height
- *Journal:* Clinical Endocrinology
- *Volume:* [Epub ahead of print]
- *Impact factor:* 3.17
- *Journal's aims and scope:* "Clinical Endocrinology publishes papers and reviews which focus on the clinical aspects of endocrinology, including the clinical application of molecular endocrinology. It features reviews, original papers, commentaries, cases of the month, book reviews and letters to the editor. Clinical Endocrinology is essential reading not only for those engaged in endocrinological research but also for those involved primarily in clinical practice."

Abstract

Background: There is evidence suggesting that first-born children and adults are anthropometrically different to later-borns. Thus, we aimed to assess whether birth order was associated with changes in growth and metabolism in childhood.

Methods: We studied 312 healthy pre-pubertal children: 157 first-born and 155 later-borns. Children were aged 3–10 years, born 37–41 weeks gestation, and of birth weight appropriate-for-gestational-age. Clinical assessments included measurement of children's height, weight, fasting lipid and hormonal profiles, and DXA-derived body composition. Data are adjusted means and 95% confidence intervals.

Results: First-borns were taller than later-borns ($p<0.0001$), even when corrected for parents' heights (0.31 vs 0.03 SDS; $p=0.001$). There was an incremental height decrease with increasing birth order, so that first-borns were taller than second-borns ($p<0.001$), who were in turn taller than third-borns ($p=0.007$). Further, among sibling pairs both height SDS ($p=0.009$) and corrected height SDS ($p<0.0001$) were lower in second- vs first-born children. Consistent with differences in stature, first- ($p=0.043$) and second-borns ($p=0.003$) had higher IGF-I concentrations than third-borns. Both first- ($p<0.001$) and second-borns ($p=0.004$) also had reduced abdominal adiposity (lower android fat to gynoid fat ratio) when compared to third-borns. Other parameters of adiposity and blood lipids were unaffected by birth order.

Conclusions: First-borns were taller than later-born children, with an incremental height reduction from first to third birth order. These differences were present after correction for genetic height, and associated to some extent with alterations in plasma IGF-I. Our findings strengthen the evidence that birth order is associated with phenotypic changes in childhood.

Introduction

There is a trend towards smaller families with an increasing number of couples having fewer children. As a result, there has been a steady increase in the population of first-born children relative to later-borns, and first-borns now represent over 60% of all births in the developed world[1,2]. The trend towards smaller families began in the mid-20th century, but over the past two decades more couples are choosing to have only one or two children[2]. Fertility rates have consequently fallen to a mean of 1.56 live births per woman in Europe, reflecting the trend towards smaller families as well as an overall reduction in birth rates[1]. Changes in family size

have occurred for a number of reasons, such as increased availability of contraception, economic pressures, and advanced maternal age at first childbirth[2]. This is notwithstanding the impact of official national policies, such as the "one child" policy in China that has been in existence for over thirty years[3].

There is evidence that birth order affects offspring phenotype and disease risk. Taller stature in first- compared to later-borns has been previously described in children[4,5,6], adolescents[7], and adults[8-11]. However, all of these studies were conducted in populations including subjects from a broad range of socio-economic groups[4-6] and/or children's heights were not corrected for parental height[4]. First-born adults are at higher risk of obesity than later-borns[7,9,12,13]. Compared to later-borns, first-born children were also shown to have a higher risk of type 1 diabetes[14] and higher blood pressure[15]. One adult study has described a less favourable lipid profile in first-borns[11], but there are no data on the potential effects of birth order on blood lipids in childhood. Thus, we aimed to determine whether first-born children would have different height, body composition, metabolism, and hormonal profiles compared to later-born children.

Methods

Ethics approval

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee (Ministry of Health, New Zealand). Written informed consent was obtained from parents or guardians, as well as verbal or written consent from each child as was appropriate to their age.

Subjects

We undertook a large project examining the effects of parental and prenatal factors in the offspring. From this larger project, we have examined the impact of conception with ovarian stimulation drugs on the growth and metabolism of children[16]. Children conceived after ovarian stimulation were asked to invite family friends and school friends who were naturally conceived to participate in the study as controls[16], so that these controls were recruited randomly by study participants. Thus, in this current study we assessed only the entire naturally conceived cohort that was recruited from this larger project.

Only healthy, developmentally normal, pre-pubertal children aged 3–10 years, born full-term (37–41 weeks gestation) were studied. All children were of New Zealand European ethnicity,

naturally conceived, born of singleton pregnancies, and of birth weight appropriate-for-gestational-age (birthweight >-2 and <2 standard deviation scores (SDS)). Exclusion criteria also included signs of puberty (Tanner stage 2 breast development in girls and testicular volume >3 ml in boys or evidence of adrenarche), receiving medication that could affect insulin sensitivity or growth, and having a first degree relative with diabetes. Children were also excluded if born to mothers with gestational diabetes, pre-eclampsia, gestational or pre-existing hypertension, chronic illnesses, or prolonged maternal drug use (including tobacco). All participants were of higher socio-economic status according to their residential address and the "decile score" of the school they attended[17]. A decile score reflects the socio-economic status of the school communities, where decile 1 indicates lowest and 10 highest socio-economic status[17]. All participants in the study were of decile 9 or 10 socio-economic status

Study parameters

All clinical assessments were carried out at the Maurice & Agnes Paykel Clinical Research Unit (Liggins Institute, University of Auckland). Standing height was measured using a Harpenden stadiometer. Each child was measured between 7.30 and 8.00 am by the same paediatrician, using standard techniques for accurate height measurement as per Schilg *et al.*[18]. Children's weight and body composition were assessed using dual-energy x-ray absorptiometry (DXA Lunar Prodigy 2000; General Electric, Madison, WI, USA). Apart from the total body fat percentage, other DXA-derived parameter of interest was abdominal adiposity, which was expressed as the android fat to gynoid fat ratio. This ratio is provided by the manufacturer's software, based on an automated sectioning of specific areas of the body[19]. A number of studies in children have shown that proportionally greater adiposity in the upper body (i.e. android fat) is associated with an increased risk of adverse metabolic outcomes[20-22]. Each child had a bone age X-ray to determine biological maturity, which was blindly assessed by a single paediatric endocrinologist using pre-established standards[23]. Maternal and paternal height and weight were measured, and body mass index (BMI) was calculated. Maternal obstetric history was also recorded to clarify parity and identify the birth order of each subject.

Children's birth weight, height, BMI, and parental heights were transformed into SDS[24]. Mid-parental height SDS (MPHSDS) was calculated for each child[25]. Children's heights SDS were then individually corrected for their genetic potential (parents' heights), using the formula: child's height SDS minus MPHSDS. Parents' BMI were transformed into SDS, and

the mean parental BMISDS (MPBMISDS) was calculated for each child[26]. The SDS system expresses the extent of the deviation of a given value from the standard population mean. Each SDS is calculated as the individual value minus the mean value for the reference population (given gender and age), divided by the standard deviation of the reference population. Thus, the mean reference point is zero[24].

Following an overnight fast, morning blood samples were drawn to measure serum glucose, insulin, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, insulin-like growth factor-I (IGF-I), IGF-II, IGF binding protein-3 (IGFBP-3). An insulin resistance score (HOMA-IR) was also computed using fasting glucose and insulin concentrations[27].

Assays

Glucose concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany), with an inter-assay coefficient of variation (CV) of 2.1%. Insulin concentrations were measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL, USA) by microparticle enzyme immunoassay, with a CV of 5.7%. Total cholesterol, HDL-C, and LDL-C concentrations were measured using a Hitachi 902 autoanalyser, with CV of 8.9%, 11.4, and 10.1, respectively. Commercially available ELISA kits E20, E30, E03A, E07, and E09 (Mediagnost, Reutlingen, Germany) were used for quantitative determination of serum IGF-II, IGF-II, IGFBP-3, leptin, and adiponectin, respectively; assay sensitivities were 0.09, 0.02, 0.1, 1.0, and 0.6 ng/ml, with CV of 3.1, 5.0, 9.6, 6.7, and 3.0%, respectively.

Statistical analysis

Mean age was compared using one-way ANOVA and sex ratio with Fisher's exact test, both in Minitab v.16 (Pennsylvania State University, State College, PA, USA). Children were separated into two groups according to their birth order: first-borns and later-borns. Comparisons between birth order groups were carried out using linear mixed models in SAS v.9.2 (SAS Institute, Cary, NC, USA). All models accounted for important confounding factors, mainly gender, birth weight SDS, gestational age, and maternal age. All models included family identification number as a random factor, to account for the clustering of siblings. Other factors were controlled for as required, depending on the outcome response of interest: for lipids, hormones, and outcomes associated with glucose homeostasis – age and

BMISDS were included; for anthropometric data – age and the appropriate parental factor (i.e. MPBMISDS or MPHSDS); and for blood pressure parameters – height and total body fat percentage. For a smaller number of outcomes, data were re-analysed with subjects split into three groups according to birth order (first-, second-, and third-borns).

The interaction effect between birth order and gender was tested in all models. Outcomes were only assessed separately for boys and girls when there was an indication of a differential response to birth order between genders. In addition, main outcomes were analysed separately for all sibling pairs of first- and second-born children. Where appropriate, data were log-transformed to approximate a normal distribution. Data are provided as means and 95% confidence intervals adjusted for the confounders in multivariate models (back-transformed were appropriate).

Results

In all, 343 eligible children volunteered to participate, but 31 were subsequently excluded: 22 were born prematurely or small-for-gestational-age, 5 were pubertal, 3 children had mothers with gestational diabetes or glucose intolerance, and one child was on medications known to influence growth. Thus, a total of 312 children took part in the study: 157 first-borns (50%) and 155 later-borns (50%) (Table 3.1; Figure 3.1). Later-borns were comprised of 119 second-born and 36 third-born children (Figure 3.1). 68 children were sibling pairs (n=136) and 12 families had three siblings who participated in the study (n=36).

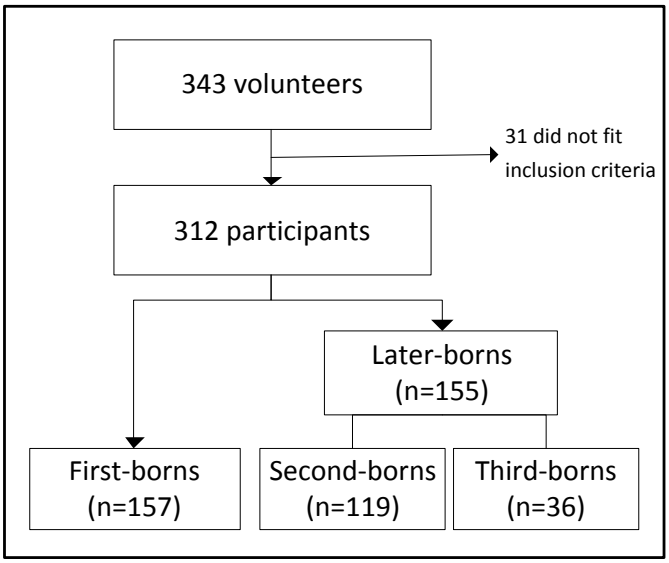

Figure 3.1. Summary of the study's recruitment process

Table 3.1. Baseline characteristics and fasting serum endocrine and metabolic parameters in first-born versus later-born children.

Age and MPHSDS data are mean ± SD; other data are means and 95% confidence intervals adjusted for other confounding factors (including birth parameters and age) in the multivariate models. Abdominal adiposity is the android fat to gynoid fat ratio.

	First-borns	Later-borns	p-value
n	157	155	
Sex ratio (boys)	54%	54%	
Age (years)	8.1 ± 2.1	6.5 ± 1.9	**<0.0001**
Chronological age – bone age (years)	-0.19 ± 0.93	-0.11 ± 0.69	0.49
Gestation (weeks)	39.8 (39.6–40.0)	39.6 (39.4–39.8)	0.13
Birth weight (kg)	3.48 (3.41–3.55)	3.60 (3.53–3.67)	**0.011**
HtSDS	1.08 (0.93–1.23)	0.67 (0.52–0.82)	**<0.0001**
MPHSDS	0.77 ± 0.72	0.89 ± 0.77	0.17
HtSDS–MPHSDS	0.42 (0.29–0.55)	0.13 (0.00–0.26)	**<0.0001**
BMISDS	-0.09 (-0.27–0.08)	-0.05 (-0.23–0.13)	0.84
Abdominal adiposity	0.59 (0.56–0.62)	0.63 (0.60–0.66)	0.11
Total body fat (%)	17.9 (16.8–18.9)	16.6 (16.5–18.7)	0.65
Total cholesterol (mmol/l)	4.33 (4.21–4.46)	4.41 (4.28–4.53)	0.56
LDL-C (mmol/l)	2.47 (2.36–2.58)	2.56 (2.44–2.67)	0.35
HDL-C (mmol/l)	1.37 (1.32–1.43)	1.39 (1.33–1.45)	0.64
Triglycerides (mmol/l)	0.73 (0.69–0.78)	0.76 (0.71–0.81)	0.88
Insulin sensitivity (HOMA-IR)	1.22 (1.10–1.35)	1.24 (1.12–1.36)	0.79
IGF-I (µg/l)	109 (101–118)	112 (103–120)	0.67
IGF-II (µg/l)	739 (723–755)	747 (731–763)	0.51
IGFBP-3 (µg/l)	2554 (2401–2707)	2635 (2478–2792)	0.41

The characteristics of the study population are outlined in Table 3.1. First-borns were of similar gestational age, but of lower birth weight than later-borns with a mean birth weight difference of 120 grams (p=0.011; Table 3.1). First-borns were older than later-borns (p<0.0001; Table 3.1), while second-born children were older than third borns (p<0.05; Table 3.2).

First-borns were taller than later-borns (p<0.0001), even when corrected for parents' heights (p<0.001; Table 3.1). In fact, there was an incremental height decrease from first- to third-borns. Thus, first-borns were taller than second-borns (p<0.001), who were in turn taller than third-borns (p=0.007; Table 3.2; Figure 3.2). Further, when sibling pairs were assessed, both height SDS (p=0.009) and corrected height SDS (p<0.0001) were lower in second-borns

compared to first-born children (Figure 3.3). Importantly, there were no differences in children's biological maturity (as assessed by bone age estimation) among groups (Table 3.1 & Table 3.2).

Although total adiposity was unaffected by birth order, abdominal fat (android fat to gynoid fat ratio) was reduced in both first- ($p<0.001$) and second-borns ($p=0.004$) compared to third-born children (Table 3.2). IGF-I concentrations were higher in both first- ($p=0.043$) and second-borns ($p=0.003$) compared to third-borns (Table 3.2). Conversely, blood lipids and other hormonal parameters were unaffected by birth order (Table 3.2).

There were no sex-dependent differences between boys and girls according to birth order. Exploratory analyses among first-borns on main study outcomes showed no differences between first-borns who had siblings (n=109; 69%) compared to those who were an only child (n=48; 31%).

Table 3.2. Baseline characteristics and fasting serum endocrine and metabolic parameters in first-, second-, and third-born children.

Age and MPHSDS data are mean ± SD; other data are means and 95% confidence intervals adjusted for other confounding factors (including birth parameters and age) in the multivariate models. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 for comparisons with first-borns; †p<0.05, and ††p<0.01 for comparison with third-borns. Abdominal adiposity is the android fat to gynoid fat ratio.

	First-borns	Second-borns	Third-borns
n	157	119	36
Age (years)	8.1 ± 2.1	6.7 ± 1.8****†	5.8 ± 2.1****
Sex ratio (boys)	54%	54%	51%
Chronological age – bone age (years)	-0.19 ± 0.93	-0.15 ± 0.72	0.02 ± 0.53
Gestation (weeks)	39.8 (39.6–40.0)	39.6 (39.4–39.9)	39.4 (39.0–39.8)
Birth weight (kg)	3.48 (3.41–3.55)	3.59 (3.51–3.66)*	3.66 (3.52–3.81)*
HtSDS	1.08 (0.93–1.23)	0.67 (0.50–0.83)***	0.70 (0.39–1.00)*
MPHSDS	0.77 ± 0.72	0.90 ± 0.74	0.87 ± 0.91
HtSDS–MPHSDS	0.42 (0.29–0.55)	0.19 (0.06–0.32)***††	-0.14 (-0.37–0.08)****
BMISDS	-0.09 (-0.27–0.08)	-0.07 (-0.26–0.12)	0.04 (-0.32–0.40)
Abdominal adiposity	0.59 (0.56–0.62)	0.61 (0.58–0.64)†	0.71 (0.64–0.77)**
Body fat (%)	17.9 (16.8–18.9)	17.5 (16.3–18.6)	18.3 (16.1–20.5)
IGF-I (µg/l)	109 (101–118)	115 (106–124)††	96 (80–113)*
IGF-II (µg/l)	739 (723–755)	752 (735–769)	725 (693–757)
IGFBP-3 (µg/l)	2554 (2401–2707)	2647 (2487–2808)	2554 (2272–2836)

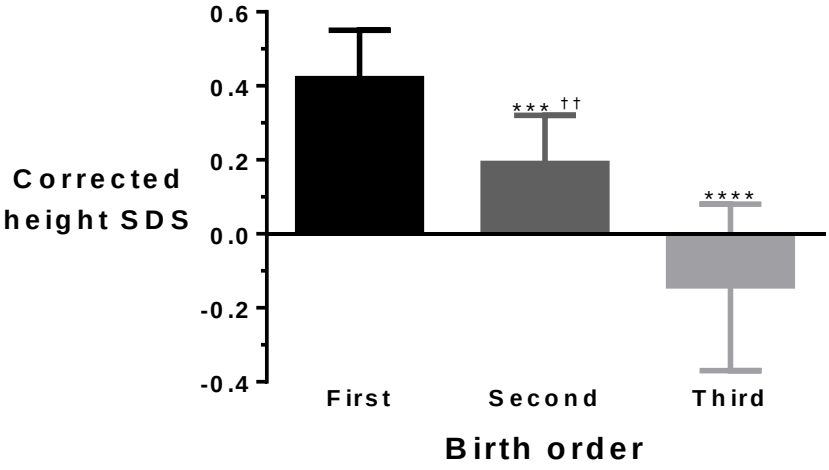

Figure 3.2. Height SDS corrected for mean parental height among first- (n=157), second- (n=119), and third-born (n=36) children.

Data are means and 95% confidence intervals, adjusted for other confounding factors in the multivariate model. ***p<0.001, ****p<0.0001 vs first-borns; ††p<0.01 vs third-borns.

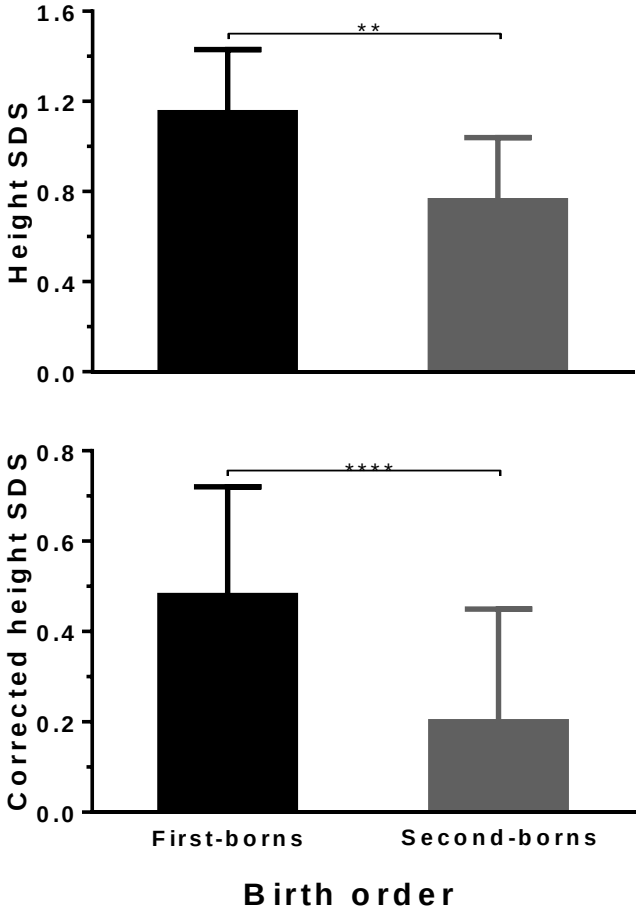

Figure 3.3. Height SDS and height SDS corrected for mean parental height from subgroup analyses on first- and second-born children from sibling pairs in the same family (n=136).

Data are means and 95% confidence intervals, adjusted for other confounding factors in the multivariate model. **p<0.01 and ****p<0.0001.

Discussion

We found that first-born children were approximately 2.5 centimetres taller than later-borns, with birth order also having a graded effect on height, with an incremental height reduction from first to third birth order. These differences equated to a mean height decrease of 1.3 cm from first- to second-borns, and a further decrease of 2.0 cm from second- to third-born children. Further, in keeping with their taller stature, first- and second-borns had higher IGF-I concentrations than third-borns. This latter observation is consistent with our study findings, as IGF-I is an important mediator of childhood growth[28].

The major strengths of our study were our contemporary cohort (born after the year 2000), consisting exclusively of children of higher socio-economic status (where nutrition was plentiful, irrespective of birth order) and of single ethnicity (New Zealand European). Importantly, each child's height was also individually corrected for accurately measured parental heights (i.e. genetic potential). Further, children across birth order groups had similar BMI, meaning that height differences were not driven by childhood obesity[29]. Notably, as there were no differences in biological maturity between groups (as assessed by bone age X-rays), our study indicates that the observed differences in height between birth order groups are more likely to persist into adulthood[25].

Previous studies assessing the impact of birth order on childhood height also found that first-borns were 1–2 cm taller than later-born children[4-6]. However, their data were not corrected for parental heights, which are the most important predictors of offspring height[25,30]. In addition, these childhood studies included participants from all socio-economic groups, and only two studies included limited adjustments for socio-economic status[4,6]. The latter has a strong effect on height, with 1–2 cm differences observed between children of lower and higher socio-economic groups[31], with more marked effects (2–6 cm) on adult height[30]. This issue is further compounded when we consider that greater family size (i.e. increasing birth order) is strongly associated with lower socio-economic status[32]. A large British study provided evidence that number of siblings also affects childhood height, independently of socio-economic status[33]. However, the latter study did not assess possible effects of birth order, and our data showed no differences in stature between first-borns who were an only child versus first-borns with siblings.

Adult studies demonstrating taller stature in first-borns have been conducted in developing countries[7,9-11], where increasing family size reduces the availability of nutrition in childhood[34]

and may explain the shorter stature of later-born offspring[31]. The only adult study conducted in a developed country comprised a cohort of adults born in post-war England, a time of limited availability of food[8].

While children in all birth order groups in our study had similar BMI and total body fat percentage, third-born children had higher abdominal fat than first- and second-borns. Increased abdominal fat is a risk factor for the metabolic syndrome[35], and third-born children may be at a greater risk of this disease than both first- and second-borns. The only other childhood study assessing the effect of birth order on childhood adiposity found that first-borns had a greater BMI than later-borns at 4 years of age, but similar BMI and body composition in adolescence[7]. Our findings also contrast with several adult studies that have found that first-borns had greater BMI and/or increased adiposity when compared to later-borns[9,11-13]. As our cohort consisted of pre-pubertal children, it remains possible that further differences in adiposity between first- and later-born children could emerge in adolescence or adulthood.

We found no effects of birth order on blood lipid profiles in childhood. Although this is the first study to examine such effects in children, one adult study found that first-borns had less favourable lipid profiles compared to later-borns[11]. As our cohort included pre-pubertal children, possible birth order effects on lipids may be yet to emerge.

There is no clear single explanation for the observed taller stature of first-born children or the graded height reduction with increasing birth order, and several mechanisms may be responsible for these differences. Taller stature in first-born children may be mediated by increased levels of growth hormone (GH) and IGF-I receptors compared to later-borns[36]. A study comparing first- and later-born sheep revealed marked differences in the hepatic GH–IGF axis[36]. Compared to later-borns, first-born lambs had a persistent increase in hepatic GH-receptor mRNA[36], as well as up-regulated hepatic IGF-I receptor mRNA[36]. These receptor differences were accompanied by a trend towards a higher crown to rump length in first-born one-month-old lambs, but unfortunately no details on any subsequent phenotypic differences were provided[36]. These findings suggest that first-borns may have increased GH and IGF-I receptor responsiveness, and increased growth may accompany these receptor differences. It is possible that these findings provide some insight into the mechanism responsible for the height differences observed between first- and later-born children.

Implantation and placentation seem to differ between first and later pregnancies, and first-born fetuses may receive a reduced nutrient supply *in utero* compared to their later-born siblings[37,38]. However, "fetal restriction" is associated with shorter stature in childhood[38,39]. Therefore this mechanism is not consistent with our findings on taller stature among first-born children.

Alternatively, differences in implantation and placentation between first- and later-borns may trigger more subtle changes in the early fetal environment. These include possible epigenetic changes (i.e. alterations in gene expression not caused by changes in DNA sequence[40]) in imprinted genes regulating childhood growth. Gene imprinting is a regulatory process that takes place at gametogenesis, embryogenesis, and placentation. Imprinted genes may be altered by subtle changes in the early fetal environment, subsequently affecting phenotype[40]. Birth order has been previously described to alter imprinted genes involved in growth, such as H19 and IGF-II[41]. Therefore, it is possible that birth order may lead to alterations in placental imprinted genes, leading to subsequent changes in childhood growth.

Possible limitations of this study include the age differences between birth order groups, but children's heights and BMI were converted to SDS to minimize possible effects of age and gender on auxological outcomes. Furthermore, not only were other measured outcomes corrected for age in statistical models (where appropriate), but there were also no differences in biological maturity among groups. There was also a relatively small number of third-born children (n=36), but this group was still large enough for important differences between birth order groups to be detected. In addition, as our cohort comprised a homogenous group of children of same ethnicity and higher socio-economic status, our findings may not be directly applicable to the general population. However, this homogeneity means that we eliminated these two important factors known to affect phenotype and metabolism in childhood, so that the effects of birth order could better evaluated.

In conclusion, we observed that first-born children were taller than later-borns, with an incremental height reduction observed from first to third birth order. Overall, the mechanisms responsible for these differences remain unexplained and further investigation is required. Given the continuing worldwide trend towards smaller families, the proportion of first-borns in the population is likely to continue to grow. As a result, the possible effects of birth order on childhood and adult phenotype and disease risk warrant further evaluation.

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Chapter 4. Increasing maternal age is associated with taller stature & reduced abdominal fat in their children

Preface

This chapter contains an unaltered reproduction of an article submitted to the journal PLoS One. The article is currently undergoing peer-review, and we are yet to receive feedback from reviewers.

- *Authors:* Savage T, Derraik JGB, Miles HL, Mouat F, Hofman PL, Cutfield WS
- *Title:* Increasing maternal age is associated with taller stature and reduced abdominal fat in their children
- *Journal:* PLOS One
- *Impact factor:* 4.40
- *Journal's scope:* "PLoS ONE features reports of original research from all disciplines within science and medicine. By not excluding papers on the basis of subject area, PLOS ONE facilitates the discovery of the connections between papers whether within or between disciplines."

Abstract

Background: Maternal age at childbirth continues to increase worldwide. We aimed to assess whether increasing maternal age is associated with changes in childhood height, body composition, and metabolism.

Methods: 277 healthy pre-pubertal children, born 37–41 weeks gestation were studied. Assessments included: height and weight corrected for parental measurements, DXA-derived body composition, fasting lipids, glucose, insulin, and hormonal profiles. Subjects were separated according to maternal age at childbirth: <30, 30–35, and >35 years.

Results: Our cohort consisted of 126 girls and 151 boys, aged 7.4 ± 2.2 years; maternal age at childbirth was 33.3 ± 4.7 years (range 19–44 years). Children of mothers aged >35 and 30–35 years at childbirth were taller than children of mothers aged <30 years by 0.26 (p=0.002) and 0.23 (p=0.042) SDS, respectively. There was a reduction in childhood BMISDS with increasing maternal age at childbirth, and children of mothers aged >35 years at childbirth were 0.61 SDS slimmer than those of mothers <30 years (p=0.049). Children of mothers aged 30–35 (p=0.022) and >35 (p=0.036) years at childbirth had abdominal adiposity reduced by 10% and 13%, respectively, compared to those in the <30 group. Children of mothers aged 30–35 years at childbirth displayed a 19% increase in IGF-I concentrations compared to offspring in <30 group (p=0.042). Conversely, IGF-II concentrations were lower among the children born to mothers aged 30–35 (6.5%; p=0.004) and >35 (8.1%; p=0.005) compared to those of mothers aged <30 years. Girls of mothers aged 30–35 years at childbirth also displayed improved HOMA-IR insulin sensitivity (p=0.010) compared to girls born to mothers aged <30 years.

Conclusions: Increasing maternal age at childbirth is associated with a more favourable phenotype (taller stature and reduced abdominal fat) in their children, as well as improved insulin sensitivity in girls.

4.1. Introduction

Over the past three decades, maternal age at first childbirth has increased by approximately 4 years in most developed countries [1]. This shift in reproductive behaviour means that most children are currently born to mothers aged more than 30 years [1]. The reasons for the

postponement of childbirth include increased availability of contraception, greater educational and career opportunities for women, economic pressures, and personal choice [2]

There is growing interest in the possible contribution of increasing maternal age to the future health of the offspring. Increasing maternal age has been linked to greater risk of type 1 diabetes [3], as well as higher blood pressure in childhood[4] and higher rates of type 2 diabetes[5] in adulthood. Increasing maternal age is also associated with increased rates of obstetric and perinatal complications, including fetal loss, pre-eclampsia, premature delivery, and low birth weight [6]

Older mother also have children with increased rates of chromosomal and other genetic disorders, which are thought to be largely due to a decline in oocyte quality [7]. The timing of the onset and rate of this decline in oocyte quantity and quality is a subject of on-going debate [7]. Nonetheless, there is a substantial reduction in female fertility from 30 years of age, which may signal the start of oocyte decline [8]. However, some authors suggest that oocyte quality and quantity declines most sharply after 35 years of age [9], while others describe a gradual decline from menarche to menopause [10]. Increasing age is also associated with physiological changes in the mother's reproductive system, such as changes in gonadotropin and other hormone levels [11,12].

These alterations in the early fetal environment may contribute to changes in the physical characteristics and disease risk of the offspring, but it is unknown whether maternal age directly affects the growth or metabolism of their children. Thus, in this study we aimed to assess whether increasing maternal age would be associated with changes in height, body composition, as well as lipid and metabolic profiles in the offspring in childhood.

4.2. Methods

4.2.1. Ethics statement

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee (Ministry of Health, New Zealand). Written informed consent was obtained from parents or guardians, as well as verbal or written consent from each child as was appropriate to their age.

4.2.2. Subjects

We undertook a large project examining the effects of parental and prenatal factors in the offspring. From this larger project, we have examined the impact of conception with ovarian stimulation drugs on the growth and metabolism of children [13]. Children conceived after ovarian stimulation were asked to invite family friends and school friends who were naturally conceived to participate in the study as controls [13], so that these controls were recruited randomly by study participants. Thus, in this current study we assessed the entire naturally conceived cohort that was recruited from this larger project (Figure 4.1).

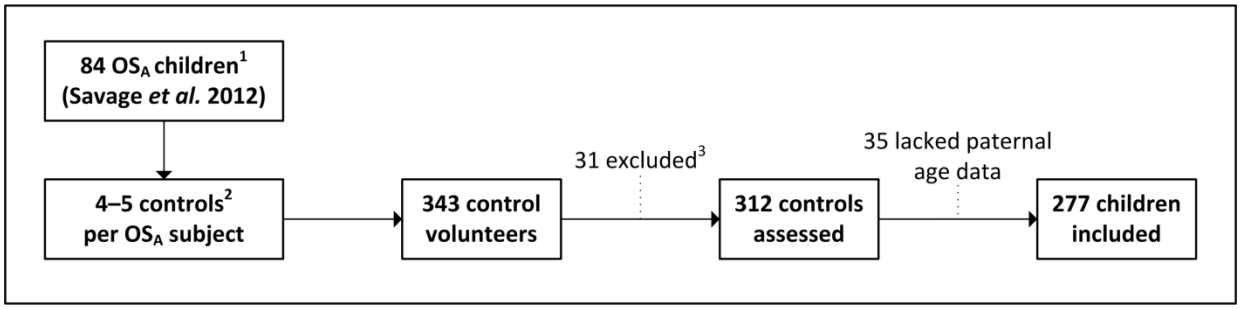

Figure 4.1. Summary of the study's recruitment process.
[1] OS_A children had been conceived via ovarian stimulation, and were examined in Savage *et al.* [13]. [2] Controls were friends of OS_A children to ensure similar age group, ethnicity, and socio-economic status. [3] 22 children were born small-for-gestational age and/or premature; five were pubertal; three were born of a mother with gestational diabetes/glucose intolerance; and one child was on medication known to influence growth.

Only healthy, developmentally normal, pre-pubertal children aged 3–10 years, born 37–41 weeks gestation were studied. All children were of New Zealand European ethnicity, naturally conceived, born to singleton pregnancies, and of birth weight appropriate-for-gestational-age (birthweight >-2 and <2 standard deviation scores (SDS)). Exclusion criteria also included signs of puberty (Tanner stage 2 breast development in girls and testicular volume >3 ml in boys or evidence of adrenarche), receiving medication that could affect insulin sensitivity or growth, and having a first degree relative with diabetes. Children were excluded if born to mothers with gestational diabetes, pre-eclampsia, gestational or pre-existing hypertension, chronic illnesses, or prolonged maternal drug use (including tobacco). All participants were of higher socio-economic status according to their residential address and the "decile score" of the school they attended [14]. A decile score reflects the socio-economic status of the school communities, where decile 1 indicates lowest and 10 highest

socio-economic status [14]. The "decile score" is a comprehensive assessment of community affluence, which takes into account a number of factors such as household income, parental occupation, parents' educational qualifications, number of occupants per dwelling size, and government welfare benefits [15,16]. All participants in the study were from schools of decile 9 or 10.

4.2.3. Study design

All clinical assessments were carried out by a single researcher at the Maurice & Agnes Paykel Clinical Research Unit (Liggins Institute, University of Auckland). Standing and sitting height were measured using a Harpenden stadiometer. Children's weight and body composition were assessed using dual-energy X-ray absorptiometry (DXA Lunar Prodigy 2000; General Electric, Madison, WI, USA). Apart from the total body fat percentage, the DXA-derived parameter of interest was abdominal adiposity, which expressed as the android fat to gynoid fat ratio and provided by the manufacturer's software based on an automated sectioning of specific areas of the body [17]. A number of studies in children have shown that proportionally greater adiposity in the upper body (i.e. android or male fat) is associated with adverse metabolic outcomes [18,19,20].

The parameters measured included total body fat percentage, android fat percentage (abdominal fat – measured from lower end of ribs and the line drawn through the hip joint), and gynoid fat percentage (fat on hips and thighs – measured from the line drawn through the hip joints and the middle of thighs). The ratio of the android fat mass to the gynoid fat mass (android/gynoid fat) was calculated [21]. Each child also had a bone age X-ray to assess biological maturity, which was blindly assessed by a single paediatric endocrinologist using pre-established standards [22].

Maternal and paternal height, weight, and body mass index (BMI) were recorded. Maternal obstetric history was also recorded to clarify parity, and relevant medical history. Children's birth weight, height, BMI, and parental height were transformed into SDS [23,24,25]. Mid-parental height SDS (MPHSDS) was calculated for each child [26]. Children's heights SDS were then individually corrected for their genetic potential (parents' heights), using the formula: child's height SDS minus MPHSDS. Parents' BMI were transformed into SDS, and the mean parental BMISDS (MPBMISDS) was calculated for each child [27]

Following an overnight fast, blood samples were drawn from each child for assessment of total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, insulin-like growth factor 1 (IGF-I), IGF-II, IGF binding protein 3 (IGFBP-3). Children also had glucose and insulin levels measured, and insulin sensitivity evaluated using the homeostasis model assessment of insulin resistance (HOMA-IR) [28]

Plasma insulin was measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL, USA) by microparticle enzyme immunoassay (Abbott Diagnostics, Wiesbaden, Germany) with an inter-assay coefficient of variation (CV) of <5%. Glucose, triglycerides, total cholesterol, HDL-C, and LDL-C concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany) with an inter-assay CV of 1.2% for glucose, and <5% for total cholesterol, triglycerides, HDL-C, and LDL-C. Commercially available ELISAs (R&D Systems, Minneapolis, MN, USA) were used to measure plasma IGF-I (DSL-100, intra-assay CV 2.8%, inter-assay CV 9.2%), IGFBP-3 (DSL-10-6600, intra-assay CV 3.1%, inter-assay CV 9.9%), and IGF-II (Meddiagnost, Reutlingen Germany; E-30, intra-assay CV 1.9%, inter-assay CV 6.3%).

4.2.4. Statistical analysis

To examine the possible non-linear effects of maternal age on measured outcomes, subjects were separated according to maternal age at childbirth using two maternal age thresholds: 30 [8] and 35 years [9]. Subjects were divided into 3 groups: children born to mothers aged less than 30 years of age (<30), 30 to 35 years (30–35 years), and greater than or equal to 35 years (>35).

Demographics of the study cohort were presented as means ± standard deviation, and compared between groups using one-way ANOVA. Random effect mixed models were used to compare outcomes among maternal age groups, and accounted for important confounding factors including gender, birth weight SDS, gestational age, birth order, and paternal age. The maternal identification number was considered as a random factor to account for the clustering of siblings. Other factors were controlled for as required, depending on the outcome response of interest: for lipids, hormones, and outcomes associated with glucose homeostasis – age and BMISDS were included; for anthropometric data – the appropriate parental factor (i.e. MPBMISDS or MPHSDS). Outcome data were presented as estimated

marginal means with associated 95% confidence intervals. The differences between groups were estimated and tested using pairwise comparisons.

The interaction effect between group and gender was tested in all models. Outcomes were only assessed separately for boys and girls when there was an indication of a differential response to maternal age between genders. Response variables on glucose homeostasis were log-transformed to approximate normality. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc. Cary NC, USA). All statistical tests were two-sided and maintained at a 5% significance level.

4.3. Results

A total of, 343 children volunteered to participate, but 31 were excluded: 22 children were born small-for-gestational age and/or premature, five were pubertal, 3 were born to a mother with gestational diabetes/glucose intolerance, and one child was on medication known to influence growth (Figure 4.1). Of the remaining 312 controls, a further 35 had to be excluded to incomplete parental age data (Figure 4.1). Thus, our study cohort consisted of 277 children (126 girls and 151 boys) aged 3–10 years (7.4 ± 2.2). The offspring of 196 mothers were included in this study, as there were 71 sibling groups of 2 or 3 children. Maternal age at the time of child birth ranged from 19–44 years (33.3 ± 4.7 years; Figure 4.2), and this maternal age distribution is representative of New Zealand European families of higher socio-economic status [29].

Age, sex ratio, birth weight SDS, and gestational age were similar among groups (Table 4.1). There were no differences in maternal BMI, mean parental BMI, or duration and rate of breast feeding among groups. In addition, children in all maternal age groups had similar biological maturity as assessed by bone age X-rays (all $p>0.79$; Table 4.1).

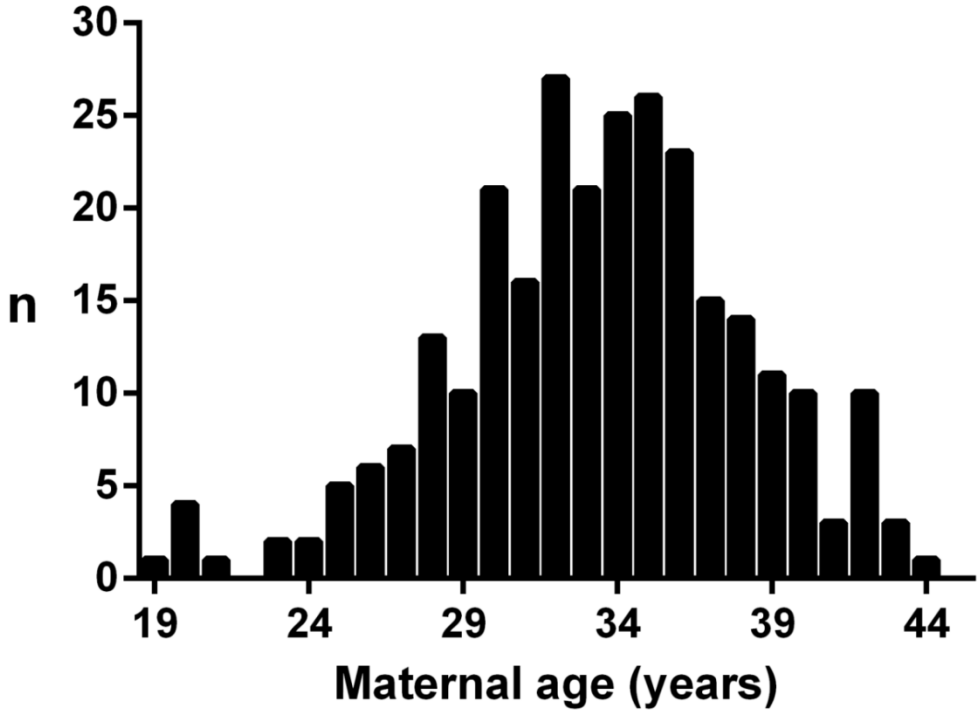

Figure 4.2. Distribution of maternal age at childbirth in the study cohort.

Table 4.1. Demographics of the study cohort according to their mother's age at childbirth.
Data are means ± standard deviation. ***p<0.001 for comparisons with <30 group; †††p<0.001 for comparison with >35 group.

	Maternal age at childbirth		
	<30 years	**30–35 years**	**>35 years**
n	62	103	112
Sex ratio (% boys)	55	58	50
Age (years)	7.6 ± 2.3	7.6 ± 2.0	7.0 ± 2.2
Bone age – chronological age (years)	-0.21 ± 0.97	-0.10 ± 0.80	-0.19 ± 0.71
Maternal age (years)	26.9 ± 2.8	32.7 ± 1.4***†††	38.0 ± 2.4***
Gestational age (weeks)	39.7 ± 1.2	39.8 ± 1.2	39.5 ± 1.2
Birth weight (kg)	3.54 ± 0.51	3.58 ± 0.53	3.50 ± 0.44

4.3.1. Anthropometry

When corrected for their genetic potential, children of mothers aged 30–35 and >35 years were 0.26 (p=0.002) and 0.23 (p=0.042) SDS taller than children of mothers aged <30 (Figure 4.3; Table S1). There was a reduction in childhood BMI SDS with increasing maternal age at childbirth, so that children born to mothers aged >35 years were 0.61 SDS slimmer than those of mothers <30 years (p=0.049) (Figure 4.3; Table S1). Increasing maternal age at childbirth was also associated with improved fat distribution in the offspring, so that children in both 30–35 (p=0.022) and >35 groups (p=0.036) had abdominal adiposity which was lower by 10% and 13%, respectively, compared to children of mothers aged <30 years at childbirth (Figure 4.3; Table S1).

A leaner phenotype was more apparent among boys born to mothers aged 30–35 and >35 years. In this group, increasing maternal age at childbirth was associated with a decrease in total body fat percentage, which was 12.9% in boys born to mothers aged >35 years compared to 14.7% (p=0.053) and 16.6% (p=0.015) in the boys of mothers aged 30–35 and <30 years at childbirth, respectively. Among girls, total body fat percentage was not different among groups (19.8% for <30 years, 18.5% for 30–35 years, and 19.1% for >35 years; all p>0.42).

4.3.2. Metabolic and hormonal profiles

Children of mothers aged 30–35 years at childbirth displayed a 19% increase in IGF-I concentrations compared to the offspring of mothers aged <30 years (p=0.042; Table 4.2; Table S1). Conversely, IGF-II concentrations were lower among the children of mothers aged 30–35 (6.5%; p=0.004) and >35 (8.1%; p=0.005) compared to those of the mothers aged <30 years at childbirth (Table 4.2 & Table 4.4). There were no observed effects of maternal age at childbirth on their children's lipid profile (Table 4.2 & Table 4.4).

Maternal age at childbirth did not affect parameters of glucose homeostasis among boys. However, girls of mothers aged 30–35 years at childbirth displayed improved insulin sensitivity (p=0.010) as expressed by lower HOMA-IR values (Table 4.3 & Table 4.5).

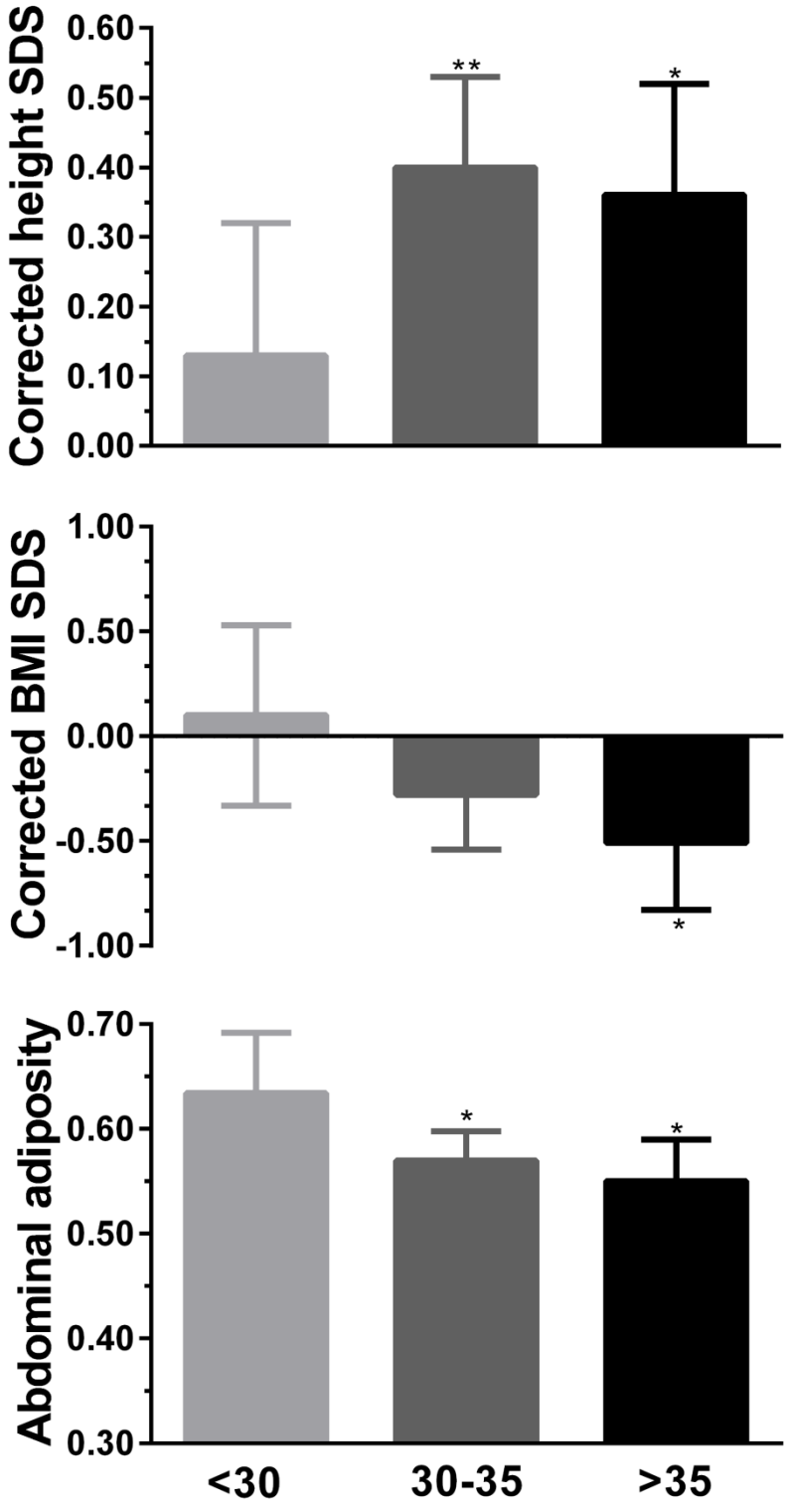

Figure 4.3. Corrected height, corrected BMI, and abdominal adiposity in the offspring of mothers of different ages at childbirth.

Height and BMI standard deviation scores are corrected for mean parental height and mid-parental BMI SDS, respectively. Abdominal adiposity is represented by the android fat to gynoid fat ratio. Data are means and 95% confidence intervals, adjusted for other confounding factors in multivariate models, including paternal age. *p<0.05 and **p<0.01 vs children of mothers aged <30 years at childbirth.

Table 4.2. Lipid and IGF profiles in childhood according to maternal age at childbirth.
Data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models (including paternal age). *p<0.05 and **p<0.01 vs children of mothers aged <30 years at childbirth.

	Maternal age at childbirth		
	<30 years	**30–35 years**	**>35 years**
n	62	103	112
Lipid profile			
Total cholesterol (mmol/l)	4.35 (4.12–4.51)	4.33 (4.20–4.47)	4.32 (4.15–4.51)
LDL-C (mmol/l)	2.52 (2.31–2.74)	2.57 (2.45–2.69)	2.51 (2.35–2.67)
HDL-C (mmol/l)	1.41 (1.31–1.52)	1.34 (1.28–1.40)	1.37 (1.29–1.45)
LDL-C : HDL-C	1.77 (1.58–2.00)	1.89 (1.77–2.02)	1.83 (1.68– 2.00)
Hormones			
IGF-I (µg/l)	97 (81–113)	115 (106–125)*	111 (99–123)
IGF-II (µg/l)	790 (759–823)	739 (721–757)**	726 (702–749)**
IGFBP-3 (ng/ml)	2803 (2501–3104)	2682 (2508–2855)	2596 (2373–2818)

Table 4.3. Parameters of glucose homeostasis among boys and girls according to maternal age at childbirth.
Data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models (including paternal age). *p<0.05 and **p<0.01 for comparisons with <30 group.

	Maternal age at childbirth		
	<30 years	**30–35 years**	**>35 years**
Boys (n)	34	65	52
Insulin sensitivity (HOMA-IR)	0.99 (0.79–1.24)	0.98 (0.87–1.11)	0.97 (0.82–1.14)
Fasting glucose (mmol/l)	4.70 (4.54–4.86)	4.80 (4.70–4.90)	4.85 (4.71–4.98)
Fasting insulin (mU/l)	4.79 (3.90–5.88)	4.52 (4.04–5.07)	4.47 (3.84–5.20)
Girls (n)	28	47	51
Insulin sensitivity (HOMA-IR)	1.49 (1.14–1.95)	1.00 (0.86–1.17)**	1.14 (0.95–1.37)
Fasting glucose (mmol/l)	4.93 (4.74–5.14)	4.70 (4.59–4.81)*	4.75 (4.62–4.89)
Fasting insulin (mU/l)	6.75 (5.30–8.61)	4.85 (4.22–5.57)*	5.42 (4.86–6.41)

Table 4.4. Height, body composition, lipid profile, and hormonal profiles in childhood according to maternal age at childbirth.

Data are 95% confidence intervals for the differences between estimated marginal means, adjusted for other confounding factors in the multivariate models (including paternal age). Respective p-values are provided in brackets.

	Maternal age at childbirth		
	30–35 vs <30	**30–35 vs >35**	**<30 vs >35**
Corrected height SDS	0.10 – 0.42	-0.10 – 0.17	-0.45 – -0.01
	(0.002)	(0.64)	**(0.042)**
Corrected BMI SDS	-0.84 – 0.08	-0.17 – 0.62	0.01 – 1.21
	(0.10)	(0.25)	**(0.049)**
Abdominal adiposity	-0.090 – -0.010	-0.165 – 0.163	0.005 – 0.086
	(0.022)	(0.74)	**(0.036)**
Lipid profile			
Total cholesterol (mmol/l)	-0.26 – 0.27	-0.22 – 0.23	-0.34 – 0.35
	(0.99)	(0.97)	(0.99)
LDL-C (mmol/l)	-0.19 – 0.28	-0.14 – 0.26	-0.29 – 0.32
	(0.69)	(0.55)	(0.93)
HDL-C (mmol/l)	-0.19 – 0.04	-0.12 – 0.07	-0.10 – 0.20
	(0.21)	(0.65)	(0.51)
LDL-C : HDL-C	-0.09 – 0.43	-0.16 – 0.28	-0.45 – 0.22
	(0.20)	(0.60)	(0.51)
Hormones			
IGF-I (µg/l)	0.7 – 36.2	-11.1 – 19.2	-37.4 – 8.3
	(0.042)	(0.61)	(0.21)
IGF-II (µg/l)	-86 – -16	-16 – 43	20 – 109
	(0.004)	(0.38)	**(0.005)**
IGFBP-3 (ng/ml)	-437 – 195	-177 – 348	-212 – 625
	(0.45)	(0.52)	(0.33)

Table 4.5. Parameters of glucose homeostasis among boys and girls according to maternal age at childbirth.
Data are 95% confidence intervals for the ratios between estimated marginal means, adjusted for other confounding factors in the multivariate models (including paternal age). Respective p-values are provided in brackets.

	Maternal age at childbirth		
	30–35 vs <30	**30–35 vs >35**	**<30 vs >35**
Boys			
Insulin sensitivity (HOMA-IR)	0.78 – 1.27	0.82 – 1.26	0.75 – 1.39
	(0.96)	(0.90)	(0.90)
Fasting glucose (mmol/l)	0.98 – 1.06	0.96 – 1.03	0.92 – 1.02
	(0.29)	(0.57)	(0.22)
Fasting insulin (mU/l)	0.74 – 1.19	0.83 – 1.23	0.80 – 1.42
	(0.62)	(0.91)	(0.64)
Girls			
Insulin sensitivity (HOMA-IR)	0.50 – 0.91	0.69 – 1.12	0.90 – 1.89
	(0.009)	(0.28)	(0.16)
Fasting glucose (mmol/l)	0.91 – 0.98	0.95 – 1.03	0.98 – 1.10
	(0.036)	(0.54)	(0.20)
Fasting insulin (mU/l)	0.55 – 0.95	0.72 – 1.12	0.89 – 1.75
	(0.019)	(0.32)	(0.20)

4.4. Discussion

Our study shows that increasing maternal age at childbirth is associated with a more favourable phenotype in their children. This includes an increase in height, a reduction in abdominal fat, as well as improved insulin sensitivity in girls.

On average, children of mothers aged 30–35 and >35 years at childbirth were 1.5 cm taller than the offspring of mothers aged less than 30 years. This difference in stature was accompanied by higher serum IGF-I concentrations in the children born to older mothers who were taller than children born to mothers aged <30 years. This corroborates our findings, as IGF-I is an important mediator of childhood growth [30]. Importantly, our finding of taller stature with increasing maternal age was present after correction for genetic height, the most important determinant of childhood height [26]. Further, we also corrected for other factors known to influence childhood height, including gestational age, birth weight [31], and birth order [32], as well as accounting for socio-economic status through our cohort selection process [33]. As children in all maternal age groups had similar biological maturity as assessed by bone age X-rays, it is likely that the observed height differences will persist into adulthood [26].

Increasing maternal age was also associated with a decreased BMI and a reduction in abdominal fat in their children. Increased abdominal fat is a component of the metabolic syndrome in childhood and adulthood [34]. Thus, we suggest that children born to mothers over 30 years of age may be at a lower risk of metabolic disease and obesity compared to those of younger mothers. The observed improvement in insulin sensitivity among girls born to older mothers would support this assertion, as a reduction in insulin sensitivity is predictive of the metabolic syndrome in adulthood [35,36].

Children born to younger mothers also had higher IGF-II concentrations that may be associated with their increased adiposity, as elevations in serum IGF-II concentrations are associated with increased body fat [37].[1] Causal factors for childhood obesity have been extensively investigated [38], but previous studies have found no impact of maternal age on childhood BMI [39,40,41]. However, in those studies, maternal age was just one of many secondary study outcomes in populations from all socio-economic groups [38]. Since both maternal age at childbirth and obesity risk are strongly associated with socio-economic status

[1] Interestingly, the *IGF2* gene is expressed solely from the paternal allele [61].

[2,42], previous studies might have been unable to accurately detect subtle effects of maternal age on childhood BMI or body composition.

There are a very limited number of studies examining the physical and metabolic outcomes in the offspring associated with maternal age at childbirth [3,4,5]. These studies described subtle increases in blood pressure [4] and type 1 diabetes risk [3] in childhood, and an increase in type 2 diabetes risk in adults [5]. These findings contrast to our observations of a more favourable offspring phenotype in childhood associated with increasing maternal age at childbirth. However, previous studies included either crude adjustments in their analyses for socio-economic status [4] or none at all [3,5], which is important as lower socio-economic status is known to be associated with increased blood pressure and type 2 diabetes risk [43]. Most importantly, all three studies included subjects who were born small-for-gestational-age, prematurely, and/or of low birth weight [3,4,5], groups at a greater risk of developing hypertension and type 2 diabetes [44,45]. Although Cardwell et al. and Lawlor et al. adjusted for birth weight and gestational age in their analyses [3,4], Lammi et al. did not [5]. Lower birth weight is associated with higher blood pressure later in childhood and adolescence [46,47], and birth weight decreases with increasing maternal age at childbirth [6]. Thus, birth weight rather than maternal age may account for Lowler et al.'s observations of higher offspring blood pressure in childhood with increasing maternal age at childbirth, as postulated by the authors themselves [4]. However, Cardwell et al. did speculate on a number of possible explanations for the increased risk of type 1 diabetes in children born to older mothers, including immunological changes in the mother and subtle genetic changes in the offspring [3]. However, the authors concluded that the mechanisms responsible remain unclear [3].

Similarly, there is no clear single explanation for the observed changes in childhood growth, body composition, and metabolism with increasing maternal age at childbirth in our study. It is possible that pre-natal and/or post-natal environmental factors are responsible for our observations. Increasing maternal age is a well-known risk factor for chromosomal disorders in children [7]. However, it is also likely that more subtle gene alterations, such as epigenetic changes, take place. Epigenetic changes are alterations in gene expression not caused by changes in DNA sequence [48], and may be associated with alterations in phenotype [49]. Increasing age is associated with an increased frequency of epigenetic modifications in both somatic cells [50] and oocytes [51,52,53]. Thus, it is possible that epigenetic changes that

occur in maternal oocytes with increasing age are responsible for our findings on childhood growth, body composition, and metabolism.

Increasing maternal age is associated with several physiological changes, including subtle increases in maternal follicle-stimulating hormone (FSH) [54], testosterone and oestrogen [55,56] levels. Such hormonal changes have been associated with alterations in maternal oocyte DNA, as well as alterations in post-natal growth [57] and metabolism [58] in the offspring. Thus, it is possible that changes in maternal hormones with increasing age alter the in utero environment, leading to programmed changes in childhood phenotype.

It is recognised that variations in the post-natal child-rearing environment across the socio-economic spectrum affects childhood growth and body composition [33,59]. Higher socio-economic status is associated with a taller and slimmer phenotype in childhood; while children reared in lower socio-economic environments tend to be shorter and fatter [33,60]. Since our cohort was comprised of a homogenous group of children from higher socio-economic families, the child-rearing environment is less likely to explain our findings. The absence of a clear explanation for our findings and those of other studies [3,4,5] highlights the need for investigation of the possible mechanisms responsible for changes in phenotype and metabolism in the offspring associated with maternal age at childbirth.

Limitations to our study include a relatively small cohort of 277 healthy pre-pubertal children from approximately 200 mothers. In addition, we studied a homogenous group of children (same ethnicity and higher socio-economic status), which may limit application of our study findings to the general population, particularly to those of lower socio-economic status. However, this homogeneity also meant that we eliminated much of the phenotypic and metabolic variability associated with socio-economic status, thus enabling us to better address the likely effects of maternal age on measured outcomes.

4.5. Conclusion

Our study showed that increasing maternal age at childbirth is associated with taller stature and reduced abdominal fat in their offspring in mid-childhood, as well as improved insulin sensitivity in girls. The triggers and mechanisms responsible for these differences are unclear, but may include a combination of maternal age-related changes in the prenatal and post-natal environment. Our study suggests that the worldwide trend towards increasing maternal age is unlikely to underpin the increase in obesity rates in childhood.

4.6. References

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Chapter 5. Increasing paternal age at childbirth is associated with taller stature & less favourable lipid profiles in their children

Preface

This chapter contains an unaltered reproduction of an article revised and re-submitted to the journal Clinical Endocrinology.

- *Authors:* Savage T, Derraik JGB, Miles HL, Mouat F, Hofman PL, Cutfield WS
- *Title:* Increasing paternal age at childbirth is associated with taller stature and less favourable lipid profiles in their children
- *Journal:* Clinical Endocrinology
- *Impact factor:* 3.17
- *Journal's aims and scope:* "Clinical Endocrinology publishes papers and reviews which focus on the clinical aspects of endocrinology, including the clinical application of molecular endocrinology. It features reviews, original papers, commentaries, cases of the month, book reviews and letters to the editor. Clinical Endocrinology is essential reading not only for those engaged in endocrinological research but also for those involved primarily in clinical practice."

5.1. Abstract

Background: Paternal age at childbirth has been increasing worldwide, and we assessed whether this increase affects growth, body composition, and metabolism in their children.

Methods: We studied 277 children (aged 3–10 years) born to fathers aged 19.8–51.8 years. Assessments included: height and weight adjusted for parental measurements, DXA-derived body composition, fasting lipids, glucose homeostasis, and hormonal profiles.

Results: Children born to fathers aged 31–35 (p=0.009) and >35 years (p=0.021) were 2 cm taller than those of fathers aged ≤30 years. Children of fathers aged >35 years at childbirth had a lower BMI (-0.32 SDS) than offspring of fathers aged 31–35 (-0.01 SDS; p=0.043) and ≤30 (0.22 SDS; p=0.019). There were marked effects of paternal age at childbirth on childhood blood lipids. LDL-C concentrations in children born to fathers aged >35 years were 11% and 21% higher than in children of fathers aged 31–35 and ≤30 years, respectively (p<0.01). Total cholesterol to HDL-C ratio was also higher among the children of fathers aged 31–35 (12%; p=0.014) and >35 (16%; p=0.004) years at childbirth compared to the ≤30 group. In addition, HOMA-IR in girls (but not boys) born of fathers aged 31–35 (0.99) and >35 years (1.11) indicated better insulin sensitivity compared to offspring in the ≤30 group (1.63; p<0.05).

Conclusions: Increasing paternal age at childbirth is associated with a more favourable phenotype in their children (taller and slimmer, with better insulin sensitivity in girls), but with a less favourable lipid profile.

5.2. Introduction

There has been a major shift in reproductive behaviour over the past several decades with an increasing number of couples having children in their thirties and forties.[1] While the increase in maternal age at childbirth has received much attention, an upwards shift in paternal age at childbirth is also taking place. In most developed countries, the age at first fatherhood has increased from an average age of 29 to 32 years between 1980 and 2000.[2] As a result, in 2003, more than 40% of children in the UK were born to fathers aged 35 years or older, and this trend towards older age at fatherhood shows no signs of abating.[2] Many reasons may account for the trend towards postponement of fatherhood, including economic pressures and personal choice (see review by Roberts et al[3]).

Some studies suggest that male fertility and sperm DNA quality start to decline after approximately 35 years of age.[4] Such a decline leads to an increased risk of spontaneous gene mutations, DNA damage, and possibly epigenetic changes in sperm genes. However, unlike maternal age, there appears to be no clear threshold beyond which male gamete quality starts to decline, or when the risk of adverse childhood outcomes definitively increases with increasing paternal age at childbirth. The available evidence suggests that 20–30 years may represent the lowest paternal age related-risk of adverse health outcomes in the offspring,[5] with this risk becoming greater thereafter. There is some evidence that a paternal age at childbirth of approximately 35–37 years could mark the start of a non-linear increase in the risk to the offspring.[5,6]

Increasing paternal age at childbirth is associated with a small, but appreciable risk of genetic and birth defects, as well as a slight increase in offspring risk of autism, adult psychiatric disorders, and some childhood cancers.[2,7] A recent study found that increasing paternal age is associated with a higher rate of obesity amongst offspring in adulthood.[8] Increasing maternal age is associated with alterations in offspring height and body composition[9] and type 1 diabetes risk.[10]. However, the possible effects of paternal age on childhood growth and metabolism have not previously been examined. Therefore, we aimed to assess whether increasing paternal age at childbirth would be associated with changes in the height, body composition, metabolism, and hormonal profiles in the offspring in childhood.

5.3. Methods

5.3.1. Study cohort

We undertook a large project examining the effects of parental and prenatal factors in the offspring. From this larger project, we have examined the impact of conception with ovarian stimulation drugs on the growth and metabolism of children.[11] Children conceived after ovarian stimulation were asked to invite 4–5 family friends and school friends who were naturally conceived to participate in the study as controls,[11] so that these controls were recruited by study participants, and were of similar age group, ethnicity, and socio-economic status. Thus, in this current study we assessed the entire naturally conceived cohort that was recruited from this larger project (between October 2010 and October 2012).

Only healthy, developmentally normal, pre-pubertal children aged 3–10 years, born 37–41 weeks gestation were studied. All children were of New Zealand European ethnicity,

naturally conceived, born of singleton pregnancies, and of birth weight appropriate-for-gestational-age (birth weight >-2 and <2 standard deviation scores (SDS)). Exclusion criteria also included signs of puberty (Tanner stage 2 breast development in girls and testicular volume >3 ml in boys or evidence of adrenarche), receiving medication that could affect insulin sensitivity or growth, as well as having a first degree relative with pre-diagnosed diabetes. Children were excluded if born to mothers with gestational diabetes, chronic illnesses, or prolonged maternal drug use (including tobacco). All participants were of higher socio-economic status according to their residential address and the "decile score" of the school they attended. A decile score reflects the socio-economic status of the school communities, and it is a comprehensive assessment of community affluence. It takes into account a number of factors such as household income, parental occupation, parents' educational qualifications, number of occupants per dwelling size, and government welfare benefits. All participants in the study were of decile 9 or 10 (highest) socio-economic status.

5.3.2. Clinical assessments

All clinical assessments were carried out by a single researcher at the Maurice & Agnes Paykel Clinical Research Unit (Liggins Institute, University of Auckland). Standing height was measured using a Harpenden stadiometer. Children's weight and body composition were assessed using dual-energy X-ray absorptiometry (DXA Lunar Prodigy 2000; General Electric, Madison, WI, USA). Apart from total body fat percentage, the DXA-derived parameters of interest were percentage truncal fat, android fat to gynoid fat ratio (an indicator of abdominal adiposity), and bone mineral density (L1–L4). Each child also had a bone age X-ray to assess biological maturity, which was blindly assessed by a single paediatric endocrinologist using pre-established standards.[12]

Maternal and paternal height, weight, and body mass index (BMI) were measured and recorded. Maternal obstetric history was also recorded to clarify parity, and relevant medical history. Children's birth weight, height, and BMI were transformed into SDS.[13,14] Mid-parental height SDS (MPHSDS) was calculated for each child.[15] Children's heights SDS (HtSDS) were then individually corrected for their genetic potential (parents' heights), using the formula: "HtSDS–MPHSDS". Parents' BMI were transformed into SDS, and the mean parental BMISDS (MPBMISDS) was calculated for each child.

Following an overnight fast, blood samples were drawn from each child for assessment of total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein choleterol (LDL-C), triglycerides, insulin-like growth factor I (IGF-I), IGF-II, IGF binding

protein 3 (IGFBP-3). Children also had glucose and insulin levels measured, and insulin sensitivity evaluated using the homeostasis model assessment of insulin resistance (HOMA-IR).[16]

Plasma insulin was measured using an Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL, USA) by microparticle enzyme immunoassay (Abbott Diagnostics, Wiesbaden, Germany) with an inter-assay coefficient of variation (CV) of <5%. Glucose, triglyceride, total cholesterol, HDL-C, and LDL-C concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by enzymatic colorimetric assay (Roche, Mannheim, Germany) with an inter-assay CV of 1.2% for glucose, and <5% for total cholesterol, triglycerides, HDL-C, and LDL-C. Commercially available ELISAs (R&D Systems, Minneapolis, MN, USA) were used to measure plasma IGF-I (DSL-100, intra-assay CV 2.8%, inter-assay CV 9.2%), IGFBP-3 (DSL-10-6600, intra-assay CV 3.1%, inter-assay CV 9.9%), and IGF-II (Meddiagnost, Reutlingen Germany; E-30, intra-assay CV 1.9%, inter-assay CV 6.3%).

5.3.3. Ethics approval

Ethics approval for this study was provided by the Northern Y Regional Ethics Committee (Ministry of Health, New Zealand). Written informed consent was obtained from parents or guardians, as well as verbal or written consent from each child as was appropriate to their age.

5.3.4. Statistical analysis

Children in our study were divided into paternal age (at childbirth) subgroups approximating potentially relevant paternal age thresholds, while also reflecting age ranges known to be important for maternal age effects. As a result, to examine the possible non-linear effects of paternal age at childbirth on measured outcomes, subjects were stratified into three groups: children born to fathers aged less than or equal to 30 years of age (≤30), 31 to 35 years (30–35 years), and greater than 35 years (>35).

Mean ages among groups were compared using one-way ANOVA in Minitab v.16 (Pennsylvania State University, State College, PA, USA). Other comparisons between paternal age groups were carried out using linear mixed models in SAS v.9.3 (SAS Institute, Cary, NC, USA), which included paternal identification number as a random factor to account for the clustering of siblings. All models accounted for important confounding

factors, namely gender, birth weight SDS, gestational age, and birth order, and maternal age. Other factors were controlled for as required, depending on the outcome response of interest: for lipids, hormones, and outcomes associated with glucose homeostasis – children's age and BMISDS were included; for anthropometric data – the appropriate parental factor (i.e. MPBMISDS or MPHSDS). Following a global test, pairwise comparisons were carried out to identify specific differences between groups. Subgroup analyses were also run including only data on the recruited siblings.

The interaction effects between paternal age groups and gender were tested in all models, and outcomes only assessed separately for boys and girls if there was indication of a differential response between genders. Data on parameters associated with glucose homeostasis were log-transformed to approximate a normal distribution. Age data are provided as means ± standard deviation; other data are means and 95% confidence intervals adjusted for confounders in the multivariate models, back-transformed where appropriate.

5.4. Results

A total of 343 children volunteered to participate, but 31 were excluded: 22 children were born small-for-gestational age and/or premature, five were pubertal, three were born of a mother with gestational diabetes/glucose intolerance, and one child was on medication known to influence growth (Figure 5.1). Of the remaining 312 controls, a further 35 had to be excluded due to lack of paternal age data (Figure 5.1). Thus, our study cohort consisted of 277 children (126 girls and 151 boys) aged 7.4 ± 2.2 years (range 3–10 years). The offspring of 196 fathers were included in this study, as there were 71 sibling groups of 2 or 3 children (n=153). Paternal age at childbirth was 35.4 ± 5.4 years (range 19.8–51.8) (Figure 5.2).

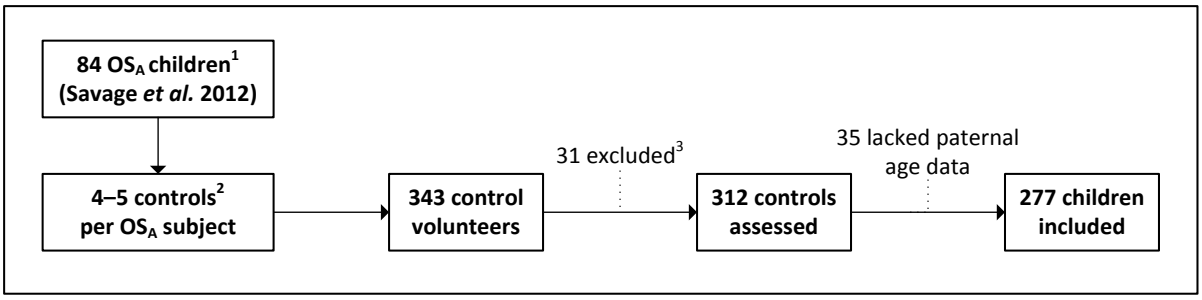

Figure 5.1. Summary of the study's recruitment process.
[1] OSA children had been conceived via ovarian stimulation, and were examined in Savage et al. {Savage, 2012 #1276}; [2] Controls were friends of OSA children to ensure similar age group, ethnicity, and socio-economic status; [3] 22 children were born small-for-gestational age and/or premature; 5 were pubertal; 3 were born of a mother with gestational diabetes/glucose intolerance; and 1 child was on medication known to influence growth.

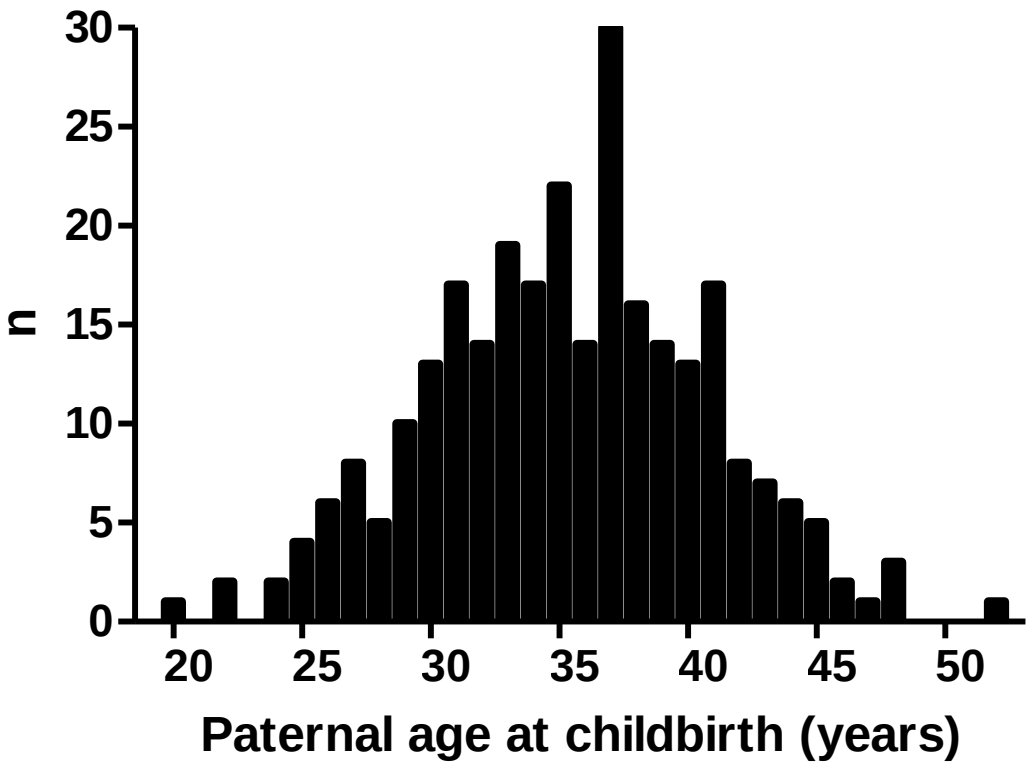

Figure 5.2. Distribution of paternal age at childbirth in the study cohort.

Age, sex ratio, birth weight, and gestational age were similar among paternal age subgroups (Table 5.1). There were also no differences between subgroups in parental anthropometric characteristics, including height SDS and BMISDS (data not shown). Importantly, children across paternal age subgroups had similar duration and rates of breast feeding (data not shown), and there were no differences in biological maturity between subgroups based on bone age X-rays (Table 5.1).

Table 5.1. Birth outcomes and biological maturity in our study cohort according to paternal age at childbirth.
Age data are means ± SD; other data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models, including either maternal age. Biological maturity is represented by the bone age minus the chronological age.

	Paternal age at childbirth		
	≤30 years	**31–35 years**	**>35 years**
n	59	90	128
Sex ratio (males)	59%	58%	50%
Age (years)	7.55 ± 2.22	7.59 ± 2.22	7.12 ± 2.15
Gestational age (weeks)	39.8 ± 1.3	39.8 ± 1.1	39.5 ± 1.2
Birth weight (kg)	3.58 (3.43–3.73)	3.56 (3.47–3.65)	3.47 (3.39–3.56)
Biological maturity	0.01 (-0.36–0.39)	-0.21 (-0.42–0.01)	-0.17 (-0.40–0.07)

5.4.1. Anthropometry

When heights were corrected for genetic potential (HtSDS–MPHSDS), children of fathers aged 31–35 (0.35 SDS; p=0.009) and >35 (0.39 SDS; p=0.019) years at childbirth were taller than those of fathers aged ≤30 years (0.12 SDS) (Figure 5.3). There was also some evidence of reduced adiposity in the offspring with increasing paternal age at childbirth (Figure 5.3). BMISDS among children born of fathers aged >35 years (-0.32 SDS) was lower than in the offspring of fathers aged 31–35 (-0.01 SDS; p=0.043) and ≤30 (0.22 SDS; p=0.019) years (Figure 5.3). Truncal fat (central adiposity) was lower in children of fathers aged >35 years at childbirth than in the offspring of fathers aged ≤30 years (12.2 vs 15.0 %; p=0.029) (Figure 5.3). There were no differences between groups in total body fat, android to gynoid fat ratio, or bone mineral density (data not shown).

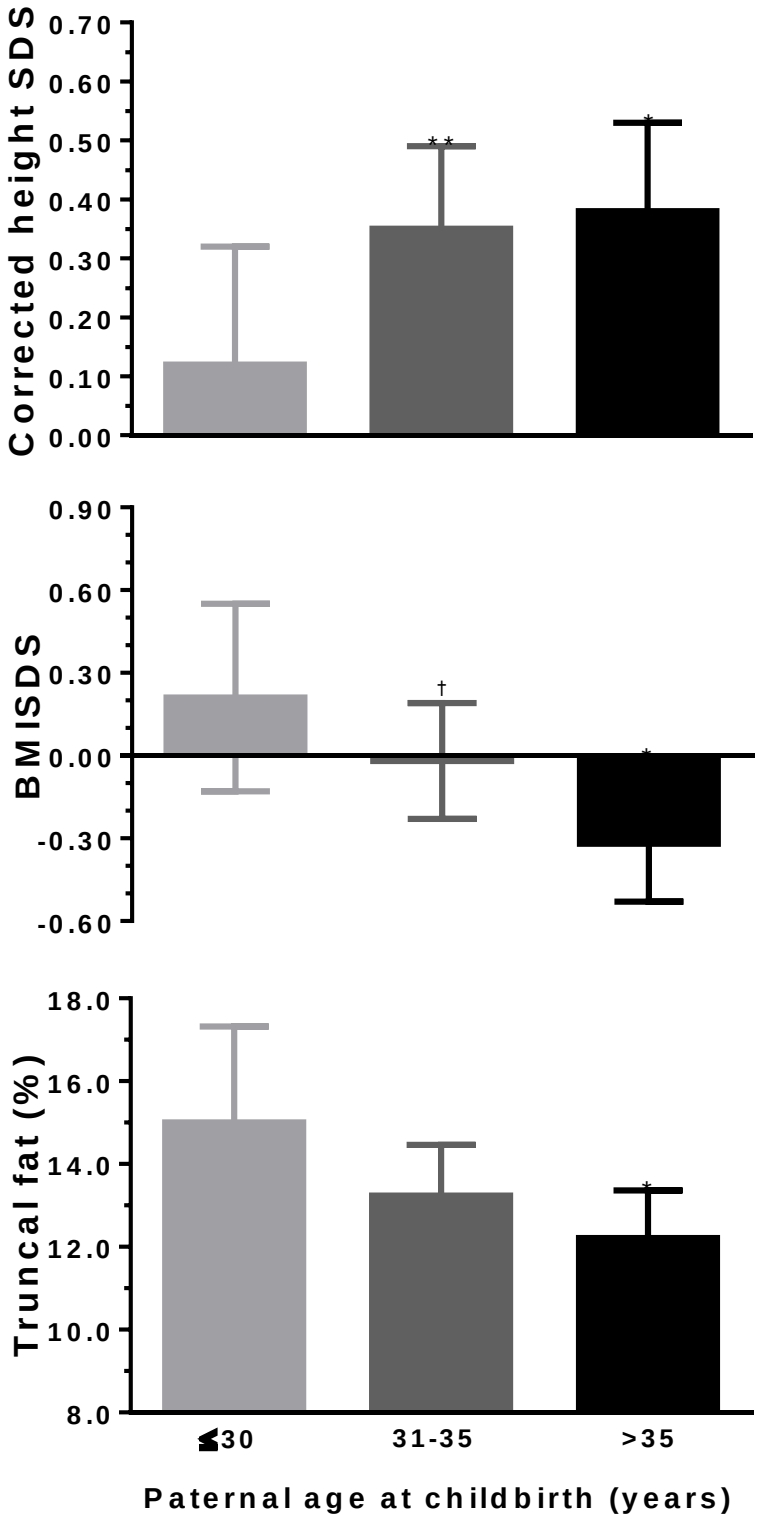

Figure 5.3. Height standard deviation score (SDS) corrected for mean parental height SDS and measures of adiposity among the children of fathers of different ages at childbirth.

Data are means and 95% confidence intervals, adjusted for other confounding factors in multivariate models, including maternal age. *p<0.05 and **p<0.01 vs children of fathers aged ≤30 years at childbirth; †p<0.05 vs children of fathers aged >35 years. BMI SDS, body mass index SDS.

5.4.2. Lipid profiles

Increasing paternal age at childbirth was strongly associated with less favourable childhood plasma lipid profiles in the offspring. Plasma total cholesterol concentrations among children of fathers aged >35 years at childbirth (4.52 mmol/l) were higher than in both the 31–35 (4.18 mmol/l; p=0.002) and ≤30 (4.20 mmol/l; p=0.046) groups. Similarly, LDL-C concentrations were higher among the children born to fathers aged >35 years at childbirth (2.65 mmol/l) compared to children of fathers aged 31–35 (2.38 mmol/l; p=0.005) and ≤30 (2.22 mmol/l; p=0.003) years (Figure 5.4).

HDL-C concentrations in the children of fathers aged 31–35 years at childbirth were lower than in the ≤30 group (1.31 vs 1.46 mmol/l; p=0.012), but similar to the >35 group (1.37 mmol/l; p=0.19). However, the total cholesterol to HDL-C ratio was increased among the children of fathers aged 31–35 (3.37; p=0.014) and >35 (3.47; p=0.004) years at childbirth compared to the ≤30 group (3.00), due to higher LDL-C concentrations (Figure 5.4). An identical pattern was also observed for the LDL-C to HDL-C ratio.

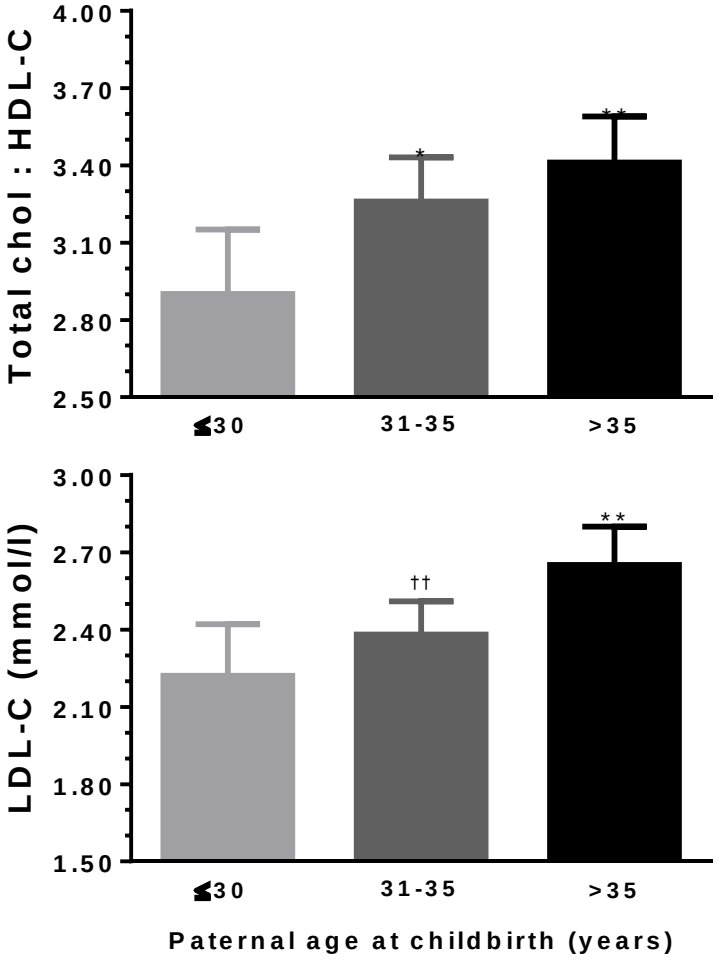

Figure 5.4. Lipid profiles among the children of fathers of different ages at childbirth.
Data are means and 95% confidence intervals, adjusted for other confounding factors in multivariate models, including maternal age. *p<0.05 and **p<0.01 vs children of fathers aged ≤30 years at childbirth; ††p<0.01 vs children of fathers aged >35 years.HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Total chol, total cholesterol.

5.4.3. Growth factors and glucose homeostasis

There were no significant differences in IGF-I and IGFBP-3 concentrations among groups, but paternal age had a sex-dependant effect on IGF-II (Table 5.2). Among girls, IGF-II concentrations were lower in the daughters of fathers aged 31–35 (p=0.002) and >35 (p=0.009) years at childbirth compared to the ≤30 group (Table 5.2). In contrast, the exact opposite pattern was observed among boys (p<0.05; Table 5.2).

There was also a sex-dependent effect on glucose homeostasis, with paternal age at childbirth affecting girls but not boys (Table 5.2). Fasting insulin concentrations were lower in girls born to fathers aged 31–35 (p=0.005) and >35 (p=0.042) years at childbirth compared to the girls born to younger fathers (Table 5.2). Thus, girls in the 31–35 and >35 groups were more insulin sensitive (i.e. had lower HOMA-IR) than girls born to fathers aged ≤30 years at childbirth (p=0.003 and p=0.040, respectively; Table 5.2).

Table 5.2. Growth factors and parameters of glucose homeostasis among boys and girls according to paternal age at childbirth.

Data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models (including paternal age). *p<0.05 and **p<0.01 vs children of fathers aged ≤30 years at childbirth. Data for outcomes with a differential response between boys and girls are provided separately for each sex.

	Paternal age at childbirth		
	≤30 years	**31–35 years**	**>35 years**
Study cohort (n)	62	103	112
IGF-I (µg/l)	119 (103–136)	107 (97–118)	108 (97–118)
IGFBP-3 (ng/ml)	2481 (2179–2782)	2615 (2429–2802)	2816 (2616–3016)
Boys (n)	35	52	64
IGF-II (µg/l)	693 (652–734)	741 (715–767)*	753 (723–783)*
Insulin sensitivity (HOMA-IR)	0.95 (0.0.77–1.18)	0.99 (0.87–1.13)	0.98 (0.85–1.14)
Fasting glucose (mmol/l)	4.75 (4.59–4.92)	4.83 (4.73–4.94)	4.78 (4.66–4.89)
Fasting insulin (mU/l)	4.51 (3.70–5.50)	4.49 (3.96–5.08)	4.63 (4.06–5.29)
Girls (n)	24	38	64
IGF-II (µg/l)	823 (775–868)	736 (708–765)**	741 (715–767)**
Insulin sensitivity (HOMA-IR)	1.63 (1.21–2.20)	0.99 (0.83–1.19)**	1.11 (0.95–1.30)*
Fasting glucose (mmol/l)	4.83 (4.62–4.06)	4.71 (4.58–4.84)	4.78 (4.66–4.90)
Fasting insulin (mU/l)	7.38 (5.63–9.69)	4.84 (4.11–5.69)**	5.22 (4.51–6.04)*

5.4.4. Siblings

Analyses of specific outcomes among sibling groups corroborated most findings on the larger cohort, although weaker p-values were obtained in view of the reduction in *n* (from 277 to 153). There was a gradual increase in HtSDS–MPHSDS with increasing paternal age at childbirth, and non-significant data suggesting a steady reduction in adiposity (Figure 5.5). Regarding lipid profiles, children born to older fathers had higher LDL-C concentrations as well as higher total cholesterol to HDL-C ratio (Figure 5.5).

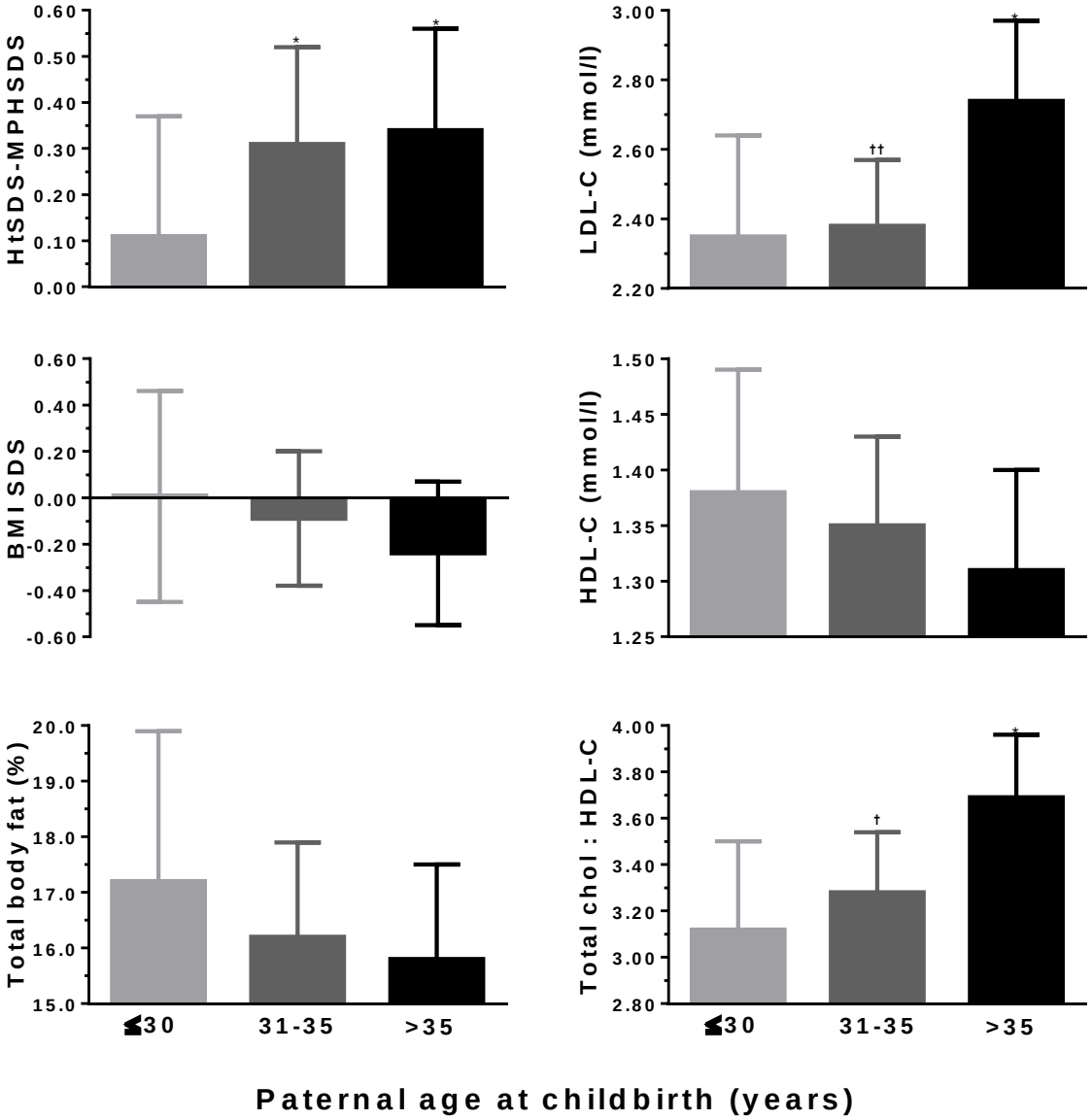

Figure 5.5. Anthropometry and lipid profile among siblings born of fathers of different ages (n=153).
Data are means and 95% confidence intervals, adjusted for other confounding factors in multivariate models, including maternal age. *p<0.05 vs children of fathers aged ≤30 years at childbirth; †p<0.05 and ††p<0.01 vs children of fathers aged >35 years.

5.4.5. Maternal age

There was a high correlation between paternal and maternal ages ($r=0.75$; $p<0.001$). As a result, similar stratified analyses were carried out, examining maternal age groups (stratified as per the paternal age thresholds) with paternal age included as a continuous variable. These data are provided in Table 5.3. The observed patterns of progressive improvement in adiposity and worsening lipid profiles were not observed for maternal age (Table 5.3). However, there was a significant increase in HtSDS–MPHSDS from the youngest group to those born to mothers aged 31–35 years ($p=0.012$; Table 5.3), with paternal age (as a continuous variable) remaining associated with HtSDS–MPHSDS ($p=0.033$).

5.5. Discussion

Our study shows that increasing paternal age at childbirth is associated with taller stature and reduced adiposity, but a less favourable lipid profile in their children. These changes were associated with a relatively young paternal age at childbirth (35.4 years), when one considers that in the USA for example, birth rates among fathers aged 35–39 and 40–44 increased by approximately 50% over the last three decades.[17] The influence of paternal age at childbirth on height or metabolism in their children has not been described previously.

Children born to fathers over 30 years of age were approximately 2 cm taller than those of fathers aged ≤30 years. Notably, this height difference was present after correction for genetic height, the most important determinant of childhood height.[15] In addition, children in the paternal age subgroups had similar biological maturity according to bone age X-rays. Thus, the taller stature among children of fathers aged 30 years or more was unlikely to be associated with earlier pubertal development, and the observed height differences are likely to persist into adulthood.[15]

Table 5.3. Study outcomes among the children of mothers of different ages at childbirth.

Data are means and 95% confidence intervals, adjusted for other confounding factors in multivariate models, including paternal age. Data are means and 95% confidence intervals adjusted for other confounding factors in the multivariate models. *p<0.05 vs children of fathers aged ≤30 years at childbirth; †p<0.05 and ††p<0.01 vs children of fathers aged >35 years.

	≤30 years	31–35 years	>35 years
n	77	126	74
Anthropometry			
HtSDS–MPHSDS	0.18 (0.00–0.35)	0.37 (0.24–0.51)*	0.39 (0.21–0.56)
Height SDS	0.77 (0.49–1.04)	0.88 (0.71–1.05)	1.00 (0.73–1.27)
BMI SDS	-0.18 (-0.47–0.12)	-0.12 (-0.31–0.06)	-0.02 (-0.32–0.27)
Total body fat (%)	-0.14 (-0.42–0.14)	-0.03 (-0.21–0.15)	0.14 (-0.14–0.43)
Truncal fat (%)	-0.16 (-0.43–0.11)	-0.02 (-0.19–0.15)	0.17 (-0.11–0.44)
Glucose homeostasis			
HOMA-IR			
Boys	0.91 (0.75–1.10)	1.02 (0.91–1.14)	0.97 (0.78–1.21)
Girls	1.24 (0.97–1.59)	1.06 (0.91–1.23)	1.20 (0.97–1.59)
Fasting insulin (mU/l)			
Boys	4.41 (3.68–5.28)	4.62 (4.16–5.12)	4.54 (3.72–3.54)
Girls	5.76 (4.59–7.23)	5.07 (4.43–5.80)	5.70 (4.68–6.94)
Hormonal concentrations			
IGF-I (ng/ml)	101 (87–115)	114 (105–122)	113 (98–127)
IGF-II (ng/ml)			
Boys	761 (725–797)	732 (709–755)	705 (662–748)
Girls	781 (742–821)	729 (705–754)*	770 (736–805)
IGFBP-3 (ng/ml)	2691 (2428–2955)	2647 (2480–2815)	2705 (2434–2976)
Lipid profile			
Total cholesterol (mmol/l)	4.40 (4.19–4.63)	4.28 (4.15–4.42)	4.35 (4.14–4.58)
LDL-C (mmol/l)	2.52 (2.33–2.72)	2.46 (2.35–2.58)	2.40 (2.22–2.59)
HDL-C (mmol/l)	1.43 (1.34–1.52)	1.30 (1.25–1.36)*†	1.43 (1.33–1.52)
Total cholesterol : HDL-C	3.14 (2.93–3.37)	3.37 (3.23–3.52)	3.13 (2.91–3.36)

As paternal age at childbirth increased, their children displayed a reduction in BMI and truncal fat. Since BMI in childhood is predictive of adult BMI, our findings suggest that the slimmer children of older fathers may have a lower risk of obesity in adulthood.[18] Increased truncal fat is a component of the metabolic syndrome, so that the children born to fathers aged over 30 years may be at a lower risk of metabolic disease and obesity. Importantly, the observed improvement in insulin sensitivity seen among girls born of older fathers would support this hypothesis, as a reduction in insulin sensitivity is predictive of the metabolic syndrome in adulthood.[19] Nonetheless, there are conflicting reports regarding the effects of paternal age at childbirth on offspring obesity. In contrast to our study, a recent large investigation found an increased risk of obesity in young adult offspring in association with increasing paternal age. However, this was only observed when groups at the extreme of the paternal age spectrum were compared (<20 vs >50 years). [8] Furthermore, unlike our study, they only examined males and parental BMI was not accounted for in their analyses to correct for genetically-determined obesity.[8]

However, increasing paternal age at childbirth was also associated with less favourable lipid profiles in their children. Specifically, children of fathers over 30 years of age had higher total cholesterol to HDL-C ratios compared to the children of younger fathers. Childhood lipid profiles worsened as paternal age at childbirth increased further, so that the children of fathers aged over 35 years had higher total cholesterol (due to higher LDL-C concentrations) than children of fathers aged ≤35 years. Childhood lipid profiles track or accentuate into adulthood.[20] It is therefore possible that the less favourable lipid profiles in these children may deteriorate further later in life, placing them at a greater risk of cardiovascular disease in adulthood.

There is no clear explanation for our observed findings in children in association with increasing paternal age at childbirth. This is particularly so for the observation of less favourable lipid profiles but lower adiposity in offspring with increasing paternal age, which is contradictory to findings on lipid profiles and adiposity in children and adults.[21,22]. Thus, the triggers and mechanisms responsible for the influence of paternal age on offspring phenotype and metabolism require further investigation. Consequently, the factors leading to a taller and slimmer phenotype are potentially different to those associated with a less favourable lipid profile. Factors responsible for these differences may be pre-natal, such as epigenetic changes in paternal gametes or paternal environmental factors (diet and toxins), or due to differences in the post-natal child-rearing environment. Similarly, while higher IGF-II

concentrations among the girls born to youngest fathers are likely a result of their greater adiposity[23], the opposite pattern for IGF-II concentrations observed among boys cannot be easily explained by differences in adiposity.

Sperm DNA may undergo subtle gene alterations with increasing paternal age, including epigenetic changes[24] that may be associated with phenotypic changes in the offspring. Increasing age is associated with a greater frequency of epigenetic modifications in both somatic[25] and germ cells.[24] Such gene changes may link advanced paternal age at childbirth with increased rates of autism and schizophrenia in the offspring.[26,27] It is therefore possible that increasing age leads to epigenetic changes in paternal sperm genes regulating growth and metabolism. We speculate that such gene changes may be subsequently transmitted to the offspring, leading to programmed changes in phenotype and metabolism in childhood.

Increasing paternal age is also associated with longer duration of exposure to common environmental toxicants (e.g. air pollution, and pesticides used on commonly consumed foods), which can affect sperm quality or induce epigenetic changes. Paternal alcohol consumption can also induce epigenetic changes in sperm,[28] with possible consequences to the offspring. Although not yet demonstrated, it is possible that such environmental factors may lead to phenotypic changes and other alterations in offspring outcomes. In addition, even paternal dietary habits may affect the offspring.[29] For example a recent animal study found that a pre-conceptional paternal diet rich in carbohydrates was associated with adverse lipid profiles in the offspring.[30]

The post-natal child-rearing environment across the socio-economic spectrum is also known to affect phenotype and other health outcomes in childhood.[31,32] The fact that increasing maternal age was also associated with greater stature suggests that environmental factors could have a role affecting offspring height. A recent study on nearly 80,000 British children showed that increasing maternal age was associated with improved health and better developmental outcomes in the offspring in early childhood.[33] Children reared in families of higher socio-economic status tend to be taller and slimmer than those from lower socio-economic backgrounds.[34,35] However, our homogenous cohort of children was entirely from higher socio-economic families, meaning that our findings are unlikely to be explained by differences in child-rearing environment. Further, a more favourable child-rearing environment in association with increasing parental age would not explain the contrasting finding of a less favourable lipid profile in the offspring of older fathers.

A possible limitation of our study is that we studied a homogenous group of children (same ethnicity and higher socio-economic status), which may limit application of our study findings to the general population, particularly to those of lower socio-economic status. We also acknowledge that our measure of socio-economic status ('decile' scores) integrates a number of affluence variables, but does not provide detailed information about individual parent's income and education. Nonetheless, we believe that our relative homogenous cohort has eliminated much of the phenotypic and metabolic variability associated with both ethnicity and socio-economic status, allowing us to better address the potential effects of paternal age on measured outcomes. It is also important to note that the observed associations with paternal age do not necessarily prove causality. Prospective studies are needed to specifically assess possible mechanisms through which paternal age may affect growth and metabolism in the offspring in childhood.

5.6. Conclusion

Our study showed that increasing paternal age at childbirth was associated with taller stature and reduced adiposity, but a less favourable lipid profile in their children. Further investigation of the possible triggers and mechanisms responsible for these findings is warranted. As paternal age at childbirth continues to increase worldwide, it is important to further evaluate the possible long-term effects on offspring health.

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Chapter 6. Discussion

6.1. Brief summary of findings

- Conception with the help of fertility medications (OS_A) was associated with a reduction in childhood height and BMI, and with a slightly more favourable metabolic profile
- Naturally conceived children of subfertile parents did not differ either phenotypically or metabolically to children of fertile parents.
- Increasing birth order was associated with a gradual reduction in childhood height, as well as subtle changes in serum IGF-I levels in tandem with this height reduction.
- Increasing maternal and paternal ages at childbirth were associated with a taller and slimmer phenotype in their children. Advancing maternal age was also associated with alterations in childhood IGF-II levels in tandem with their slimmer phenotype. In contrast, advancing paternal age was associated with less favourable lipid profiles in the offspring.

The above findings have been discussed in detail, and compared to other studies in the individual manuscript chapters. Therefore, this discussion chapter will focus on potential underpinning triggers and mechanisms. The relevance of our finding will also be discussed in a broader context, and areas for future research will be suggested.

This project has assessed the impact on offspring of a major shift in human reproductive behaviour, which has occurred over the past several decades. These behavioural changes have been observed in most developed countries, and include an increase in maternal age at childbirth by more than 4 years (from 26 to over 30 years of age). In tandem, paternal age at childbirth has similarly increased by more than 3 years, and currently stands at approximately 32 years of age on average. As a result, nowadays more than half of all children in the developed world are born to a father aged more than 32 years and a mother aged over 30.

The reduction in family size and the consequent increase in the proportion of first-born children have occurred over a similar period of time, with an increasing number of couples having just one or two children. The other important change in human reproductive behaviour over the past 30 years has been the dramatic increase in the uptake of fertility treatment, so

that up to 1 in 20 children born today were conceived with the help of ovarian stimulation fertility treatment.

The studies in this thesis have tackled the consequences of these changes in parental factors on children's phenotype, hormonal profiles, and metabolism. A common theme discussed in each individual study is the possible role of the early fetal environment, and epigenetics as a potential underpinning mechanism. In recent years, the putative role of epigenetics has been proposed as a potential mechanism for much of the observed changes in phenotype, as well as disease susceptibility in response to alterations in the early fetal environment. Some evidence to this regard has been obtained in elegant animal studies, but robust data supporting the role of epigenetics in such phenotypic changes in humans remains to be established. Furthermore, there are no studies linking a continuum of epigenetic changes to genes and changes in gene expression, to alterations in circulating hormones, as well as changes in phenotype. Nevertheless, epigenetics remains one of the more likely mechanisms responsible for our observations, and therefore deserves consideration. Thus, the potential role of epigenetics and the early fetal environment will remain an important focal point in this discussion.

6.2. The influence of maternal & paternal ages at childbirth on the growth & metabolism of children

There are no previous studies assessing the impact of paternal age on childhood height and metabolism, and studies assessing the impact of maternal age at childbirth on these outcomes are limited. We found that increasing maternal and paternal ages at childbirth were each associated with an increase in childhood height. While the changes were not identical, increasing age of mother or father at childbirth were also associated with a reduction in BMI and/or a more favourable body composition in their children.

6.2.1. Maternal age at childbirth

We found that a maternal age at childbirth greater than 30 years was associated with a taller and slimmer phenotype in their children. While the importance of a slight increase in childhood height to long-term health is unclear, these children also displayed an important reduction in abdominal adiposity. This is because abdominal fat is one of the most important predictors of obesity-related disease, and is a predictor of the metabolic syndrome (Weiss &

Caprio 2005; Weiss *et al.* 2004). Furthermore, differences in childhood body composition and BMI are likely to persist or accentuate in adulthood (Freedman *et al.* 2005; Wells *et al.* 2007), which means children born to mothers aged over 30 years may be at a reduced risk for later obesity.

In keeping with their lower abdominal fat, we found lower IGF-II levels in children born to mothers over 30 compared to children of younger mothers. It is worth noting that we adjusted IGF-II values for total body fat in our statistical analysis (amongst other relevant parameters, such as age and gender), thus ensuring observed differences in IGF-II levels were not simply representative of increased adiposity (Faienza *et al.* 2010; Sandhu *et al.* 2003). This is important as serum IGF-II is correlated with adiposity (Sandhu *et al.* 2003).

It is possible that serum IGF-II concentrations provide a crude estimate of *IGF2* gene expression activity (Faienza *et al.* 2010). Indeed, recent investigations revealed that *IGF2* gene expression may have an important role in obesity and metabolic disease, even in childhood (Faienza *et al.* 2010). Thus, it is possible that a combination of lower IGF-II and reduced abdominal fat in children born to mothers aged over 30 years may reduce their risk of obesity and metabolic disease in later life. In contrast, children born to younger mothers (aged <30 years) may be at increased risk for such health issues.

6.2.2. Paternal age at childbirth

Increasing paternal age at childbirth was also associated with an increase in childhood height, with children born to fathers over 30 years of age being almost 2 cm taller than those born to younger fathers. Importantly, this finding was present after adjustment for maternal age, birth order, genetic height, and other confounders. Similar to the maternal age subgroups, children in all 3 paternal age groups had similar birth parameters. Importantly, the taller children actually had lower BMI and tended to have reduced adiposity compared to the shorter children of younger fathers, meaning that their taller stature was not attributable to adiposity (Ong *et al.* 2009). The clinical significance of their slightly taller stature is unknown.

The most striking finding in our study assessing the impact of paternal age at childbirth on childhood outcomes was the marked effect on children's lipid profiles. Children born to fathers aged over 30 years of age had a less favourable lipid profile than children born to younger fathers. These findings were unexpected, particularly given that these children with relatively poorer lipid profiles were slimmer with a more favourable body composition.

While the differences in lipid parameters were not clinically significant, the observed differences remain important. For example, the total cholesterol to HDL-C ratio in children born to fathers aged under 30 years was 3.00 compared to the ratio of 3.47 in children of fathers aged over 35. This is striking, particularly considering the American Heart Association suggests that 3.5 is the optimum total cholesterol to HDL-C ratio for adults (Lloyd-Jones *et al.* 2009). Similarly, children of fathers aged more than 35 years had a mean total cholesterol of 4.5 mmol/l and LDL-C of 2.6 mmol/l. Again, while these values are within normal clinical limits, they are strikingly higher than those of children born to fathers aged less than 30 years (4.2 and 2.2 mmol/l, respectively). Childhood lipid profiles tend to track or more likely accentuate into adulthood (Webber *et al.* 1991), and elevations in total cholesterol, total cholesterol:HDL-C ratio, and LDL-C are all strongly associated with cardiovascular disease risk (Lloyd-Jones *et al.* 2009). Thus, it appears likely that children born to fathers aged more than 30 years (particularly to those older than 35 years) may be at an increased risk for poorer lipid profiles and consequent cardiovascular disease in later life.

However, in contrast to their poorer lipid profiles, the children of fathers aged more than 30 years at childbirth actually had lower BMI and reduced adiposity than the children of younger fathers. While it is not entirely clear if a reduction in body fat in childhood highly correlates with future obesity risk (Freedman *et al.* 2004; Maynard *et al.* 2001; Schaefer *et al.* 1998), it is established that a lower BMI in childhood is predictive of adult BMI (Freedman *et al.* 2005; Wang & Beydoun 2007).

Therefore, it is more likely that these children, who have poorer lipids, will have a lower risk of obesity as adults compared to the children of younger fathers. Also, we found that they are slightly taller than their peers born to fathers aged less than 30 years. So, overall, a paternal age at childbirth over 30 years is associated with children who are taller and leaner, but with a relatively poorer lipid profile.

6.2.3. Dual roles and relatively young maternal and paternal ages impacting offspring phenotype

As already stated, the findings of the impacts of maternal and paternal ages at childbirth on their children's phenotype and metabolism share the similarities of a taller and slimmer offspring born to mothers and fathers aged over 30 years. Importantly, data analyses in each study were appropriately adjusted for maternal or paternal ages. Thus, it is likely that both maternal and paternal ages have separate effects on offspring outcomes in childhood.

Our finding of phenotypic changes in childhood in the offspring born of relatively young mothers (over 30 years of age) was somewhat unexpected. As previously outlined, older maternal ages over 35 or 37 years are associated with a marked increase in the risk of obstetric and perinatal problems, as well as a dramatic increase in the risk of chromosomal disorders in offspring (Broekmans *et al.* 2009). This led us to expect that perhaps only maternal ages greater than 35 years would have been associated with differences in childhood phenotype. However, it seems that changes in offspring disease risk associated with increasing maternal age do not have clear age thresholds.

The slight increase in risk of type 1 diabetes and of elevated blood pressure in the offspring associated with increasing maternal age is essentially linear (Cardwell *et al.* 2010; Lawlor *et al.* 2004). Nevertheless, there is previous evidence of changes in some maternal factors, even from a relatively young age of 30 years. Such changes are signalled by a decline in female fertility, accompanied by an increase in maternal hormones such as FSH (Broekmans *et al.* 2007). Therefore, it is likely that the triggers and mechanisms responsible for these changes are complex and multi-factorial.

Similarly, our findings of alterations in offspring phenotype and lipid profiles at paternal ages at childbirth of 30 and 35 years were somewhat surprising. Much older paternal ages at childbirth (over 40 or 50 years) have the strongest association with offspring risk of autism (Reichenberg *et al.* 2006) and adult-onset psychiatric disorders (Peedicayil 2010). Indeed, it is often perceived that a paternal age over 40 years may be important in terms of risk to offspring (de La Rochebrochard *et al.* 2003). This is well illustrated by the upper age limit of 40 years for sperm donation in Europe and the USA, which appears to be largely based on the risk of gene mutations to offspring (de La Rochebrochard *et al.* 2003; Tomlinson *et al.* 2008). However, the latter risk increases exponentially over time, and there seems to be a non-linear increase in risk to offspring from a paternal age of 35 or 37 years (Aitken *et al.* 2004; Kong A 2012; Singh *et al.* 2003; Slama *et al.* 2005; Vestergaard *et al.* 2005; Wunsch & Gourbin 2002).

Therefore, it is possible that a paternal age younger than previously thought may be important to offspring risk. Indeed, an increased risk of epilepsy in offspring has been observed in children born to fathers aged over 35 years (Vestergaard *et al.* 2005). Similarly, three studies found that a paternal age over 30 or 35 years at childbirth was associated with a minor increase in the risk of type 1 diabetes in children (Bingley *et al.* 2000; Cardwell *et al.* 2005;

Stene *et al.* 2000). Thus, the paternal ages at which we observed an impact on offspring phenotype and metabolism, while relatively young, may add further to the debate on the paternal age threshold associated with the lowest risk to offspring. Again, similar to maternal age, triggers and mechanisms responsible for the differences in offspring are likely complex and multi-factorial.

Some of the possible triggers and mechanisms responsible for these observed differences in childhood outcomes with increasing maternal and paternal ages have been explored briefly in the respective manuscripts. I will explore some of the possible triggers and mechanisms more thoroughly, as well as mention possible alternatives.

6.2.4. Parental age and possible pre-natal triggers and mechanisms

6.2.4.1. Maternal age

As outlined in the thesis introduction, increasing maternal age is associated with many changes in maternal physiology, as well as genetic changes in oocytes (both pre- and post-conception). Seemingly subtle changes, such as oxidative stress and alterations in maternal hormone levels, may also contribute to this process. These changes may trigger alterations in oocyte or fetal epigenetics, which in turn, could alter offspring phenotype. It is possible that such changes are responsible for the observed effects of maternal age at childbirth on offspring auxology and hormone levels in childhood.

Increasing maternal age is associated with well-recognised deterioration in the quality of the maternal oocyte, leading to an increased risk of oocyte aneuploidy (Steuerwald *et al.* 2007). However, increasing maternal age is also likely associated with more subtle genetic alterations in the oocyte, such as epigenetic changes (Grøndahl *et al.* 2010; Hamatani *et al.* 2004). Epigenetic changes may lead to altered phenotypic outcomes, as explained in the introduction of this thesis. Epigenetic alterations in oocyte may be triggered by several different maternal age-related changes. These may occur as part of the normal aging process (Richardson 2003; Salpea *et al.* 2012) or due to age-related epigenetic changes in oocytes themselves (Grøndahl *et al.* 2010; Hamatani *et al.* 2004; Steuerwald *et al.* 2007). Oocyte aging itself has been associated with alterations in several genes, such as those involved in mitochondrial function and oxidative stress, as well as alterations in the expression of genes involved in chromatin structure, DNA methylation, and genome stability (Hamatani *et al.* 2004; Steuerwald *et al.* 2007; Yue *et al.* 2012). Thus, pre-ovulatory oocyte aging (which

occurs in tandem with increasing maternal age) is associated with alterations in gene expression.

An interesting concept of post-ovulatory aging has also been explored, although most studies have focused on the impact of such aging to oocytes extracted for external fertilisation for the process of IVF (Emery *et al.* 2005; Nagy *et al.* 2006). These studies have demonstrated a reduction in oocyte integrity in such oocytes, after a relatively brief period of 1 to 2 days (Emery *et al.* 2005; Reichman *et al.* 2010). While this is important to the IVF process, such information may also shed light on another type of oocyte aging that occurs with increasing maternal age. There is evidence that the frequency of sexual intercourse amongst couples reduces with increasing age (Dunson *et al.* 2002; Weinstein & Stark 1994). After ovulation, the 'window of fertility' for a woman is approximately 5 to 10 days (Wilcox *et al.* 1995). Thus, if a woman has sexual intercourse less frequently with increasing age, it is likely that her oocyte will experience an increased duration of post-ovulatory aging prior to fertilisation. The possible consequences to the oocyte have been outlined in detail by Takahashi *et al.* (Takahashi *et al.* 2011), and include reactive oxygen species leading to mitochondrial injury, as well as abnormal oocyte intracellular calcium regulation. Such processes may lead to alterations in oocyte epigenetics or other changes.

Oxidative stress

Another possible source of age-related alterations in oocytes is oxidative stress, which increases with age (Finkel & Holbrook 2000) and impacts on oocyte gene quality (Robaire *et al.* 2012). This may lead to a reduction in genetic regulatory processes in the oocyte, such as altered DNA repair mechanisms as well as possible epigenetic changes (Lim & Luderer 2010). Oocyte mitochondrial dysfunction has also been associated with oxidative stress, which may lead to reduced oocyte competence (Steuerwald *et al.* 2007).

Maternal hormones

Increasing maternal age is also associated with greater endogenous production of FSH, which rises in tandem with a diminishing oocyte pool (Broekmans *et al.* 2009). It is well recognised that rates of spontaneous twinning increase with increasing maternal age (Abel & Kruger 2012; Hoekstra *et al.* 2008). This is a result of a form of "endogenous ovarian hyperstimulation", whereby elevated levels of FSH are associated with an increased frequency in the ovulation of more than one oocyte (Prior 2005). There is some evidence from assisted reproductive technology that exogenous FSH administration is associated with

changes in the DNA methylation of oocytes (Market-Velker *et al.* 2010; Sato *et al.* 2007). Thus, it is possible that higher levels of endogenous FSH with increasing maternal age may also lead to epigenetic or imprinting changes in oocyte genes (Market-Velker *et al.* 2010; Sato *et al.* 2007).

Other possible causes of the differences observed in children with increasing maternal age are changes in pregnancy levels of maternal testosterone and other steroid hormones. Two studies have shown an age-related increase in maternal testosterone during pregnancy (Troisi *et al.* 2003; Wang & vom Saal 2000), but another investigation found decreased maternal testosterone levels with increasing maternal age (Carlsen *et al.* 2003). A study in sheep found that administration of testosterone to pregnant ewes reduced fetal growth but increased postnatal growth (Crespi *et al.* 2006). There were also changes in offspring levels of growth factors and growth hormone receptors after testosterone exposure *in utero* (Crespi *et al.* 2006). Therefore, it is possible that subtle age-related increases in maternal testosterone levels during pregnancy may contribute to the height differences seen in children born to older mothers.

Maternal testosterone and oestrogen levels also change with increasing age (Panagiotopoulou *et al.* 1990; Troisi *et al.* 2003), and there is evidence from animal studies that changes in levels of such hormones during pregnancy may alter fetal and postnatal growth (Crespi *et al.* 2006; Wang & vom Saal 2000), as well as insulin sensitivity (Eisner *et al.* 2000).

Uterine aging

Senescence (biological aging) of the uterus and other female reproductive structures may alter the fetal environment with increasing maternal age (Zenke & Chetkowski 2004). Age-related changes to the uterus may alter the vascular flow to the fetus of older mothers (Cano *et al.* 1995). A change in vascular flow to the fetus (such as placental insufficiency leading to low birth weight) is a risk factor for childhood insulin resistance and shorter stature (Chaussain *et al.* 1994). Thus, alterations in uterine vascular flow in older mothers may lead to programmed changes in the fetus, which could manifest as physical differences in childhood. Indeed, it is well recognised that birth weight reduces with increasing maternal age (Lee *et al.* 1988), and it is thought that this change is due to alterations in placental efficiency (Fretts *et al.* 1995; Harman & Talbert 1970). However, nutrient restriction to the fetus is associated with lower birth weight and an increased risk of shorter stature in

childhood (Hack *et al.* 1995). This contrasts with our finding of taller stature in children with increasing maternal age, making this association less likely to explain our observations.

It is possible that any one or a combination of these maternal age-related changes could trigger alterations in oocyte or fetal epigenetics, imprinted genes, as well as other described cellular changes in oocyte. It is also possible that changes in hormone levels or uterine physiology could alter offspring programming and phenotype. If alterations in maternal factors trigger epigenetic changes, it is possible that such alterations could lead to changes in imprinted genes regulating offspring growth or other genes. Imprinted genes, such as H19 are involved in childhood growth and are vulnerable to imprinting changes (Reik *et al.* 2001a). IGF-2 gene expression is also vulnerable to epigenetic changes (Giannoukakis *et al.* 1993), which are not only associated with alterations in fetal growth (Fowden *et al.* 2006), but have also been linked to differences in body composition in childhood (Ong *et al.* 2002a) and adulthood (Fowke *et al.* 2010).

6.2.4.2. Paternal age at childbirth

Some possible triggers and mechanisms which may be responsible for the changes seen in children born to fathers of different ages have already been outlined in the manuscript. However, it is worthwhile exploring these in some more detail, and addressing other possible triggers and mechanisms which may be responsible for these the observations in our study.

Increasing paternal age is associated with changes in male gamete quality, and this has been discussed in Chapter 1. Such changes may impact on sperm genes and other factors, with possible consequences to offspring phenotype.

Aging of the male gamete

Changes in the male gamete in association with increasing age have been the subject of far less research than those in female gametes. Furthermore, such age-related changes in male gametes are far less dramatic than those seen in female gametes. Alterations also appear to occur gradually in males, rather than at relatively clear age threshold as in women. Further, it is important to note that, although the changes that take place in male gametes with increasing age are relatively subtle, these may still impact on offspring outcomes.

Similar to the effects of maternal age, male gametes deteriorate with increasing age, with the occurrence of single gene defects being the most widely recognised risk associated with increasing paternal age (Crow 1997). The exponential increase in the occurrence of single

gene defects in sperm with increasing male age illustrates the vulnerable nature of sperm genes to the passage of time (Kong A 2012).

Epigenetics

There is evidence of epigenetic changes and imprinting errors occurring in sperm genes with increasing paternal age (Curley *et al.* 2011; Oakes *et al.* 2003). These age-related changes include hyper-methylation, altered chromatin packaging and integrity (Zubkova & Robaire 2006), amongst other epigenetic alterations (Kokkinaki *et al.* 2010; Wyrobek *et al.* 2006). Indeed, a positive correlation between paternal age and levels of DNA methylation in newborns has been described in humans (Adkins *et al.* 2011). However, such changes in genes have yet to be linked to any phenotypic differences in offspring. Nevertheless, there is increasing circumstantial evidence that epigenetic changes in sperm genes may be the underlying mechanism leading to increased risk of a number of diseases in the offspring of older fathers, including schizophrenia, bipolar disorder, and autism (Farrer *et al.* 1991; Peedicayil 2010; Perrin *et al.* 2007). Thus, while this field of research remains in its early stages, it seems likely that epigenetic changes in paternal sperm genes may be important mediators of later disease risk, and may also affect phenotype in the offspring.

Environmental toxicants

Increasing age inevitably leads to an increased duration of exposure to environmental toxicants. Thus, for example, a 20-year-old father has far less cumulative exposure to toxicants than a father aged 40 years. Common exposures include alcohol intake and air pollution, as well as exposure to agrochemicals (through consumption of common foodstuffs).

The possible role of common environmental toxicants in alterations in sperm genes is becoming increasingly apparent (Perera & Herbstman 2011). Indeed, the potential role of such toxicants in epigenetic changes in germ cells (as well as possible consequences to the fetus *in utero*) is of concern (Perera & Herbstman 2011). Since it is inevitable that increasing paternal age would be associated with an increased accumulation of toxicant exposure, older fathers are more likely to experience any consequent epigenetic changes in germ cells (Tarin *et al.* 1998).

Male diet & behaviour

Another male factor that is potentially important to offspring programming is preconception male diet (Ferguson-Smith & Patti 2011; Lim & Song 2012). A recent animal study revealed

that a carbohydrate-rich paternal diet led to up-regulation of lipid biosynthesis genes in offspring (Carone *et al.* 2010). Men's BMI tends to increase after the age of 30 years (Flegal & Troiano 2000), and this weight gain is likely related to excessive carbohydrate consumption. Therefore, it is possible that such dietary changes in men over 30 could lead to programmed changes in the lipid metabolism of their offspring. While most studies are focusing on the impact of male diet on semen quality, motility, and morphology (Attaman *et al.* 2012), human studies assessing the possible impact of male diet on offspring phenotype and disease risk would be fascinating.

Oxidative stress & telomere length

Reactive oxygen species are free radicals that can cause oxidative stress-induced damage to nucleic acids, proteins, and other cell components, which are thought to accumulate with age (Harman 2006) The possible role of age-related increases in these reactive oxygen species in sperm remains largely unknown, but they may possibly contribute to sperm DNA damage (Aitken *et al.* 2003; Robaire *et al.* 2012).

An interesting paternal age-related change in DNA that takes place is an associated increase in offspring telomere length (De Meyer *et al.* 2007; Njajou *et al.* 2007). Sperm telomeres increase in length with increasing male age, and it is proposed that this leads to the increased telomere length in offspring of older fathers (De Meyer *et al.* 2007). Shorter telomeres are increasingly evident in disease states, and longer telomeres may be a biomarker for longevity (Nordfjäll *et al.* 2009). This change is of interest as it appears to be a positive change in sperm DNA with increasing age, which may confer an advantage to offspring. While an increase in offspring DNA telomere length has been associated with increasing paternal age (Nordfjäll *et al.* 2009), the consequences to offspring (if any) are still unknown (Eisenberg 2011).

6.2.5. Parental age and the post-natal environment

While all families in our cohort were of higher socio-economic status, it is possible that increasing age confers different maternal and paternal characteristics and behaviour, even in a relatively homogenous group. As a result, maternal and paternal age-related differences in the post-natal environment of child rearing need to be considered as possible mechanisms responsible for the phenotypic changes in the offspring observed in our study.

6.2.5.1. Diet and exercise

Men and women who have children when they are under the age of 30 years may have inherently different attitudes to child-rearing compared to those who have children at older ages (Bornstein *et al.* 2006; Ragozin *et al.* 1982). Such differences in attitudes may impact on childhood dietary and exercise habits. Indeed, increasing age of mothers and fathers is associated with a better diet in their children (Brown *et al.* 2008; Northstone & Emmett 2005), and this is often due to greater parental knowledge about food choices (Robinson & Godfrey 2008). As healthier food choices help protect against obesity (Summerbell *et al.* 2005), such maternal and paternal age-related behaviours may help explain why children of older mothers and fathers in our cohort had reduced adiposity.

However, in contrast, increasing age of mothers and fathers is associated with a reduction in their own exercise levels (Macera *et al.* 2005), which, in turn, leads to reduced levels of activity and exercise in their children, who tend to emulate their parents' behaviour (Mitchell *et al.* 2011). Increasing age of parents is also associated with a reduction in active childhood play, irrespective of the parents' own activity levels (MacDonald & Parke 1986; Moore *et al.* 1991). As exercise and obesity are intrinsically linked (Summerbell *et al.* 2005), we would therefore expect that increasing age of parents would lead to increased adiposity in their children.

On balance, increasing maternal and paternal ages at childbirth are associated with improved diet, but reduced exercise levels in their children. Thus, it seems likely that any dietary improvements in children of older parents would be offset by a reduction in their physical activity levels. Nonetheless, even if parents' child-rearing behaviours differed slightly with increasing age, it is unlikely to fully explain the observed changes in height, BMI, body composition, hormone levels, and lipid profiles with increasing maternal and paternal ages at childbirth.

6.2.6. Conclusion

Our study findings show that advancing maternal and paternal ages at childbirth are associated with taller and slimmer phenotype in their children. The striking changes in childhood lipid profiles associated with increasing paternal age at childbirth are in stark contrast to this taller, leaner phenotype. This is important as changes in phenotype in childhood may have long-term implications.

It is possible that these alterations in childhood phenotype and metabolism are triggered by a combination of the many changes that occur in male and female gametes or the early fetal environment in association with advancing maternal and paternal ages. We assessed a cohort who was likely to have a similar child-rearing environment, based on similar socio-economic status. Although parents' ages may have led to subtle differences in the environment of child-rearing, such differences are unlikely to explain the observed differences in childhood phenotype and metabolism.

While we found that children born to parents aged over 30 years were taller and slimmer, it is important that this is not seen as reassuring to prospective parents aged over 30, and particularly for prospective mothers aged over 35 years old. The established risks to offspring of increasing maternal age at childbirth, particularly that of chromosomal disorders (Broekmans *et al.* 2009), outweighs any benefit of having a child with a taller and leaner phenotype. In comparison to the risks of increasing maternal age at childbirth, increasing paternal age at childbirth is associated with a much lower risk of severe outcomes in the offspring, but these risks still exist and include autism and gene defects (Kong A 2012). Furthermore, our finding of an association between increasing paternal age and less favourable lipid profiles in children provides further reasons for caution about the possible risks of increasing paternal age at childbirth. Future research will help clarify the additional risks and possible benefits to offspring of increasing maternal and paternal ages at childbirth.

6.2.7. Directions for future research

Clearly, the impact of maternal and paternal ages at childbirth is under-researched. While our study was cross-sectional, it is clear that large prospective studies would best evaluate the effects of maternal and paternal ages at childbirth on childhood outcomes. Ideally, prospective parents would be enrolled prior to conception, so that all facets of their pre-pregnancy diet, behaviour, physiology and other influential factors could be examined. Longitudinal follow up throughout pregnancy and subsequent on-going assessments of parents and children would provide a wealth of important information.

If such comprehensive studies detected pivotal parental factors affecting offspring outcomes, the information could be utilised to introduce interventions to modify offspring risks. However, these studies require enormous commitment of resources, time and take decades to yield meaningful results. There are nonetheless, many large longitudinal studies on-going

worldwide that could possibly address a number of important questions on this topic. In the meantime, contemporary studies could address more specific research questions.

Future studies need to tackle specific areas of public health interest. Our findings suggest that children born to mothers and fathers aged less than 30 years may be at risk of future obesity, and we need to clarify this risk across the socio-economic spectrum. Detailed information on childhood diet and exercise levels in association with maternal and/or paternal age at childbirth would be of great interest and relevance. Such data could better target already limited public health resources and education towards more specific groups, when initiating interventions to reduce childhood obesity and future disease risk. Furthermore, more in depth assessment of the association between maternal and paternal ages at childbirth with adult lipid profiles and type 2 diabetes risk are clearly warranted.

Also, as previously discussed, the limited data assessing the impact of maternal age at childbirth on adult offspring mortality indicated that increasing maternal age is associated with reduced life-span in the offspring (Liu *et al.* 2011b). Work on paternal age and offspring mortality is equally limited (Gavrilov & Gavrilova 2000), and was based on historic data. Given the dramatic rise in maternal and paternal ages at childbirth over the past few decades, it is important to adequately assess possible long-term risks to offspring.

Exploration of the relative contributions of maternal and paternal ages at childbirth to the phenotypic changes in offspring needs to be clarified. This could be tackled in several ways. A first step might be to utilise animal models of divergent ages, which would provide important data and help direct the design of further human studies. Secondly, offspring of parents with widely divergent ages could be studied, thereby clarifying the effect of the older parent on offspring outcomes. Finally, epidemiological studies could also address this question, but would require a large study population and rigorous methodology.

6.3. Birth order

6.3.1. The influence of birth order on the growth and metabolism of children

We found that first-born children were approximately 2.5 centimetres taller than later-born children. We also found that birth order had a graded effect on height, with an incremental height reduction from first to third birth order. These differences equated to a mean height

decrease of 1.3 cm from first- to second-borns, and a further decrease of 2.0 cm from second- to third-born children. In addition, in keeping with their taller stature, first- and second-borns had higher IGF-I concentrations than third-borns.

We have already examined our findings and have compared them to previous studies. However, it is pertinent to broadly comment on our results, and outline their contribution to this area of research. Importantly, we observed an incremental reduction in childhood height with increasing birth order, demonstrating effects beyond that of first-borns compared to later-borns. Ours is the first study to assess the gradual impact of birth order in childhood outcomes. Further, while several studies have previously examined the impact of birth order on childhood height, we were the first to do so while minimising important confounders; while one previous study has assessed the impact of birth order on childhood BMI (Wells *et al.* 2011), ours is the first study on a homogenous group in a developed country. In addition, there are no previous data assessing the impact of birth order on childhood metabolism.

We detected no differences in adiposity or metabolism with increasing birth order, which are important negative findings *per se*. The increased BMI described in adult first-borns (Reynolds *et al.* 2010; Stettler *et al.* 2000) means that the transition from a slim first-born child to a heavier first-born adult phenotype occurs after the onset of puberty. The possible impact of birth order on adult lipid profiles also needs further assessment, as only one limited study has tackled this issue to date (Siervo *et al.* 2010).

6.3.2. Possible triggers and mechanisms

Although some possible triggers and mechanisms have been outlined briefly in the respective manuscripts, it is pertinent to expand on some of these possibilities, as well as include some possible alternative mechanisms.

6.3.2.1. Pre-natal factors

Fetal restriction

Several authors suggest that the *in utero* fetal restriction model is the most likely mechanism responsible for the effect of birth order on offspring outcomes (Dahly & Adair 2010; Gluckman & Hanson 2004b; Reynolds *et al.* 2010; Siervo *et al.* 2010; Wells *et al.* 2011). Limited data suggest that relative fetal restriction occurs in first pregnancy (see Chapter 1), as implantation and placentation are less invasive, there is reduced efficiency in the delivery of nutrition to the first-born fetus (Gluckman & Hanson 2004b; Prefumo *et al.* 2006). Therefore,

the placenta invades the uterine wall more efficiently in later pregnancies, leading to a more effective flow of nutrients to the later-born fetus (Khong *et al.* 2003; Litwin *et al.* 2010; Prefumo *et al.* 2006). Indeed, this mechanism is thought to be at least partially responsible for the recognised slightly lower birth weight (approximately 200 g) of first-borns compared to later-borns described in large population studies (Oken *et al.* 2003). A difference in birth weight between first- and later-borns is also seen in many other mammal species, including sheep (Hyatt *et al.* 2010), mice (Reading 1966 1142), and seals (Bowen *et al.* 1994).

It is recognised that *in utero* restriction (leading to, for example, a fetus being born SGA) is associated with increased risk of obesity (Chernausek 2012), insulin resistance (Hofman *et al.* 2004; Veening *et al.* 2002), and increased blood pressure (Levy-Marchal & Jaquet 2004; Yiu *et al.* 1999) in childhood (Hofman *et al.* 1997). However, children exposed to nutrient restriction *in utero* (e.g. SGA children) tend to be shorter than their peers in childhood and adulthood (Chernausek 2012). Therefore, while the fetal restriction model is likely important in the overall context of birth order, it is not does explain the observed height reduction with increasing birth order in our study. Therefore, alternative mechanisms need to be considered.

Epigenetics and imprinting

A possible explanation for the taller stature of first- compared to later-born children may be that the differential placentation between first and subsequent pregnancies is associated with epigenetic changes in imprinted genes regulating growth. As imprinted genes are abundantly expressed in the placenta, they are also vulnerable to changes in the placental or external environment (Nelissen *et al.* 2011). Indeed, imprinting control mechanisms appear to be more sensitive to environmental cues in placental than embryonic or fetal tissues (Fortier *et al.* 2008; Wei *et al.* 2010).

Placental imprinted genes vulnerable to changes include those involved in growth, such as *IGF2* and *H19* (Bourque *et al.* 2010). Placental expression of *H19* has been found to be increased in pregnancies complicated by fetal growth restriction compared to non-restricted pregnancies (Koukoura *et al.* 2011). Since first-borns are relatively growth restricted *in utero*, they may have altered *H19* expression. Therefore, this could lead to altered programming of their post-natal growth, and possibly explain their taller stature. However, this assertion is speculative at this stage.

Offspring hormones

Alternatively, it is possible that this differential growth between first- and later-born children may be mediated by differences at the level of the growth hormone receptor in the offspring (Geary *et al.* 2003; Hyatt *et al.* 2007). First- and later-born sheep have marked differences in their hepatic GH–IGF axis, and first-borns also have up-regulated hepatic IGF-I receptor mRNA (Hyatt *et al.* 2007). These receptor differences were accompanied by a trend towards a higher crown to rump length in first-borns at age one month. Unfortunately, details on phenotype were not provided beyond this age (Hyatt *et al.* 2007).

These data suggest that first-borns may have increased GH and IGF receptor responsiveness compared to later-borns, providing some insight into a possible mechanism for the taller stature of first-borns. However, the triggers for these changes remain elusive. While studies assessing the existence of such changes in humans are practically challenging, it is certainly an area which warrants investigation.

Maternal hormones

Maternal hormone levels in pregnancy (including oestrogen) have been found to be higher in primiparous mothers compared to multiparous mothers (Bernstein *et al.* 1986; Panagiotopoulou *et al.* 1990). Such differences include higher cord blood oestrogen and progesterone (Maccoby *et al.* 1979), and dehydroepiandrostenedione sulphate (DHEAS) concentrations (Troisi *et al.* 2003) in first- compared to later-borns. Indeed, the elevated breast and testicular cancer risk in first- compared to later-born adults is hypothesised to be linked to this *in utero* exposure, with some limited evidence to support it (Hsieh *et al.* 1991; Prener *et al.* 1992).

Animal studies yield some evidence that elevations of androgens and/or oestrogens *in utero* are associated with changes in fetal and post-natal growth (Crespi *et al.* 2006; Wang & vom Saal 2000). However, maternal hormone levels in pregnancy are influenced by many factors, including maternal age and birth weight (Troisi *et al.* 2003), both of which also influence post-natal growth. Therefore, it is uncertain if differences in maternal hormone levels during pregnancy are a likely mechanism for phenotypic differences in offspring associated with birth order.

Maternal pregnancy diet

There is ample experimental evidence that maternal diet in pregnancy influences offspring phenotype and outcomes (Dolinoy *et al.* 2006; Samuelsson *et al.* 2008). Overall, a balanced

healthy diet promotes normal fetal and post-natal growth. There is evidence that women gain more weight in the first than in any subsequent pregnancies, and that weight gain is positively correlated with birth weight (Seidman *et al.* 1989).

The quality of maternal diet in relation to parity has been the subject of limited research (Godfrey *et al.* 1996; Knudsen *et al.* 2007; Scholl & Chen 2009). Interestingly, the limited data comparing diet in first and later pregnancies suggest that first-time mothers have a healthier diet and are more likely to take vitamin and/or micronutrient supplements (Scholl *et al.* 1997). Such differences in maternal diet during pregnancy may explain phenotypic differences, such as the taller stature of first-born children.

Maternal psychological stress

Maternal stress levels tend to be higher in first than in subsequent pregnancies (Paarlberg *et al.* 1996), and many animal and human studies showed that maternal stress can affect the fetus, and lead to alterations in offspring phenotype and disease risk (reviewed by Vaughan *et al.* 2011). Evidence suggests that these changes in the maternal environment are associated with changes in placental imprinted genes, including those involved in growth (Vaughan *et al.* 2011). However, to date such changes have only been associated with fetal growth restriction and other adverse outcomes, none of which are associated with an increase in post-natal height (Mulder *et al.* 2002; Rondo *et al.* 2003). Therefore, this mechanism is most unlikely to explain the taller stature of first-borns.

6.3.2.2. Post-natal factors

Tanner (1989) suggested that first-borns benefit from a nutritionally plentiful environment, as they are an 'only child', at least for a while. This proposed mechanism to explain the taller stature of first-borns is relevant in poorer socio-economic groups, where family resources are limited. As previously discussed, although undernutrition has a marked effect on height (Bhutta *et al.* 2008), it does not explain the taller stature of first-borns in our cohort, as all children were reared in an plentiful environment, irrespective of birth order.

The taller stature of first-borns may also be related to an excessive 'catch-up' weight and height gain from birth (Dunger *et al.* 2007; Ong *et al.* 2009; Ong *et al.* 2002b). Ong, Dunger *et al.* (Dunger *et al.* 2007; Ong *et al.* 2002b) demonstrated that first-borns who were of lower birth weight had an increased risk of more rapid weight (and consequently height) gain, and therefore taller stature in mid-childhood (Dunger *et al.* 2007; Ong *et al.* 2002b). This is not

surprising given the known association between elevated BMI and taller stature in childhood (Metcalf *et al.* 2011). In contrast, the taller first-borns in our cohort had a similar BMI and body composition to the shorter later-borns. Furthermore, our findings persisted even after correction for birth parameters in the statistical model. These are critical differences between our study and both Dunger *et al.* (2007) and Ong *et al.* (2002b), as we demonstrated an effect of birth order and not birth weight or obesity.

Other post-natal environmental differences among birth orders have been proposed in psychological studies (Paulhus *et al.* 1999; Sulloway 2001; 2007), where the first-born and the 'middle child' are often the subjects of investigation. Numerous publications have outlined the differing psychological aspects related to birth order within a family, but none appear to impact on phenotype or disease risk, at least not in childhood. However, it is possible that the psychological impact of birth order affects behaviour (e.g. higher rates of smoking in later-born adults (Argys *et al.* 2006)), and may need to be considered in future studies.

6.3.3. Conclusion

Our findings of differing height and subtle changes in adiposity and serum growth factor levels add weight to the assertion that first-borns are phenotypically different to later-borns. The triggers and mechanisms responsible are unclear, but are not explained by the often quoted fetal restriction model. However, it seems likely that a pre-natal trigger is responsible for this effect. The animal study finding altered GH and IGF-I mRNA receptor responsiveness in association with birth order may provide the best insight into a possible mechanism mediating the taller first-born phenotype.

First-borns represents between 60 to 70% of the newborn population in the Western World today. Further investigation on the possible differences in phenotype and disease risk between first-born and their siblings in childhood and adulthood is warranted. This is particularly the case given that the trend towards couples having smaller families looks likely to continue, and is likely to escalate. Therefore, it is likely that first-borns will represent more than half the population over the age of 40 or 50 years in the coming decades. If birth order brings altered disease risk and phenotype with it, then it is critical that we have as much information as possible on such risks to better inform public health policy.

6.3.4. Directions for future research

Further study is required to assess the implications of the emerging predominance of first-borns in the population. Prospective, longitudinal studies assessing pre-pregnancy parental behaviour and long-term follow up of offspring throughout child-rearing would best assess the impact of birth order. These studies require rigorous design to tackle the many complex variables and confounding factors associated with birth order. However, in practical terms, the heterogeneity of social, economic, and environmental factors in such studies are challenging and difficult to untangle.

Such large population studies in humans are worthwhile, but valuable information could be gained from animal studies enabling a controlled environment to investigate specific birth order effects on offspring outcomes. Clearly, animal models on sheep or primates who tend to have singleton offspring are required for such studies. Human studies need to target specific questions which are modifiable, and may impact on health and disease risk factors of offspring in relation to birth order. For example, maternal behaviours (such as diet, exercise, and sleep) during first and subsequent pregnancies are likely to differ, but little is known about such differences. It is also unknown if these differences impact on offspring outcomes. If pivotal maternal behavioural factors were uncovered that affect offspring outcomes, this would enable mothers and health-care professionals to modify such behaviours.

Although adult studies have found that first-borns are at risk of obesity, these were limited and paid little attention to the possible biological triggers and mechanisms responsible (Dahly & Adair 2010; Reynolds *et al.* 2010; Stettler *et al.* 2000). Indeed, there is only limited evidence of differences in implantation and placentation between first and later pregnancies (Khong *et al.* 2003; Litwin *et al.* 2010; Prefumo *et al.* 2006).

It is also important to assess the association of birth order with long-term disease risk. Surprisingly, there is only one study assessing the association between type 2 diabetes and birth order (Lammi *et al.* 2007), with limited conclusions as a result of confounders.

6.4. Ovarian stimulation

This was the first study to examine the anthropometric, metabolic, and hormonal outcomes in children conceived with the help of OS_A born at full term. It was also the first study on children conceived with the help of fertility treatment (OS_A or ART) to utilise two control groups of naturally conceived children from fertile and subfertile couples.

OS_A children were shorter than naturally conceived children by an average of 2.5 cm. This height difference was more pronounced in OS_A boys, who were on average, 3 cm shorter than naturally conceived boys. OS_A children also had a lower corrected BMI and showed subtle metabolic differences compared to controls. These findings point towards OS_A children exhibiting a different phenotype compared to naturally conceived children.

As discussed by Savage *et al.* (2012) (Chapter 2), only one study has previously assessed height in OS_A children whom tended to be taller (Makhoul *et al.* 2009). However, the study population consisted of children born of very low birth weight, and almost half of participants were twins (Makhoul *et al.* 2009). Our finding of shorter stature in OS_A children contrasts with the taller stature of ART children (Green *et al.* 2010; Makhoul *et al.* 2009; Miles *et al.* 2005; Miles *et al.* 2007; Pruksananonda 2001; Saunders *et al.* 1996). Indeed, when comparing our findings to those of Miles *et al.* (2005) (who assessed ART children using a similar experimental design), our OS_A subjects were approximately 6 cm shorter than the ART children. This suggests that the height differences between OS_A, ART, and naturally conceived children are of different and complex aetiologies.

6.4.1. OS_A versus ART

As discussed, although ART and OS_A share the process of ovarian stimulation, ART consists of several further steps, including external fertilisation, embryo culture, and embryo selection. Furthermore, the drugs used for ovarian stimulation for ART differ and are at a higher dose than those used for OS_A (van Rumste *et al.* 2008). Since OS_A children are shorter and ART children are taller than naturally conceived controls, there seems to be something inherent in the process of ovarian stimulation that leads to shorter stature.

Therefore, it seems likely that the additional steps in ART may be pivotal in the reversal of the shorter stature to taller stature. Embryo culture is associated with changes in gene imprinting and even different compositions of culture media are associated with changes in birth weights (Dumoulin *et al.* 2010). Thus, this extra step in ART is a possible mechanism

accounting for the taller stature in these children. However, it is important to remember that the embryos selected for transfer in ART are usually the 'best' one or two embryos (Ziebe *et al.* 1997). Selection criteria vary, but the biggest embryo is often selected, contributing to the taller phenotype of ART children.

Given this contrasting height outcome, it is important to try to disentangle the possible triggers and mechanisms responsible for the shorter stature of OS_A children.

6.4.2. Possible triggers and mechanisms

The likely reasons for the differences seen in OS_A children are an effect of the ovarian stimulation process on the epigenetic process of the oocyte/embryo, or a direct drug effect altering the uterine environment, cytokines, or growth factors. Other possible reasons for the observed phenotypic changes in OS_A children may be related to alterations in paternal sperm for the process of intra-uterine insemination, or an effect of maternal stress on the developing embryo.

6.4.2.1. Pre-natal

Fetal programming and epigenetics

OS_A constitutes a major departure from the normal process of gametogenesis, leading to marked changes in the early fetal environment compared to natural conception. It is possible that OS_A leads to changes in the epigenetic process in the oocyte via an increase in endogenous FSH through the actions of clomiphene, and/or due to an effect of exogenous FSH (Savage *et al.* 2012; Chapter 2). However, with the exception of one human study (Sato *et al.* 2007), the evidence of an effect of FSH on oocyte or embryo epigenetics has been extrapolated from animal models assessing the effects of ART (reviewed by van Montfoort *et al.* 2012). Several studies have attempted to elucidate if differences are present in the epigenome of ART children (Savage *et al.* 2011). Overall, these studies yielded conflicting findings of either a minor effect or no effect of ART on the epigenome and related processes such as gene methylation (Gomes *et al.* 2009; Horsthemke & Ludwig 2005; Katari *et al.* 2009; Oliver *et al.* 2011; Puumala *et al.* 2012a; Santos *et al.* 2010a; Sato *et al.* 2007; Tierling *et al.* 2010).

Placental and cord blood samples of naturally conceived infants compared to infants conceived by OS_A and ART were recently assessed for epigenetic changes (Rancourt *et al.* 2012). No differences in methylation levels at imprinting control regions were found, but

subtle differences in *H19* methylation and gene expression of OS_A and ART were detected (Rancourt *et al.* 2012). These changes require further study in order to decipher whether they are reproducible or of any significance in terms of impact on phenotype. Thus, while epigenetics and possible imprinting changes may provide the most logical explanation for our observations, the evidence is circumstantial. Furthermore, epigenetic changes attributed to the process of ovarian stimulation have not yet been correlated with phenotypic changes in offspring.

Effect of ovarian stimulation drugs on the early fetal environment

The observed differences in OS_A children may be a direct effect of clomiphene citrate and/or FSH on the oocyte or developing embryo. The possible triggers and mechanisms have been discussed in detail by Savage *et al.* (2012) (Chapter 2), but a brief summary is provided here.

FSH alters intra-uterine chemokines, cytokines, and growth factors in stimulated oocytes (Boomsma *et al.* 2010), and clomiphene is associated with a reduction in serum levels of IGF-I in women after standard treatment for ovarian stimulation (Fiad *et al.* 1998). However, it is unknown if these changes impact on the developing oocyte or embryo.

Animal studies have demonstrated subtle effects of clomiphene at a cellular level, more specifically, enhancement of cellular apoptotic processes in ovaries, fallopian tubes, villi, and decidual tissues (Chaube *et al.* 2006; Shao *et al.* 2009). Furthermore, clomiphene has many well recognised effects on the intra-uterine environment, including altered embryonic adhesion (Valbuena *et al.* 2001) and endometrial thickness (Nakamura *et al.* 1997), amongst others. With metabolites of clomiphene detectable in maternal serum more than one month after administration (Homburg 2005), it is possible that clomiphene could have effects at a subtle level in the developing oocyte or embryo leading to phenotypic changes. Further research assessing the impact of clomiphene at a cellular level and the possible link to phenotypic changes (if any) is required.

Maternal psychological stress

Maternal psychological stress is understandably higher both prior to, and during pregnancies conceived with help of fertility treatment, when compared to naturally conceived pregnancies (Harata *et al.* 2012; Oddens *et al.* 1999). However, the only studies assessing fertility treatment-related stress were in couples undergoing ART (Oddens *et al.* 1999; Schmidt 2009). While OS_A also constitutes fertility treatment and is no doubt a stressful experience, it is likely to be less stressful than ART, which is a far more invasive treatment.

Many animal and human studies showed that maternal stress can affect the fetus, leading to alterations in offspring phenotype and disease risk (reviewed by Vaughan *et al.* 2011). Evidence suggests that such changes in the maternal environment are associated with changes in placental imprinted genes, including those involved in growth (Vaughan *et al.* 2011). Maternal stress is also associated with alterations in the maternal hypothalamic-pituitary adrenal (HPA) axis, which in turn impacts fetal hormone regulation (Mulder *et al.* 2002). Subsequently, these changes can increase the risk of adverse obstetric and neonatal outcomes (Austin & Leader 2000; Mulder *et al.* 2002), as well as an increased risk of behavioural problems in childhood (Talge *et al.* 2007).

Animal studies elegantly demonstrate that maternal pregnancy stress is associated with reduced growth in offspring (Emack *et al.* 2008). In humans, severe maternal stress, psychological problems, depression, and other mood disorders during naturally conceived pregnancies have also been associated with reduced growth in offspring (Patel *et al.* 2004; Traviss *et al.* 2012). It is therefore possible that severe maternal stress may alter childhood growth, leading to the shorter stature of OS_A children. However, this is not consistent with the higher maternal stress experienced by ART mothers, who go on to have taller children. Nonetheless, it is plausible that maternal stress in OS_A could programme shorter stature in offspring, but that this programming could be reversed in ART by the powerful additional steps of embryo culture and selection. As previously outlined, these additional steps of embryo culture and selection may be very important in the generation of the height differences between OS_A and ART children. This area clearly requires further study.

Male factors – intra-uterine insemination & infertility

The process of intra-uterine-insemination (IUI) may also alter the properties of male semen, consequently affecting offspring phenotype. Over 80% of OS_A mothers in our cohort underwent IUI of father's sperm.

The technical process of IUI varies, but generally involves selection of the most motile sperm, which are removed from semen by the process of sperm washing (Qasim *et al.* 1996). Cytokines in semen are increasingly recognised as important factors in the regulation of implantation (Robertson 2007; Robertson *et al.* 2009), and these cytokines may influence fetal programming (Sjoblom *et al.* 2005). While this work is of interest, it is unknown whether the removal of seminal fluid impacts offspring outcomes, and this area warrants further research in animal models and humans.

Another possibility is that the infertility of fathers in the OS_A group is associated with transmission of altered genes to offspring, and consequent differential phenotypic outcomes. Sperm of infertile males has a higher rate of epigenetic and imprinting defects that can be transmitted to offspring (Dada *et al.* 2012) However, fathers with male factor infertility only comprised 21% of OS_A fathers in our cohort, making this a less likely mechanism for the observed changes in phenotype OS_A children. Nevertheless, the possible role of male factor infertility and its possible impact on offspring outcomes cannot be dismissed, and needs to be considered in future studies of OS_A children.

6.4.2.2. Post-natal

Overall, ART conceived children appear to fare a little better than their naturally conceived peers in terms of social adjustment and general psychological well-being (Wilson *et al.* 2011). We could not identify similar studies on OS_A children, but these outcomes in ART and OS_A children are likely to be similar. However, there is no evidence showing that ART or OS_A conceived children are reared differently to their naturally conceived peers. In particular, there is no evidence of any differences in the post-natal rearing environment that could explain the differences in height, BMI and subtle metabolic differences between OS_A and naturally conceived children.

6.4.2.3. Subfertility

While assessing outcomes in OS_A children, we also conducted another important study comparing anthropometry, metabolism, and hormonal profiles in children of subfertile and fertile parents. There were no significant differences between these two groups, either overall or when comparing sexes separately. This is the first time such a study has been conducted, and although our group of children of subfertile parents was relatively small (n=54), the study had sufficient power to detect differences in main outcomes between children of fertile and those of subfertile parents. As a result, our negative findings are particularly important, as previous studies assessing ART conceived children cautioned that their observations could be, at least partially, attributable to parental subfertility (Savage *et al.* 2011).

Parental subfertility is associated with an increased risk of low birth weight, premature delivery, congenital abnormalities, and imprinting disorders in the offspring (Doornbos *et al.* 2007; Ludwig 2009; Zhu *et al.* 2006). Therefore, it has been increasingly suggested that subfertile parents are inherently different to fertile parents, so that their children may have a

different phenotype (Ludwig 2009; Savage *et al.* 2011). Thus, we expected to find differences in anthropometric, biochemical, or hormonal parameters between children of subfertile and fertile parents, but no significant differences were observed.

Our negative findings are important, and suggest that subfertility is unlikely to be a major contributing factor the different outcomes observed in ART and OS_A children. It is nonetheless necessary to highlight that subfertile, OS_A, and ART parents all share subfertility as a common background characteristic. As a result, while subfertile parents eventually conceive naturally, parents who undergo OS_A or ART do not. Therefore, it is possible that there is something inherently different about OS_A or ART parents that may alter their children's phenotype. However, if such differences exist, they remain unknown and warrant further research.

6.4.3. Conclusion

This is the first study to assess the growth and metabolism in OS_A children born at full term. We also carried out the first study comparing growth and metabolism in children of fertile and subfertile parents. The triggers and mechanisms responsible for the shorter stature, lower corrected BMI, and subtle metabolic differences in OS_A children (compared to naturally conceived children of fertile and subfertile parents) is unclear. These may be related to altered fetal programming, likely triggered by the ovarian stimulation process leading to alterations in imprinted genes, as well as by a direct effect of potent drugs such as clomiphene.

6.4.4. Directions for future research

Even though clomiphene has been used in fertility treatment regimes for decades, it is an under-researched drug, with potentially potent effects outside that of ovarian stimulation. While efforts continue towards more "controlled ovarian stimulation" a recent review entitled "Controlled ovarian stimulation: who's kidding who?" (Macklon 2005) sums up the progress that has yet to be made in this area. Nevertheless, advances continue in the area of fertility treatment, with greater consideration that success is a singleton birth at full term. It is likely that the long-term health of fertility treatment offspring will become an area of greater focus. The vast majority of the ART conceived population are aged less than 30 years, but there is a larger and older population of OS_A conceived adults that have never been studied. The same lack of data applies to adults born of subfertile parents.

The possible involvement of epigenetics or imprinting changes in the observed differences in OS_A offspring is a complex area that required investigation. The crucial roles of process of epigenetics and imprinting at gametogenesis and embryogenesis are becoming increasingly clear, and, as technology advances in the area of epigenetics, further valuable information will be gathered in this field. However, much further work is required before a parallel between epigenetic or imprinting changes at a gene level can be correlated with phenotypic or metabolic changes in offspring. It is crucial that such work continues as it may impact on clinical practise. For example, certain doses or combinations of ovarian stimulation drugs may be associated with a threshold effect on imprinting gene regulation. If such an effect is uncovered, this would lead to changes in fertility drug doses and regimes in clinical practise.

The process of OS_A is complex and varies between patients and centres, with differing drug doses and protocols utilised. Such differences need to be considered when attempting to decipher any particular facet of OS_A as an important trigger leading to changes in OS_A children. Ideally, a multi-centre multinational longitudinal study should be utilised to tackle the many questions surrounding OS_A fertility treatment and the impact on offspring phenotype. Such studies could obtain high quality data on parental factors, treatment protocols, as well as offspring phenotype and health profiles. The next step towards correlating any phenotypic differences with epigenetic or imprinting differences could be tackled. Such research will furnish OS_A offspring, prospective parents, and health practitioners with information on any real risks associated with OS_A conception, either in the short or long term.

Finally, a central register of OS_A treated parents and of children conceived by OS_A needs to be established. The wide availability of drugs such as clomiphene (often prescribed by family doctors and on offer in the internet) makes this a challenging prospect.

6.5. Changes in height – does it matter?

The major findings common to all four studies covered in this project were observed changes in childhood height. These observations in association with parental factors may have implications for long-term health and disease risk. Importantly, in the absence of differences in biological maturity and due to the correction for parental heights, it is likely that the differences in stature will likely persist into adulthood. Furthermore, the observed changes in childhood height are likely to represent real alterations in the programming of growth, which may have additional short- and long-term effects.

6.5.1. Childhood

The height increase of approximately 2 cm observed in children born to mothers and fathers aged more than 30 years and in first-borns may not represent a clinically important height increase for all children. However, this seemingly small height increase may be important to children (and parents) on the shorter range of the height spectrum.

The height decrease of 2.5 cm observed in OS_A children (3 cm in boys) equates to a reduction of almost 0.5 SD in the growth curve. Thus, for a child genetically destined to have a height on the tenth centile by virtue of genetic potential, this 3 cm difference represents a significant reduction in stature. Further, this height reduction is likely to persist or even accentuate at final height attainment. To put a 3 cm difference in context, the height gain obtained from four years of daily growth hormone injections in children with idiopathic short stature is approximately 4 cm (Deodati & Cianfarani 2011).

There is debate regarding the possible social advantages of taller stature in childhood. There is evidence that taller children have a better quality of life (Ambler *et al.* 2012), but according to other studies childhood height has little effect on happiness or social success (Gill 2006; Voss & Sandberg 2004).

6.5.2. Height and disease risk in adulthood

Taller stature in adulthood has been associated with greater economic and social success (Harper 2000; Oreffice & Quintana-Domeque 2010; Wayne & Cooper 1987). However, as higher socio-economic status is associated with taller stature, that factor rather than height itself may be responsible for the economic and social success (Bowles & Gintis 2002).

Although these are relevant issues, of even greater interest is whether changes in height in childhood are predictive of health and disease risk in later life.

Although an area of relatively limited research, it appears that taller stature may be associated with a higher rate of chronic disease, increased morbidity with age, and a consequent shorter lifespan (Samaras & Storms 1992; Samaras *et al.* 2003). It is speculated that the longer lifespan of women may be at least in part, attributable to their relative shorter stature compared to men. In fact, the two correlate quite well as women are on average 8% shorter than men, and live 8% longer (Samaras *et al.* 2003). Animal studies are in line with these findings, as smaller animals within the same species (e.g. dogs) tend to live longer (Li *et al.* 1996; Samaras *et al.* 2003)

Taller stature per se has been found to be a risk factor for adult cancer (De Stavola *et al.* 2000; Gunnell *et al.* 2001; Marchand *et al.* 1991). For example, taller adults have higher rates of breast cancer (van Den Brandt *et al.* 2000), prostate cancer (Engeland *et al.* 2003; Giovannucci *et al.* 1997), and colon cancer (Andersson *et al.* 1997; Giovannucci *et al.* 1996). Attempts to quantify this risk suggest that a height difference greater than 5–6 cm is associated with increased relative cancer risk (Ahlgren *et al.* 2004; The Emerging Risk Factors Collaboration 2012; Tretli 1989; van Den Brandt *et al.* 2000). However, even an increase of 1–2 cm in height has been found to be associated with an increased risk of cancer (Chan *et al.* 1998; Lukanova *et al.* 2001). The latter studies in particular, are important given the height difference of 2–3 cm observed in our studies.

In contrast to cancer risk however, there are reports that cardiovascular disease risk reduces with increasing height (The Emerging Risk Factors Collaboration 2012). Importantly, this finding was present after adjustment for BMI, waist circumference, diabetes, smoking status, lipid profiles, and other confounders (The Emerging Risk Factors Collaboration 2012). However, other reports find similar rates of cardiovascular disease in shorter people (Walker *et al.* 1995). Also, it is contended that the observation of lower cardiovascular disease risk in taller people may be associated with factors other than height, such as dietary differences in taller people of higher socio-economic status (Samaras & Storms 1992). Regardless, in a comprehensive review of the subject, Samaras *et al.* (2003) found that all-cause mortality rates are lower in shorter compared to taller people.

There appears to be some health risks associated with taller stature, which may offset the perceived social advantages. While speculative, it is possible that the changes in childhood

height in association with parental factors assessed in our studies may have far reaching effects in terms of future health and disease risk.

6.6. Strengths and limitations of our studies

Specific strengths and possible limitations of our studies have been discussed in the individual manuscripts. However, it is relevant to outline some of these aspects here.

6.6.1. Strengths

The single greatest strength of our studies was the assessment of a homogenous population of children (and families) of single ethnicity and higher socio-economic status. These factors are major confounders in all main outcomes assessed in this project, including height (Silventoinen 2003), BMI and body composition (Deaton 2007; Stunkard *et al.* 1986; Thomas *et al.* 1991; Wang & Lobstein 2006), as well as glucose metabolism and lipid profiles (Mahley *et al.* 2001; van den Berg *et al.* 2012). As a result, our homogenous cohort allowed us to obtain a much more reliable assessment of the effects of the parental factors in question on childhood outcomes. Thus, our project contrasts to most previous studies, which have for example assessed populations across all socio-economic groups.

Other strengths included:

- The utilisation of DXA scans to assess body composition, which has important advantages over more crude methods of assessment (Mazess *et al.* 1990; Taylor *et al.* 2000; Wong *et al.* 2002).
- All our statistical models were robust and appropriately accounted for the major confounding factors.
- Heights of all children were corrected for mid parental height. This is crucial as parental height determines over 80% of a child's height variability (Silventoinen 2003).
- Biological maturity of our subjects was assessed using bone age X-rays, which permitted us to rule out advanced (or delayed) development as the cause for the observed height differences.
- Children's BMI were also individual corrected for parental BMI, which is also an important predictor of childhood BMI (Fogelholm *et al.* 1999).

- All children and their parents were clinically assessed by the same person, thus eliminating any potential inter-individual variation in auxological measurements and other data gathering.
- Our strict exclusion criteria meant that no children born at low-birth-weight or SGA were studied, unlike numerous previous studies that failed to exclude these groups. This is important as low-birth-weight and SGA children represent a different phenotype and disease risk profile, potentially clouding the effects of parental factors on childhood outcomes.
- All assessments were carried out by a medical doctor, to maximize the accuracy of pertinent medical background information and of clinical assessment of children and parents.

6.6.2. Limitations

There were a number of general limitations of our studies:

- Our project involved cross-sectional cohort studies. Although longitudinal studies on a larger population would be ideal, cross-sectional studies such as ours yield meaningful results more rapidly, assisting in the inception and design of future prospective studies.
- Recruitment of study participants was done at random, but it is possible that our cohort might have suffered from volunteer bias. However, we only assessed healthy children of higher socio-economic status, which means that volunteers were unlikely to be seeking free health care assessment. In addition, eligible siblings invariably participate as well, implying that a child from a particular family was not volunteered out of concern for their health.
- The age distribution of fathers and mothers of recruited children were biased towards those aged in their 30s Even if our cohort suffered from volunteer bias, this is unlikely to affect our study outcomes significantly. This is because we have no reason to believe that younger or older parents would preferentially volunteer their children for a study or that a higher proportion of first or later-born children would be preferentially volunteered by their parents as participants. Also, the maternal age, paternal age and birth order distribution of our cohort approximately matched the demographics of the general population of higher socio-economic New Zealand European children and parents.

- Since our cohort consisted of a homogenous cohort of a single ethnicity and of higher socio-economic status, our results may not be directly applicable to the general population. However, as this homogeneity also allowed us to better assess the true effects of particular parental factors, our findings are most likely applicable to the wider community.
- We also had no information on the epigenetic profile or imprinting genes of the children studied or their families. Thus, it was not possible to correlate our observed changes in phenotype with potential genetic alterations.

6.7. Final remarks

We are witnessing perhaps one of the most dramatic demographic changes since the post-war baby boom. Maternal and paternal ages at childbirth are increasing at a dramatic rate, whereby almost half of the children nowadays are born to mothers aged over 30 years and fathers aged over 35 years. This upward shift in reproductive behaviour is pushing prospective parents into attempting to have children at an age when their fertility is in decline. Consequently, the uptake of fertility treatment is increasing, whereby 1 in 20 children are conceived with the help of ovarian stimulation fertility treatment alone, and this is notwithstanding the additional 1 to 3% of children conceived by ART. Couples are also having just one or two children, resulting in a dramatic increase in the proportion of first-borns in the population. This decrease in family size is a choice for some parents, but is a consequence of their increased age and declining fertility for many.

These changes in parental behaviours are inter-dependent and synergistic, and are leading an evolving demographic transition towards older age at parenthood, smaller families, and an exponential increase in the demand for fertility treatment that shows no signs of abating. The possible short- and long-term consequences of these changes in parental factors to offspring phenotype and metabolism are largely unknown and under-researched. We have uncovered that maternal and paternal ages at childbirth, birth order, and conception with the help of fertility medications impact on childhood phenotype and metabolism. These changes in parental factors are associated with a height change in childhood ranging from 1–3 cm, as well as significant alterations in childhood BMI and body composition. Furthermore, we have found that children's metabolism and hormonal profiles are also affected by changes in these clearly influential parental factors.

Offspring phenotype and metabolism are determined by the influences of genetics and the environment of rearing. Where possible, we have adjusted for heritable genetic parameters and assessed a cohort who was likely to have a similar post-natal rearing environment. This enabled us to better assess the effect of each parental factor examined in our studies.

The many changes that take place in maternal and paternal genes and physiology with increasing age make it difficult to decipher the exact triggers and mechanisms responsible for the observed changes in the offspring. Similarly, the physiological, hormonal, and other changes that take place with differing birth order are complex, and are likely to impact on the developing fetus in several different ways. OS_A has perhaps the most complex and disruptive influence on maternal oogenesis, hormonal levels, uterine environment, genes, amongst other effects. This is notwithstanding the possible alterations in paternal sperm associated with OS_A, either as part of sperm preparation for *in utero* insemination or due to the inherent subfertility of the prospective father.

Clearly, maternal and paternal ages, birth order, and OS_A are intrinsically associated with changes in the vulnerable early environment of gametogenesis or embryogenesis, or both. The vulnerability of the developing fetus to alterations in their early environment is becoming more apparent, with increasing evidence that offspring phenotype and metabolism are subject to programming *in utero*.

The implications of our findings for the long-term health and disease risk for these children remains largely unknown. While assessment of the impact of maternal age at childbirth on offspring outcomes and disease risk is the subject of some research, data on the short- and long-term effects of paternal age at childbirth is limited. The possible impacts of birth order (and in particular of being first-born) also require further study.

ART has been the subject of intense research, but it is unclear why OS_A (a common fertility treatment) has been largely neglected, with no data on its long-term effects on offspring health or disease risk. This is particularly surprising given that OS_A has been commonly utilised for more than four decades. Although the use of ART is increasing, it is likely that OS_A will continue to be widely used as a fertility treatment, particularly as funding for expensive ART treatment becomes unfeasible for already over-stretched health budgets.

Further knowledge on the possible influences of these parental factors on childhood and adult phenotype and disease risk will furnish individuals, parents, and health care professionals

with much needed information. Current practises in the USA attempting to increase public awareness about the real decline in fertility associated with increasing maternal age should be emulated and expanded. Public and possibly even school-based education should inform of the risks of chromosomal and other disorders accompanying increasing maternal age, as well as the small, but real risk of offspring gene defects with increasing paternal age. The mistaken belief that fertility treatment slows the biological clock also needs to be remedied, and replaced with knowledge of the very real decline in fertility that accompanies increasing age.

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