

The ageing ovary and uterus: new biological insights

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BACKGROUND: Advanced maternal age is associated with reduced fertility and adverse pregnancy outcomes. This review details recent developments in our understanding of the biology and mechanisms underlying reproductive ageing in women and the implications for fertility and pregnancy.

METHODS: Sociological online libraries (IBSS, SocINDEX), PubMed and Google Scholar were searched for relevant demographic, epidemiological, clinical and biological studies, using key words and hierarchical MeSH terms. From this, we identified and focused on key topics where it was judged that there had been clinically relevant advances in the understanding of ovarian and uterine ageing with implications for improved diagnostics and novel interventions.

RESULTS: Mapping of the ovarian reserve, follicular dynamics and associated biomarkers, across the reproductive lifespan has recently been performed. This now allows an assessment of the effects of environmental, lifestyle and prenatal exposures on follicular dynamics and the identification of their impact during periods of germ cell vulnerability and may also facilitate early identification of individuals with shorter reproductive lifespans. If women choose to time their family based on their ovarian reserve this would redefine the meaning of family planning. Despite recent reports of the potential existence of stem cells which may be used to restore the primordial follicle and thereby the oocyte pool, therapeutic interventions in female reproductive ageing at present remain limited. Maternal ageing has detrimental effects on decidual and placental development, which may be related to repeated exposure to sex steroids and underlie the association of ageing with adverse perinatal outcomes.

CONCLUSIONS: Ageing has incontrovertible detrimental effects on the ovary and the uterus. Our enhanced understanding of ovarian ageing will facilitate early identification of individuals at greatest risk, and novel therapeutic interventions. Changes in both ovary and

uterus are in addition to age-related co-morbidities, which together have synergistic effects on reducing the probability of a successful pregnancy outcome.

Key words: ageing / fertility / infertility / ovarian reserve / anti-Müllerian hormone

Introduction

World and female reproductive demographics are changing dramatically. Within the current demographic transition, populations are moving from an initial state of high mortality and high fertility to a state of low mortality and low fertility. As recently reviewed, these changes have caused dramatic changes in the global population size, the rate of population growth, the working-age share and the age distribution (Bloom, 2011; Lee, 2011). Combinations of medical advances, public health initiatives and social changes have been responsible for this transformation. As a consequence of this, globally, adolescents and young adults aged 15–24 years currently outnumber those aged 60 years and above by 54% but the size of these two groups will equalize by 2025, after which those over age 60 years will outnumber adolescents and young adults (Bloom, 2011). For the foreseeable future we will therefore see a large number of women capable of reproduction, but with competing demands as educational attainment increases and their fiscal contribution to an ageing population is required. These factors underlie the progressive increase in age at childbirth.

Changes in fertility with female age have been widely described in historical populations where contraception was not practiced (Schmidt et al., 2012). Although the level of fertility varies, the age patterns of decline are quite similar; the fall is not large until after age 35 years (Olsen, 1990; van Balen et al., 1997; Dunson et al., 2002). A model that imposed a common age pattern but allowed the level of marital fertility to differ among populations demonstrated that compared with that of women age 20–24 years, fertility is reduced on average by 6% for women 25–29 years, by 14% for those 30–34 years and by 31% for women 35–39 years, with a much greater decline thereafter (Menken et al., 1986). These changes in natural fertility are mirrored by IVF success rates, which, while showing some variance by country, show an initial increase in success rates through the late teens to early 20s and then a general decline from the early 30s (Nelson and Lawlor, 2011; de Mouzon et al., 2012).

At a population level postponement of parenthood and an increased maternal age will have a relatively modest change in the mean number of children but a large increase in the proportion of couples suffering from infertility and seeking assisted reproduction techniques (ART) and a large increase in pregnancies in older mothers. In addition to the well-established age-related increase in the risk of miscarriage (Nybo Andersen et al., 2000; Cleary-Goldman et al., 2005), almost all other adverse fetal and maternal outcomes are also increased with advancing maternal age. Rates of placental abruption, placenta praevia, malpresentation, low-birthweight, large for gestational age, post-partum haemorrhage and preterm and post-term delivery are ~2-fold higher in older mothers (Cnattingius et al., 1992; Aldous and Edmonson, 1993; Cleary-Goldman et al., 2005; Hoffman et al., 2007). The prevalence of pre-existing maternal

medical conditions, including hypertension, obesity and diabetes also increases with age (Health, 2004), as do pregnancy-related maternal complications, such as pre-eclampsia and gestational diabetes (Solomon et al., 1997; Duckitt and Harrington, 2005). Consequently one quarter of pregnant women aged 45 years or over will have a chronic medical disease, with hypertension affecting 25% of 45–54-year-old women (Department of Health, 2004). Obesity affects ~25% of women ages 35–54 years, with a further 33% overweight. Collectively this has resulted in many more women entering pregnancy with pre-existing morbidity or at increased risk of developing novel disease in response to the physiological demands of pregnancy. The clinical impact of this is substantial, as the risk of pregnancy-induced hypertension doubles with obesity and triples with morbid obesity ($\geq 35 \text{ kg/m}^2$), while the risk of pre-eclampsia increases 2-fold with each $5\text{--}7 \text{ kg/m}^2$ increase in prepregnancy BMI (O'Brien et al., 2003b). The prevalence of gestational diabetes is also likely to increase, partly through its association with obesity (Chu et al., 2007) and also because of a redefining of the diagnostic criteria (International Association of Diabetes and Pregnancy Study Group, 2010), the latter alone increasing the prevalence almost 3-fold. Even using historical diagnostic criteria, in a cohort of healthy women aged over 50 years receiving oocyte donation, the risk of gestational diabetes was 20%, increasing to 40% in those over 55 years (Paulson et al., 2002).

These medical co-morbidities can also all influence fetal health and are likely to compound the effect of age on the risk of pregnancy in an older mother. The extreme consequence of comorbidity or pregnancy-specific disorders is maternal mortality, which at present has continued to decline since systematic records began in 1952. The most recent triennial report from the UK reported 11.39 [95% confidence interval (CI) 10.09–12.86] deaths per 100 000 maternities from indirect and direct causes (Cantwell et al., 2011). Indirect deaths, primarily related to underlying medical or psychiatric causes, have however become more frequent with an incidence of 6.72 (95% CI 5.74–7.87) per 100 000 maternities when compared with direct deaths which had declined to 4.67 (95% CI 3.86–5.64) per 100 000 maternities. The impact of age is also striking, as overall the maternal death rate in the UK in women over 40 years is now three times higher than that in women aged <25 years (Figure 1). The objective of the present review is therefore to provide a discussion of recent findings in the biology and mechanisms underlying ovarian and uterine ageing in women, and their implications for fertility with particular focus on the establishment and decline in the finite pool of oocytes, and novel approaches questioning this concept.

Methods

Multiple strategies were used to identify relevant demographic, epidemiological, clinical and biological studies relevant to the broad topic of female

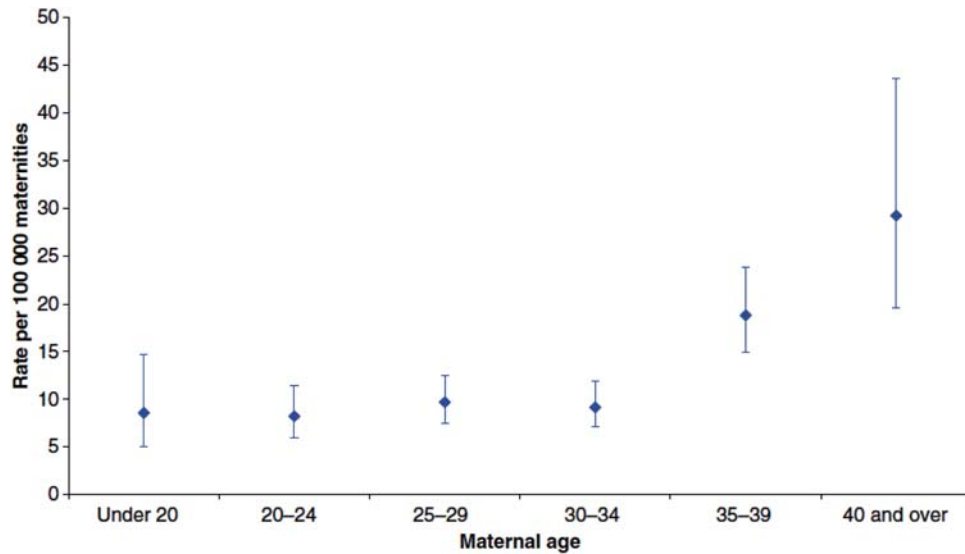


Figure 1 Maternal mortality rates by age group (years); UK: 2006–2008. Reproduced with permission from Cantwell *et al.* (2011).

reproductive ageing, without a date limit. We searched in sociological online libraries (IBSS, SocINDEX), PubMed and Google Scholar using the following key words and hierarchical MeSH terms: fertility, infertility, chromosome aberrations, reproduction, pregnancy, pregnancy complications, placenta, labour obstetric, assisted conception, maternal age, ovary, climacteric, stem cell. Additional journal articles were identified from the bibliography of studies included as well as textbooks and hand searches of other source materials including conference proceedings. Articles written in a language other than English without an available English translation were excluded from our review. From this, we identified and focused on key topics (listed in Table of Contents) where it was judged that there had been clinically relevant advances in the understanding of ovarian and uterine ageing with implications for improved diagnostics and novel interventions.

Ovarian ageing

Ovarian ageing, meaning the progressive loss of the primordial follicle pool, is strikingly different to the changes that occur in the other major organs in that the major functions of the ovary cease little more than half way through a woman's lifespan. This leads to a very long post-reproductive period in humans, and while a post-reproductive period of life is shared by a wide range of animals it appears to be only in women that it makes up such a large proportion of the overall lifespan (Hawkes *et al.*, 1998). In humans, ovarian failure at menopause has been causally associated with increased risks for the development of a long list of significant health complications, including osteoporosis, cardiovascular disease, recurrent depression and cognitive dysfunction (Prior, 1998; Buckler, 2005; Frey *et al.*, 2008). In addition, other less physically debilitating problems, such as heat intolerance and hot flushes, also negatively impact on the quality of life in peri- and post-menopausal women (Santoro, 2008). While the term premature ovarian failure has been historically widely used, it is increasingly replaced by 'primary (or premature) ovarian insufficiency' (POI; Welt, 2008; Nelson, 2009) to reflect the range of clinical

presentations and aetiologies associated with this condition. A full review of this is outwith the scope of this review, and a European Society of Human Reproduction and Embryology guideline on this important subject is currently being developed.

The evolutionary advantage of a long post-menopausal life may be related to the very long period of dependence of the human infant and child on its mother, and indeed is not truly post-reproductive as it benefits the family and offspring. There remains debate as to whether post-menopausal longevity should be seen as an adaptive trait and indeed the longevity of grandmothers may be associated with fewer grandchildren (Madrigal and Melendez-Obando, 2008; Hawkes and Smith, 2009). It also appears that female fertility carries a cost in terms of lifespan, separate from any obstetric risks, which is linked to the 'disposable soma' theory, which proposes a trade-off between longevity and investment in reproduction. Genealogical analyses where apparently accurate and complete family data are available, suggest that age at first childbirth was lowest in women who died at a younger age and highest for women who died at the oldest ages. For older women, longevity was negatively correlated with the number of children and positively correlated with the age at first childbirth (Westendorp and Kirkwood, 1998).

Female reproductive potential differs qualitatively from that of men, in that our current understanding holds that the complete complement of primordial follicles is formed during fetal life (i.e. the true ovarian reserve) and that this is then progressively depleted until reproductive senescence, i.e. the menopause. This has been challenged recently, as discussed more fully below, but the concept of a finite pool of follicles remains a cornerstone of current understanding of ovarian function.

Establishment of the primordial follicle pool

Ovarian development begins with colonization of the genital ridge from Week 5 of gestation onwards, and this is followed shortly

afterwards by sex determination (Witschi, 1948). The primordial germ cells continue to proliferate for many weeks but some start to enter meiosis from 11 weeks gestation (Bendsen et al., 2006; Le Bouffant et al., 2010). In the mouse, the onset of meiosis occurs in a synchronized wave along the axis of the gonad from embryonic Day E13 (Bullejos and Koopman, 2004). In contrast, in the human fetal ovary the onset of meiosis occurs over an extended period of time such that when primordial follicles start to occur from ~18 weeks gestation other germ cells still have a primordial phenotype and are still proliferating (Fulton et al., 2005). The human ovary thus contains a full range of developing germ cells arranged with the less differentiated cells undergoing mitosis around the edge of the ovary, with progressive stages of differentiation entry into meiosis and primordial follicle formation towards the centre of the organ (Anderson et al., 2007). Fetal life is therefore a critical time for establishing female reproductive potential and key factors involved in these processes are increasingly being identified in both mouse and human studies. These include growth factors involved in germ cell proliferation and survival (Coutts et al., 2008; Childs et al., 2010), and a number of germ cell-specific transcription factors that are essential for primordial follicle formation (Bayne et al., 2004; Pangas and Rajkovic, 2006). Meiosis is initiated by retinoic acid in the mouse, acting through its downstream target gene STRA8. The retinoic acid metabolizing enzyme CYP21B protects germ cells in the testes (Griswold et al., 2011). This model

has been clearly elucidated in the mouse, and while the key aspects of this mechanism are likely to be conserved in the human there are also some differences, exemplified by the high level of CYP21B expression in the fetal ovary as well as the testis (Le Bouffant et al., 2010; Childs et al., 2011). There are few studies describing human ovarian development in later gestation: it is believed that primordial follicle formation is essentially complete before birth with any remaining oogonia (not within primordial follicles) being removed in the first years of life (Byskov et al., 2011). The limited data available, however, contribute to our understanding of the changes in primordial follicle number across the female lifespan (Wallace and Kelsey, 2010; Figure 2).

Fetal life is a period of germ cell vulnerability

Understanding development of the human ovary provides an opportunity for greater understanding of the regulation of adult ovarian lifespan. This is exemplified by the demonstration of expression of the transcription factor FOXL2 in germ cells of the developing human ovary from soon after sex determination (Duffin et al., 2009). Mutations in FOXL2 are a cause of syndromic POI, associated with blepharophimosis/ptosis/epicanthus inversus syndrome (Crisponi et al.,

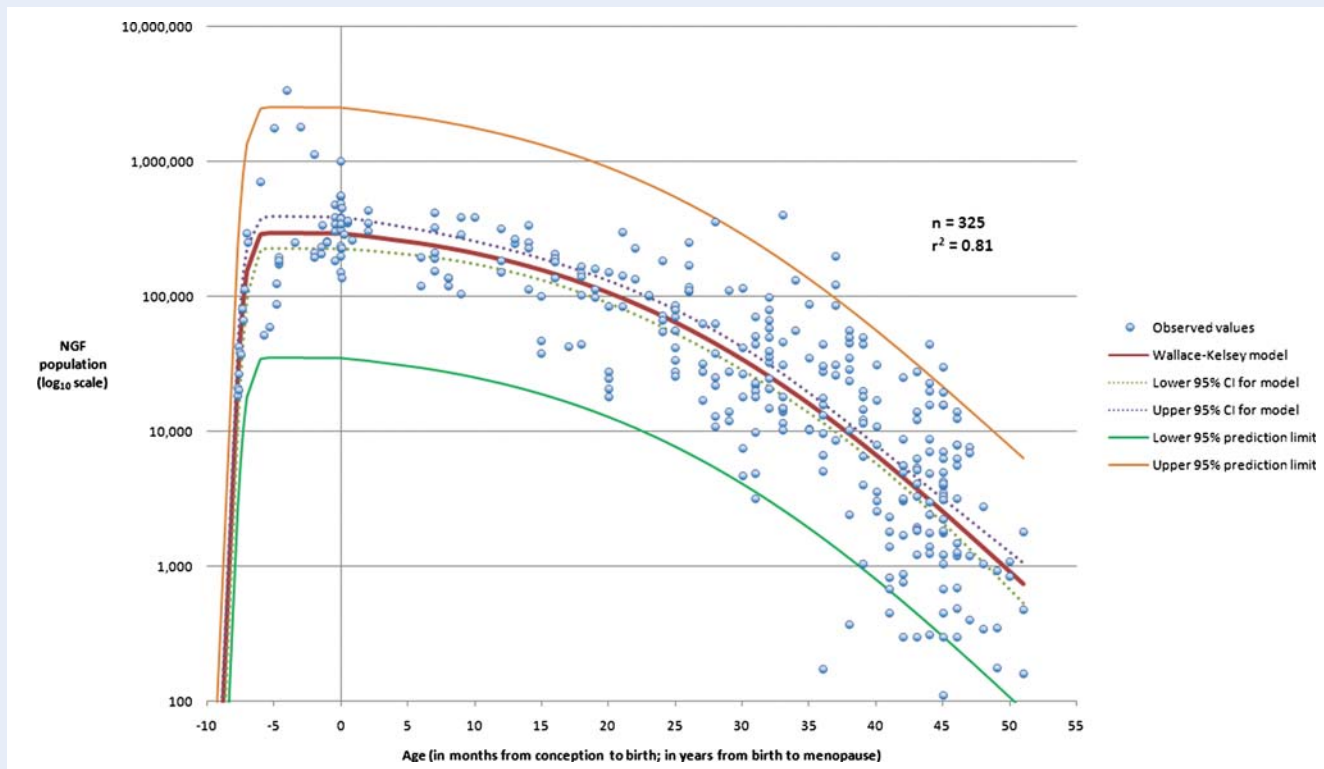


Figure 2 Decline in primordial follicle pool with increasing age. The best model for the establishment of the NGF population after conception, and the subsequent decline until age at menopause as described by an asymmetric double Gaussian cumulative model with parameters = 5.56 (95% CI 5.38–5.74), =25.6 (95% CI 24.9–26.4), =52.7 (95% CI 51.1–54.2), =0.074 (95% CI 0.062–0.085) and =24.5 (95% CI 20.4–28.6). The figure shows the dataset ($n = 325$), the model, the 95% prediction limits of the model and the 95% CI for the model. The horizontal axis denotes age in months up to birth at age zero, and age in years from birth to 51 years. Reproduced with permission from Wallace and Kelsey (2010).

2001). Recently mutations in the homeobox gene *NOBOX* have been identified in a relatively large (6.2%) proportion of women with POI (Bouilly *et al.*, 2011). *NOBOX* is one of several genes identified to be expressed specifically in the oocyte and is critical for primordial follicle formation and subsequent activation (Rajkovic *et al.*, 2004). Relationships between these master regulators have been teased out using mouse knockout models (Pangas and Rajkovic, 2006; Matzuk and Lamb, 2008), but the growing body of relevant human data described above confirms that these key factors and their interrelationships are largely conserved.

The processes of ovarian development are potentially susceptible to external as well as internal influences. The influence of maternal smoking on the son's subsequent reproductive function has been recognized for some years (Vine *et al.*, 1994; Ramlau-Hansen *et al.*, 2007), and comparable female data are now emerging. In female fetuses, smoking may have a direct toxic effect on the primordial follicle, leading to premature exhaustion of the follicular germ pool. In animal models histological analysis of ovarian tissue from the exposed offspring mice demonstrates a markedly reduced number of primordial follicles (Vahakangas *et al.*, 1985; Matikainen *et al.*, 2002), with the combination of prepregnancy and lactational exposure to polycyclic hydrocarbons associated with a 70% reduction in primordial follicle number (Jurisicova *et al.*, 2007). In humans maternal smoking has been associated with a reduction in human fetal ovarian germ and somatic cells (Lutterodt *et al.*, 2009; Mamsen *et al.*, 2010). Epidemiological studies examining the impact of maternal smoking on ovarian reserve of the offspring are consistent with a modest but significant decrease in adult fecundability following exposure to cigarette smoke *in utero* [adjusted fecundability odds ratio 0.96 (95% CI: 0.93, 0.99); Ye *et al.*, 2010].

Exposure to environmental chemicals may also be of relevance. Bisphenol A (BPA) is a ubiquitous chemical found in plastics which can disrupt prophase of meiosis I (Susiarjo *et al.*, 2007). The molecular basis for this has been elucidated in the nematode *Caenorhabditis elegans* showing that BPA disrupts the double-strand break repair processes (Allard and Colaiacovo, 2010). Intriguingly the genes involved in these repair processes are estrogen regulated, and BPA in this model has anti-estrogenic activity resulting in down-regulation of double-strand break repair gene expression in the germ line. Oocytes of the baboon fetal ovary are known to express estrogen receptor beta (Bocca *et al.*, 2008) and expression in human fetal germ cells has recently been confirmed (Fowler *et al.*, 2011). Estrogen depletion treatment using an aromatase inhibitor during primordial follicle formation in the baboon substantially reduced the number of primordial follicles that formed, with estrogen deprivation preventing breakdown of the germ cell nests that precede primordial follicle formation (Pepe *et al.*, 2006). In the bovine fetal ovary estrogen production declines dramatically at follicle formation, and also regulates follicle growth activation (Yang and Fortune, 2008).

Androgen exposure during fetal life may also have a long-term impact on ovarian function. Intrauterine androgen treatment of sheep and non-human primates results in life-long ovarian dysfunction and a metabolic phenotype with considerable similarity to polycystic ovary syndrome (PCOS; Abbott *et al.*, 2005). The relevance of this to the aetiology of PCOS is debated (Franks and Berga, 2012) but these models are of value for the investigation of this condition. Overall, these results suggest that the local steroid environment may

be important in both follicle formation and chromatin integrity during fetal life.

In summary, emerging data demonstrate that genetic mutations in key genes and external factors, such as smoking and environmental chemicals, impact on the establishment of the primordial follicle pool during fetal life, with implications for subsequent reproductive function in adult women.

The regulation of initiation of follicle growth

Post-natal ovarian function is characterized by continuous follicle activation with subsequent follicle growth through the pre-antral and antral stages until follicle depletion at the menopause. Thus, the ability to regulate therapeutically this key aspect of ovarian function would have far-reaching consequences. The antral stages are clinically detectable because of their well-established hormonal products (the inhibins and estradiol) and they can also be assessed by ultrasound but our understanding of the early stages of human folliculogenesis is limited. Growth to early antral stages occurs before, as well as after, puberty (Peters *et al.*, 1978) although later antral stages and of course ovulation do not occur until after maturation of the hypothalamo–pituitary axis at puberty with consequent increases in gonadotrophin secretion. The key issue for determination of reproductive lifespan is therefore the regulation of activation of primordial follicle growth. This appears to be a result of a balance between growth activating and inhibitory factors (Figure 3). Both groups of factors include those produced by the oocyte itself and by the surrounding somatic cells. These have been reviewed elsewhere (Zuccotti *et al.*, 2011) but a clear overall understanding of their interaction remains elusive

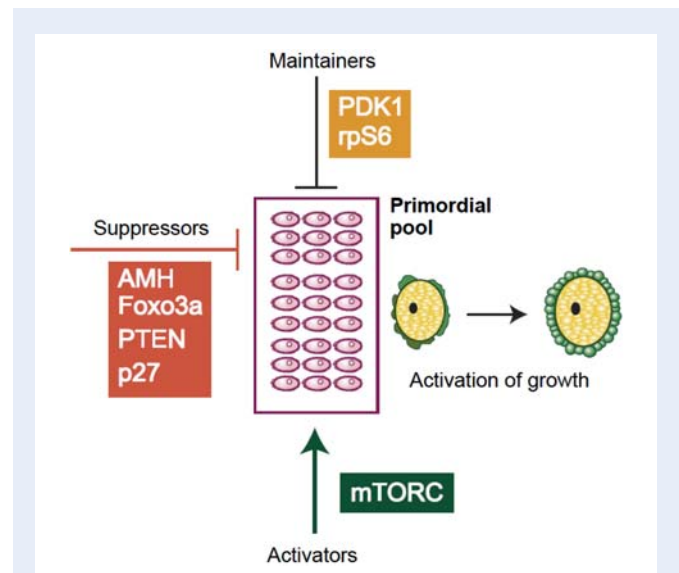


Figure 3 Mechanisms underlying activation of quiescent follicles. A summary of some of the components of the PI3K pathway that have been implicated in maintaining health of the primordial pool (black bar) and either suppressing (orange bar) or activating (green arrow) the initiation of growth. These details have been gathered through knockout mouse models (see Reddy *et al.*, 2010 for review).

at present. A central pathway, however, is the phosphatidylinositol-3'-kinase (PI3K-AKT) signalling pathway within the oocyte. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) acts as a negative regulator of this pathway and suppresses initiation of follicle development (Reddy et al., 2008). The transcription factor FOXO3 is a downstream effector of this pathway and acts to inhibit follicle recruitment (Castrillon et al., 2003). An additional component of this pathway (S6K1-RPS6) is dependent on the mammalian target of rapamycin complex I (mTORC1), a serine/threonine kinase that regulates cell growth and proliferation in response to growth factors and nutrients and also regulates primordial follicle activation (Reddy et al., 2009). Oocyte PTEN therefore suppresses, whereas mTORC promotes, follicle activation.

The role of the mTOR pathway is intriguing as it is also involved in oogenesis in *Drosophila melanogaster*. Rapamycin is an inhibitor of the TOR pathway and results in midstage loss of oogenesis in *Drosophila*, similar to the effect of nutrient deprivation (Thomson et al., 2010). Treatment of early human growing follicles with rapamycin also results in oocyte loss (McLaughlin et al., 2011), postulated to result from destruction of the oocyte by adjacent somatic cells, as is believed to be the case in *Drosophila* (Thomson and Johnson, 2010).

Oocyte quality and age

A key aspect of reproductive ageing is the decrease in oocyte quality as well as quantity. The impact of this is most strikingly demonstrated by the restoration of pregnancy rates to those seen in young women when older women use egg donation in IVF (Sauer et al., 1992; Templeton et al., 1996; Nelson and Lawlor, 2011). Successful conception is also more likely to result in miscarriage, the rate of which increases dramatically with increasing age (Nybo Andersen et al., 2000; Schmidt et al., 2012). This is largely associated with chromosomal aneuploidy in the embryo (the incidence of Trisomy 21 at age 25 years is 1 in 1500, and 1 in 16 at age 40 years), but it is likely that other biochemical processes in the oocyte may be relevant. One aspect of this may be changes in mitochondrial function with age (Bentov et al., 2011; Kujjo and Perez, 2011). All mitochondria are maternal in origin, and thus can only arise from mitochondria associated with the primordial germ cell and will remain with that cell throughout oogenesis. Ageing is associated with an increase in mitochondrial aggregation in oocytes and a reduction in cytoplasmic ATP concentrations in metaphase 2 oocytes (Tarin et al., 2001). It has been postulated that inheritance of dysfunctional aged maternal mitochondria could increase the risk of chromosomal abnormalities and indeed a range of metabolic disorders, and may account for the finding that advanced grandmaternal age is associated with an increase risk of trisomy 21 (Aagesen et al., 1984; Bentov et al., 2011).

A recent report has provided a striking demonstration that calorie restriction, known to have a range of beneficial non-reproductive effects in non-human primates (Colman et al., 2009), may result in a reduction in age-associated oocyte aneuploidy (Selesniemi et al., 2011). Adult mice that were maintained with a 40% calorie restriction showed a similar aneuploidy rate in metaphase II oocytes to that in oocytes from younger animals, as well as a restoration of oocyte yield following gonadotrophin stimulation to that of younger animals. Intriguingly, the number of non-atretic follicles per ovary was also increased by calorie restriction compared with age-matched animals,

and this applied to primordial, primary and pre-antral follicles, although in all cases the number of follicles was lower than in young animals. Calorie restriction prevented spindle and chromosome alignment defects in oocytes, and also prevented age-associated mitochondrial aggregation (Selesniemi et al., 2011). While a 40% reduction is a severe calorie restriction, the results are striking and the mechanisms involved require further elaboration. In the converse situation oocytes of overfed animals also show mitochondrial abnormalities with a reduced oocyte mitochondrial membrane potential (Wu et al., 2010) and increased cumulus and granulosa cell apoptosis. Expression of the endoplasmic reticulum stress gene ATF4 was increased in mice fed on a high-fat diet, and was also increased in human granulosa cells from obese women (Wu et al., 2010). It may therefore be that biochemical changes during oocyte ageing have similarities to those caused by metabolic abnormalities, and the importance of the mTOR nutrient sensing pathway in the follicle has been mentioned above. This indicates that the adverse effects of ageing and of metabolic disturbance, for example as caused by obesity [itself associated with increased risk of miscarriage (Boots and Stephenson, 2011)], will be particularly detrimental in combination, with clear clinical implications. Additionally, the effects of maternal peri-conceptual nutrition on the subsequent health of the offspring are increasingly recognized (Fleming et al., 2012) but outwith the scope of this review.

Genetic and lifestyle determinants of reproductive ageing

Unlike the marked secular trends in age of menarche, the age of menopause is very similar both geographically and over time. This implies a strong genetic component (estimated as contributing to >50% of interindividual variability), which is reinforced by the association between ages of menopause of women and their daughters as well as in twin studies (Torgerson et al., 1997b; Sneider et al., 1998; de Bruin et al., 2001; van Asselt et al., 2004; Murabito et al., 2005). Recently large-scale genome wide association (GWA) studies have identified novel genetic loci associated with age at menopause (He et al., 2009; Stolk et al., 2009). Single nucleotide polymorphism analysis of 278 genes in 24 000 women from the Nurses Health Study has confirmed some of these associations, highlighting steroid hormone metabolism and biosynthesis pathways, but also identifying the importance of a group of genes associated with POI (including the auto-immune regulator AIRE, FMRI, FOXL2 and the BMP antagonist NOG) with age at natural menopause (He et al., 2010).

The expression of many of these genes identified in GWA studies during fetal ovarian development highlights the importance of that period in determining reproductive lifespan and the value of understanding the mechanisms involved in the action of single-gene defects causing POI. A substantial number of these have been identified, in themselves only being identified in very few women with POI (Matzuk and Lamb, 2008). However, the above candidate gene association study (He et al., 2010) demonstrates the importance of variations in these genes as contributors to the normal as opposed to pathologically early menopause. One example of a gene whose variation can cause a range of ovarian phenotypes, from POI to milder ovarian dysfunction, is the FMRI gene, mutations in which cause

fragile X syndrome. This is the most common cause of mental retardation in boys and is caused by an expansion of the number of CGG repeats to more than 200 in the 5' untranslated region of the gene. Premutations (i.e. between 55 and 200 repeats) can also cause POI and the number of repeats can expand over generations. There is debate as to the significance of smaller repeat numbers (Bennett *et al.*, 2010). A recent analysis has suggested that women with a reduced ovarian reserve show a shift towards an increased number of CGG repeats in *FMR1* compared with women with a normal ovarian reserve, potentially indicating subtle effects on ovarian function across the spectrum of genetic variation (Karimov *et al.*, 2011). The relatively high prevalence of mutations in the *NOBOX* gene in women with POI has been mentioned above; it is likely that data will emerge identifying genetic variations in a much larger proportion of women with POI than is currently recognized (Murray *et al.*, 2011) with light consequently shed on the regulation of follicle formation, activation and early, gonadotrophin-independent, follicle growth.

Lifestyle and environmental factors by comparison appear to have only limited effects on age of menopause. A comprehensive review of a very large number of postulated factors calculated that the total contribution to age of menopause may be as low as 3% (Kok *et al.*, 2005). A recent meta-analysis suggests that smoking is associated with a decreased age of menopause of 0.90 years (95% CI 1.58–0.21; Sun *et al.*, 2011). In contrast, alcohol consumption is associated with a later age of menopause (Torgerson *et al.*, 1997a) and it is possible that this impacts on the relationship between ovarian function and cardiovascular risk. The polycyclic aromatic hydrocarbon 7, 12-dimethyl benz[a]anthracene (DMBA) is an environmental carcinogen found in cigarette smoke as well as other products of combustion. Treatment of mouse ovaries with DMBA-induced widespread primordial follicle activation and pre-antral follicle atresia both *in vitro* and *in vivo* (Sobinoff *et al.*, 2011). Widespread follicular activation also results in premature ovarian failure in other mouse models, for example the anti-Müllerian hormone (AMH), PTEN and FOXO3a knockout models (Durlinger *et al.*, 1999; Castrillon *et al.*, 2003; Reddy *et al.*, 2008). Increased activation of primordial follicles has also been postulated to contribute to the POI resulting from chemotherapy owing to loss of inhibition from larger sizes of growing follicles, themselves directly targeted by chemotherapy (Meirow *et al.*, 2010). Biochemical analysis of the effects of DMBA reveals increased AKT1 phosphorylation, mTOR activation and decreased FOXO3a expression (Sobinoff *et al.*, 2011). These results indicate that exposure to this environmental toxin may contribute to ovarian ageing through increased follicular activation acting through pathways that physiologically regulate this process, as described above (Figure 3). As well as providing a potential mechanism for the effect of cigarette smoking on advancing the age at menopause, it is possible that this result is relevant to targeting these pathways therapeutically, either positively or negatively.

Assessment of ovarian reserve and changing follicle dynamics from birth to the menopause

Our understanding of the ovarian reserve and early follicle dynamics has been hindered by the absence of a good biomarker. Estradiol and inhibin A are largely produced by the dominant and pre-ovulatory

follicle and thus largely reflect ovulatory activity. Inhibin B is produced by smaller ovarian follicles and is thus a step closer towards being a useful marker of early follicular activity but its serum concentration still shows substantial changes across the menstrual cycle (Illingworth *et al.*, 1996), reflecting its association with gonadotrophin-dependent follicular activity as a key regulator of FSH secretion. It is, however, becoming increasingly clear that AMH is a useful although imperfect marker of pre- and early antral follicle growth. AMH is produced by the granulosa cells of follicles from the time at which follicle growth is first initiated. AMH expression continues until follicles reach ~8 mm diameter and expression is very low in larger antral follicles (Weenen *et al.*, 2004; Andersen *et al.*, 2010). The relative contribution of the different follicle sizes to AMH concentrations in serum is incompletely described, as this will reflect the balance between the large number of small follicles and the smaller number of larger follicles and probably also the increasing blood supply to follicles as they grow. Early studies demonstrated that AMH concentrations fall with age in women (de Vet *et al.*, 2002) and led to rapid development of the concept that AMH may be useful to predict reproductive lifespan and time to menopause (van Rooij *et al.*, 2002). AMH concentrations are relatively, although not completely, stable during the menstrual cycle (La Marca *et al.*, 2004; van Disseldorp *et al.*, 2010), substantially increasing its utility as a marker as well as also reflecting its origin in smaller follicles. Histological data have confirmed that in humans as well as animal species the number of small growing follicles is related to the number of non-growing follicles (NGFs; Gougeon *et al.*, 1994), supporting the interpretation that AMH concentrations may in fact reflect the primordial follicle pool size; indeed this has been directly demonstrated in both mice and humans (Kevenaar *et al.*, 2006; Hansen *et al.*, 2011). In the Hansen *et al.* (2011) study AMH concentrations showed a significant correlation with the histologically determined primordial follicle pool size. AMH concentrations are not completely gonadotrophin-independent and indeed are suppressed during prolonged pharmacological gonadotrophin suppression (Anderson *et al.*, 2006) and in pregnancy (Nelson *et al.*, 2010) but it appears that there is a physiologically valid and clinically useful relationship between serum AMH concentrations and the ovarian reserve, both 'true' (the number of NGFs) and 'functional' (the number of potentially recruitable small growing follicles).

Data from very large cohorts of infertile women have now defined the decline in AMH during the second half of the reproductive lifespan (Nelson *et al.*, 2011b, c; Seifer *et al.*, 2011), with more limited data for childhood and adolescence (Hagen *et al.*, 2010, 2012). A recent analysis has compiled AMH concentrations throughout life from conception to the menopause in healthy girls and women (Figure 4; Kelsey *et al.*, 2011b). While these data are cross-sectional, this analysis reveals a number of important features which illustrate important and previously incompletely or unidentified aspects of human ovarian function. Specifically, AMH shows a clear rise in the first months of life, supporting the existence of a neonatal minipuberty in girls, similar to that well recognized in boys. This has recently been confirmed directly (Kuiri-Hanninen *et al.*, 2011). AMH concentrations then progressively rise through childhood in girls reaching a peak in the mid-20s. This rise appears to start before puberty and continue beyond with, intriguingly, an inflection during adolescence, thus there appears not to be a clear increase at the time of other endocrinological manifestations of puberty. A decrease in AMH at early

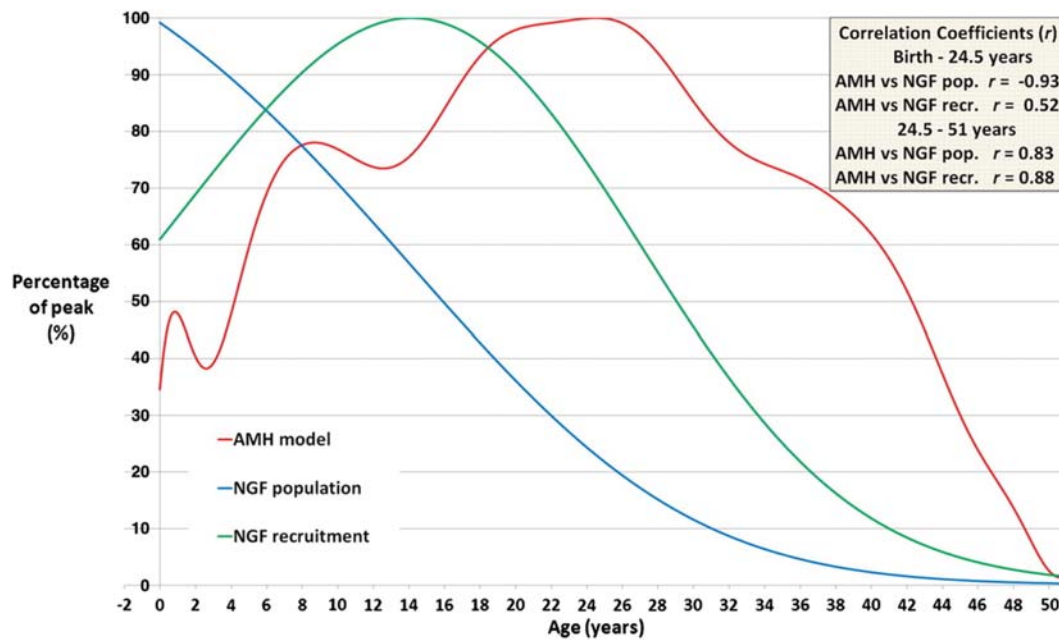


Figure 4 Comparison of serum AMH concentrations with NGF population and with NGF recruitment. The red line is the log-unadjusted validated AMH model (Kelsey et al., 2012), peaking at 24.5 years. The blue line denotes the decline in NGF population (Wallace and Kelsey, 2010), with peak population at 18–22 weeks gestation. The green line denotes the numbers of NGFs recruited towards maturation population (Wallace and Kelsey, 2010), with peak numbers lost at age 14.2 years on average. Each quantity has been normalized so that the peak occurs at 100%. Correlation coefficients (r) are given for AMH concentrations against the other two curves for birth to 24.5 years and for 24.5–51 years. Reproduced with permission from Kelsey et al. (2012).

puberty has recently been demonstrated directly in a longitudinal study (Hagen et al., 2012). These data suggest increasing follicular activity from late childhood right through to some 10 years following menarche, and indicate the existence of a prolonged period of ovarian maturation.

Our knowledge of human ovarian function and dynamics during puberty and adolescence is very limited owing to the lack of availability of relevant histological material and the inability of previous endocrinological analyses to reflect early follicle growth. Relating these changes in AMH to the number of NGFs in the ovary across life (Wallace and Kelsey, 2010) and data derived from that analysis giving the rate of loss of follicles from the non-growing pool illustrate that the rise in AMH during adolescence occurs in the face of a decline in NGF number but increasing NGF recruitment which peaks at an average age of 14 years (Kelsey et al., 2011a). Following this peak and the peak in AMH at age 24 both NGF recruitment and AMH fall in parallel (Figure 4). Disturbances in this relationship may occur in pathological states. Thus, for example girls with type 1 diabetes have higher AMH concentrations in childhood (Codner et al., 2011), which may reflect a larger primordial follicle pool but perhaps is more likely to reflect an increased number of small growing follicles. Conversely, in adult life AMH is reduced in women with type 1 diabetes (Soto et al., 2009), suggesting that there is already depletion of the primordial pool, which would be consistent with their observed increased risk of early age of menopause (Dorman et al., 2001). These data also

illustrate the wide range in AMH concentrations between individual women at any given age. This is perhaps only to be expected given the similarly wide variation in the primordial follicle pool (Faddy et al., 1992; Wallace and Kelsey, 2010) and indeed age of menopause. While menopause occurs on average at age 51 years, it varies from 40 to 60 years, i.e. the reproductive lifespan of some women is 50% longer than others and, recognizing that fertility declines a decade before the menopause (Broekmans et al., 2009), the duration of fertile life in some women will be twice that in others. The ability to identify this in young women through measurement of AMH or other as yet unidentified biomarkers is unsubstantiated at present but is likely to have significant social and medical consequences (Baird and Steiner, 2012).

Many of the above studies have also used measurements of antral follicle count (AFC), with largely similar results to measurement of AMH. The relative merits and demerits of AMH and AFC assessment have been widely debated (Jayaprakasan et al., 2010; Nelson et al., 2011a; Rosen et al., 2012) and, as with AMH, large cross-sectional studies of the decline in AFC with age are now available (Almog et al., 2011; La Marca et al., 2011). Accurate interpretation of the ovarian reserve at any given age is therefore feasible and has been widely utilized for response prediction in ART (Broer et al., 2011a). We anticipate that by combining biomarkers, including age, the most accurate estimate of the underlying primordial follicle number will be achieved.

Predicting the reproductive lifespan

The later stages of ovarian ageing are characterized by increasing menstrual cycle variability followed by an increasing likelihood of missed cycles and ultimately amenorrhoea. The menopausal transition has been extensively reviewed clinically and endocrinologically with a standardized classification proposed as the 'stages of reproductive ageing' (STRAW; Soules *et al.*, 2001). This classification includes five stages prior to the final menstrual period and two stages thereafter, accompanied by a progressive decrease in primordial follicle number (Hansen *et al.*, 2012). Although initially based on menstrual characteristics and follicular phase FSH concentration, the most recent revision incorporates AMH, inhibin B and AFC as additional supportive criteria (Harlow *et al.*, 2012). These studies all highlight the monotropic rise in FSH with ovarian ageing, a phenomenon first recognized in the mid-1970s. The rise in FSH largely reflects a decrease in inhibin B production by small antral follicles in the early follicular phase. A very large body of work has characterized these endocrinological changes in later reproductive life (Hale and Burger, 2009) but the ability of FSH and inhibin B to accurately determine the early stages is limited. The potential value of AMH as a marker of female reproductive ageing is likely to increase, particularly with the development of an international standard and harmonization of the assays (Nelson and La Marca, 2011).

Data are now becoming available from prospective studies on the use of AMH as a predictor of the menopause. In a study of 147 women aged over 40 years over a period of 6 years, initial AMH showed good prediction of not reaching the menopause (Tehrani *et al.*, 2009). This supported previous studies (van Rooij *et al.*, 2004) with the van Rooij study suggesting that inhibin B improved the predictive value of AMH. Comparable results were obtained in a study of 50 women assessed annually over 6 years with final menstrual period determined in all of them (Sowers *et al.*, 2008). Baseline AMH was associated with time to, and age at, the final menstrual period, as was the rate of decline of inhibin B, which was less predictive. It is likely that improvements in the sensitivity of currently available AMH assays may improve the accuracy of AMH assessment in the years preceding the menopause, as currently AMH becomes undetectable ~5 years before the final menstrual period (Sowers *et al.*, 2008). A more recent study analysed AMH changes over a more prolonged period of 11 years in younger women (aged 21–46 years) and found that age-adjusted AMH was highly predictive for the timing of the menopause (Broer *et al.*, 2011b). However, only 48 women had reached the menopause at the time of the second analysis. A larger study has now clearly confirmed that AMH is predictive of time to the menopause, although with wide CIs (Freeman *et al.*, 2012). Importantly, the Freeman study also demonstrates that age and AMH are independent predictors. This indicates that there are mechanisms of ageing (presumably related to the rate of follicle activation) that are independent of the growing follicle pool, in addition to the links between them, clearly demonstrated in detailed histologically based studies (Gougeon *et al.*, 1994).

The clinical implications of women being able to determine their reproductive lifespan by a blood test available to all health care professionals are potentially substantial. Certainly if women choose to time

their family based on their ovarian reserve this would redefine the meaning of family planning.

Avoiding the age-related decline in oocyte quality

Given the inexorable decline in female fertility with age, what steps can be taken to mitigate against it? In essence, two very different therapeutic approaches have been promoted to address this. The first involves assessment of embryo quality in IVF cycles of older women to screen for aneuploidy, the second to try to preserve oocytes at a young age. Both of these approaches aim to circumvent rather than prevent the problem, in the absence of, for example, pharmacological ways of influencing chromosome segregation during meiosis. Alternative approaches, such as the administration of growth hormone and dehydroepiandrosterone, have been proposed in the context of IVF to increase the number of oocytes obtained, especially in older women and 'poor responders'. Evidence as to their efficacy is unclear (Duffy *et al.*, 2010; Sunkara *et al.*, 2012), and this approach is not of relevance to reproductive ageing in general.

Screening for aneuploidy involves the biopsy of one or more blastomeres, with karyotypic analysis historically performed by fluorescence *in situ* hybridization (FISH). Unfortunately RCTs of this approach showed no evidence of a benefit in live birth rates (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007, 2011). Some of the technical aspects of those studies received adverse comment but subsequent RCTs gave similar results and the technology has largely moved on with increasing use of comparative genomic hybridization (CGH) analysis (Harper and Sengupta, 2011). Data using array CGH have recently supported the suggestion that most human aneuploidy arises from premature chromatid separation rather than non-dysjunction (Gabriel *et al.*, 2011), a model first proposed as a key component of human reproductive ageing 20 years ago (Angell, 1991, 1994). Greater understanding of the biology of the cohesins that hold chromatids together is shedding light on this key aspect of oocyte ageing (Lister *et al.*, 2010).

Despite previous adverse experiences of testing embryos at the cleavage stage, testing of blastomeres and polar bodies has been developed in an attempt to reduce the impact of mosaicism (Delhanty *et al.*, 1993; van Echten-Arends *et al.*, 2011). Analysis of the first polar body only allows partial identification of the maternal contribution, with at least as many errors arising during meiosis II as in meiosis I (Fragouli *et al.*, 2011). Failure to analyse the second polar body could lead to the non-detection of 50% of oocyte-derived chromosomal errors. Furthermore, polar body biopsy will not identify post-zygotic errors. Consequently when combined with the issue of mosaicism, the inherent option of being able to sample more tissue at blastocyst biopsy (as many as 5–10 cells) would appear to make this a more logical approach.

From a practical perspective, a blastocyst-based strategy requires sufficient high-quality embryos to reach the blastocyst stage in the first place, thereby limiting the applicability to older women with an already reduced ovarian reserve. Furthermore, vitrification of the biopsied blastocysts while awaiting the report of diagnostic testing is required, however, alternative faster non-array-based diagnostic

approaches are being developed (Treff et al., 2012). Given the lessons learned from results of RCTs on the initial use of FISH in older women, RCTs using array CGH and alternative technologies are eagerly awaited, with initial pilot studies confirming the feasibility of this approach (Geraedts et al., 2011; Magli et al., 2011).

Freezing of oocytes to avoid the decline in fertility related to age alone is essentially a social rather than a medical application. This is in contrast to women with a disease-related low ovarian reserve, for example secondary to chemotherapy. The increasing use of oocyte vitrification has advantages, with improved post-thaw viability, fertilization and clinical pregnancy rates when compared with slow freezing (Smith et al., 2010; Cobo and Diaz, 2011). It is likely that the number of women seeking oocyte storage will increase, particularly if early identification of women at risk of POI or reduced fecundability by screening with AMH is feasible (Steiner et al., 2011; Baird and Steiner, 2012). However, the financial costs and limited success rate likely from the restricted number of oocytes obtained from a single stimulation cycle mean that this approach will appeal and be available to only very few.

Another potential target to avoid age-related decline in fertility is the primordial pool of follicles. Given that the bulk of the human ovarian follicle reserve is made up of primordial follicles, this population is an ideal choice for *in vitro* growth to obtain fertilizable oocytes for potential use in ART and fertility preservation programmes (Telfer et al., 2008; Telfer and McLaughlin, 2011). The capacity of immature mammalian oocytes to develop fully *in vitro* has already been demonstrated in rodents with the birth of pups from *in vitro* grown murine primordial follicles (Eppig and O'Brien, 1996; O'Brien et al., 2003a), however, this has yet to be successfully repeated using immature human follicles. While the definition of a complete *in vitro* system has yet to be achieved, a great deal of basic scientific progress has been made using systems designed to support the partial growth of human follicles, with several developmental milestones accomplished, including follicle activation, follicle differentiation and oocyte maturation using fresh and cryopreserved human tissue (Smitz et al., 2010; Telfer and McLaughlin, 2011). An alternative strategy would be to regulate the rate of initiation of follicle growth, and thus the preservation of the primordial pool *in vivo*. This will inevitably require a much greater understanding of the molecular mechanisms underlying follicular activation (Figure 3) but is potentially a target for pharmacological intervention.

The effects of uterine ageing

In addition to the changes discussed above in oocyte quality, the ability to achieve and maintain a pregnancy will also be dependent on the uterus. This is starkly exemplified by the association of increasing maternal age and stillbirth (Huang et al., 2008; Flenady et al., 2011). This clear association remains even after controlling for co-morbidities and potential confounders, including fetal chromosomal abnormalities, multiple pregnancy, obesity, pre-eclampsia, insulin-dependent diabetes and multiple pregnancy (Fretts et al., 1995). Consequently the risk of stillbirths in mothers over the age of 40 years is approximately twice as high as that of younger mothers, however, the absolute risk would be <10 per 1000 births in most industrialized countries.

The biology underlying this association is largely undefined but potentially relates to impaired decidual and placental development and

embryo interaction with the uterus. By 12 months of age, mice demonstrate a 15% reduction in the number of implantation sites during early pregnancy and a 50% reduction in fertility at term (Holinka et al., 1979). Even when blastocysts were transferred from young donors to older hosts there was greater implantation failure (Talbert and Krohn, 1966; Gosden, 1979). Older mice show an impairment of artificially induced decidual response, which in young rodents closely resembles the normal decidual response during implantation (Shapiro and Talbert, 1974; Holinka and Finch, 1977). Although this impaired decidual response may be in part related to reduced progesterone secretion in older mice (Holinka et al., 1979), a uterine contribution is also likely. Even when progesterone is provided exogenously, the wet weight of the uterus and uterine glycogen, alkaline phosphatase and DNA content are all ~25% lower in older mice (Holinka and Finch, 1977; Finch and Holinka, 1982). Microscopic changes have also been demonstrated in response to various experimental conditions, with unilateral hyperaemia inducing vascular development and growth of the myometrium and stroma in young hamsters (3–5 months) but not older animals (13–15 months) (Sorger and Soderwall, 1981). In response to decidualogenic stimuli, older rats exhibit more lysosomes and fewer luminal microvilli in the epithelial cells (Craig, 1981). It would appear that decidual development is partly impaired by prolonged exposure to hormonal stimulation, as in aged mice those that had undergone ovariectomy at 2 months had a greater decidual response compared with controls, albeit that the controls did not have sham operations (Goodrick and Nelson, 1989). A purely age effect is, however, still present as although young (4 months) and aged (10 months) ovariectomized mice, demonstrated equivalent uterine growth in response to estrogen and progesterone, the decidual response to a standardized stimulus was reduced in the older animals (Holinka and Finch, 1977). In rodents endometrial estrogen and progesterone receptor expression have also been observed to decline with age, with concomitant increases in collagen and fibrosis (Blaha and Leavitt, 1974; Ohta, 1987).

With respect to placental development in older mothers, many studies fail to control for associated maternal characteristics. It does, however, appear that maternal age is positively associated with placental weight, even after adjusting for birthweight, parity, smoking, pre-eclampsia and diabetes (Haavaldsen et al., 2011). Whether this reflects a biological compensatory mechanism for placental dysfunction is unclear. In the small mechanistic studies which have analysed indices of placental structure, healthy older women who had a normal pregnancy outcome exhibited reduced levels of apoptosis and increased levels of trophoblast proliferation compared with younger women (Rahima and Bruce, 1987; Yamada et al., 2001). In rats, placental weight was also increased by 40–70% in the older animals (9–12 months) when compared with the young rats (3–5 months). There was no difference in birthweight, suggesting placental adaptation, with compensatory hypertrophy of the placenta required to maintain fetal growth in the face of less favourable maternal physiology. Collectively these studies suggest that decidualization and placentation may be adversely affected by maternal age, however, given that dysfunctional implantation and placental development underlies almost all adverse obstetric outcomes, high-quality translational studies harnessing the power of appropriate animal models and human basic science and clinical studies on the effect of maternal age are required.

The donor oocyte model provides an invaluable paradigm to assess the role of age-related changes in the uterus on reproductive potential. Case reports of successful pregnancy and subsequent delivery in a 70-year-old recipient of donated oocytes anecdotally demonstrate that the uterus can support pregnancy far beyond the age at which it is normally required to do so. Initial studies from donor oocyte programmes revealed similar rates of implantation, pregnancy, miscarriage and delivery rates among donor oocyte recipients of different ages (Serhal and Craft, 1989; Navot *et al.*, 1991; Sauer *et al.*, 1992). However, it has subsequently become clear that oocyte recipients have an almost 2-fold increased risk of the major perinatal complications of preterm birth and low-birthweight when compared with women using their own oocytes after adjusting for other maternal confounders (Nelson and Lawlor, 2011), in keeping with the above-described impaired decidual and placental development.

The older mother is also more likely to experience intrapartum complications, with a linear relationship between maternal age and the length of labour, the risk of emergency Caesarean delivery and operative delivery (Figure 5; Smith *et al.*, 2008), suggesting that myometrial function is impaired by advanced maternal age. With increasing maternal age there appears to be failure of autophagy within the myometrium, with older women having an increased number of cytoplasmic lipofuscin inclusions in the myometrial smooth muscle cells, and ultrastructural changes including dissociation of myofilaments, mitochondrial destruction and abnormal endoplasmic reticulum structure (Gosden *et al.*, 1978; Drampian *et al.*, 1983). Myometrial functional assessment has demonstrated a negative association of maternal age and spontaneous activity, and also an increased risk of multiphasic contractions which are associated with dysfunctional labour *in vivo* (Smith *et al.*, 2008). The basis for these structural and functional changes in the myometrium is unclear but, intriguingly, later menarche is protective and has been shown to be associated with a reduced risk of operative delivery (Smith, 2009).

These observations raise the possibility that it is the repeated exposure of myometrium and endometrium to the rise and fall of sex steroids across the menstrual cycle that is detrimental (Smith *et al.*, 2008). Historically, prior to the advent of contraception and alternatives to breastfeeding, the number of menstrual cycles would have been substantially less. Women would have conceived in adolescence, then breastfed exclusively and conceived again shortly after stopping breastfeeding, with a minimum number of menstrual cycles. In contrast, assuming menarche at 13 years, age at first birth at 29 years (the current mean age in UK) and a regular menstrual cycle, the uterus will now be exposed to 192 cycles of estrogen and progesterone prior to that first birth. With the use of the combined oral contraceptive pill, the pattern would differ but the repeated number of exposures would be similar. Average steroid exposure would perhaps even be higher, given the pharmacological doses necessary for contraceptive efficacy. We would therefore suggest the hypothesis that repeated and prolonged exposure to sex steroids induces both endometrial and myometrial uterine damage and partly underlies the age-related association with adverse outcomes.

Repeated exposures to hormonal fluctuations are known to increase the risk of disease in other hormone-sensitive tissues, such as the breast. Breast cancer risk is associated with early age of menarche, earlier development of regular ovulatory menstrual cycles, increased age of first birth, nulliparity or fewer children, not breast

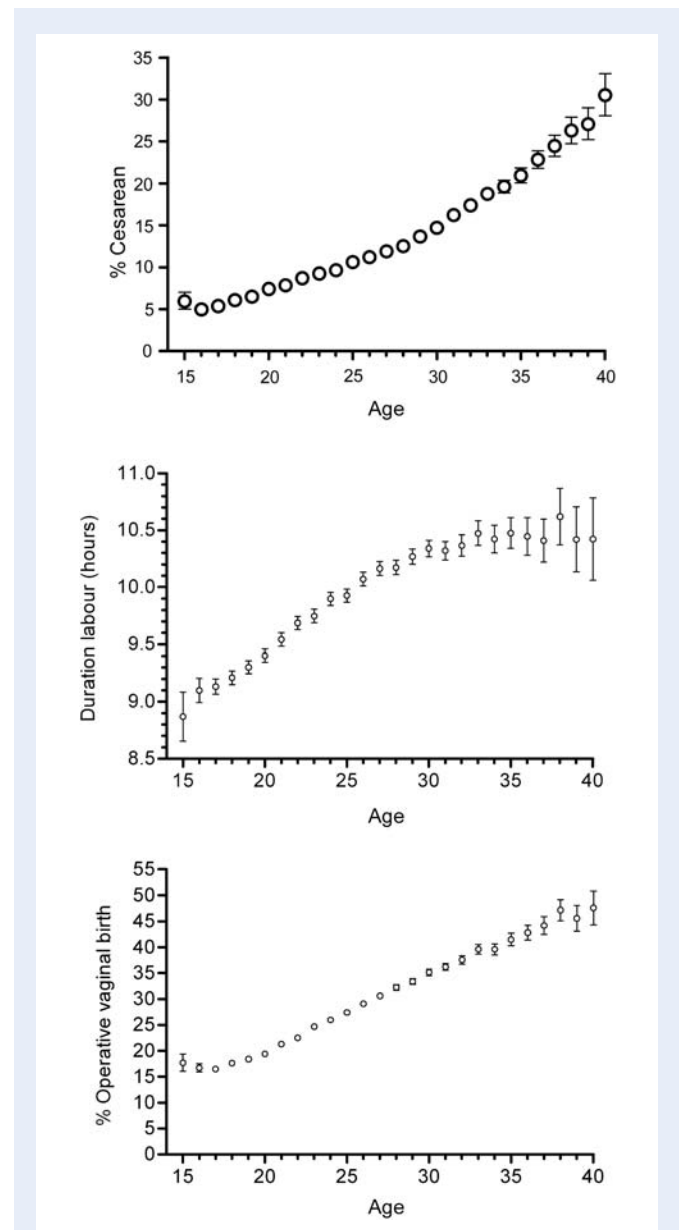


Figure 5 Maternal age and the risk of Caesarean delivery, duration of spontaneous labour and operative vaginal delivery. Top panel: proportion of women delivered by emergency intrapartum Caesarean section in relation to the age (years) of the mother ($n = 583\,847$). The bars are binomial 95% CIs. Middle panel: mean duration of spontaneous labour in relation to maternal age ($n = 409\,703$). The bars are 95% CIs of the mean. Bottom panel: proportion of nulliparous women who required operative vaginal delivery in relation to maternal age among the 518 787 women delivered by a means other than emergency Caesarean section. The bars are binomial 95% CIs. Reproduced with permission from Smith *et al.* (2008).

feeding, late age at menopause and exposure to combined hormone-replacement treatment (reviewed in Parsa and Parsa, 2009). Women with reduced exposure to menstrual cycle fluctuations, as seen with use of progestogen contraceptives, might therefore be expected to have a reduced risk of age-related pregnancy

complications, when compared with women not using hormonal contraception or exposed to the combined oral contraceptive pill.

The future: making eggs

The dogma, as outlined above, that female mammals are born with all of the oocytes they will ever possess is based on a paper from Sir Solomon Zuckerman published in 1951 (Zuckerman, 1951). Zuckerman failed to find any experimental evidence that was inconsistent with an earlier hypothesis (Waldeyer, 1870) that germ cell production in female mammals ceases prior to birth [reviewed by (Zuckerman, 1971)]. This influential paper by Zuckerman formed the cornerstone of our understanding and subsequent interpretation of experimental and clinical observations relating to ovarian development, function and failure for the next 50 years (Tilly and Telfer, 2009). The consequences of this are extremely significant, not only in the context of a loss of fertile potential but also in the broader picture of the diverse spectrum of age-related health problems that emerge in post-menopausal women linked to failure of their ovaries (Prior, 1998; Buckler, 2005). If it were possible to repopulate adult ovaries with new oocytes and follicles, the female biological clock would no longer be an unreachable target to consider for clinical intervention.

In recent years there have been some exciting and controversial developments in female reproductive biology relating to a body of evidence that ovarian follicles may be formed during adult life by a rare population of putative germline stem cells (Johnson et al., 2004; Tilly and Telfer, 2009). Publications in support of oocyte renewal during adulthood have ranged from morphometry-based studies highlighting the mathematical improbability of a non-renewable oocyte pool being established at birth in rodents (Johnson et al., 2004; Kerr et al., 2006) to studies suggesting that oocyte regeneration can occur following a pathological insult that initially depletes the resting follicle pool (Johnson et al., 2005a; Borovskaya et al., 2006). These studies reopened the debate on neo-oogenesis resulting in the publication of many critiques (Telfer et al., 2005; Johnson et al., 2005b). However, the isolation and identification of oocyte-producing germline stem cells, also called oogonial stem cells (OSC), as unequivocal proof of their existence in ovaries of adult mammals in general, and humans in particular, remained elusive. A breakthrough in identifying such a cell was made in 2009 when putative germ line stem cells were isolated from adult mouse ovaries (Zou et al., 2009) and a more recent study (White et al., 2012) has now shown that a rare population of germ line stem cells can be extracted from adult human ovaries. This new study provides compelling evidence for the existence of a population of cells within adult human and mouse ovaries that can be multiplied *in vitro* and are capable of forming oocyte-like structures based upon morphology and expression of oocyte-specific biomarkers (e.g. DDX4, KIT and LHX8; White et al., 2012). Most importantly, following a period of differentiation these cells from mouse ovaries were capable of being fertilized and forming embryos (White et al., 2012). Unfortunately because of regulatory restrictions, fertilization of these cells from human ovaries has not yet been attempted.

This new work represents a major breakthrough by identifying cells with apparent germ line potential in both mouse and human ovary. However, many challenges lie ahead and many questions require answers before a practical and convincing demonstration of developmental potential of the oocyte-like structures derived from human

cells is obtained. Clearly these 'oocyte-like' cells derived from the progenitor population *in vitro* require the somatic cell support of paracrine and junctional communication to form follicles and develop into functional oocytes. Combining these 'oocyte-like' cells with more conventional human ovarian culture models (Telfer et al., 2008) may facilitate follicle formation and growth, and enable detailed analysis and testing of any resulting oocytes to be carried out (Rodrigues et al., 2008; Telfer and Albertini, 2012). If these oocytes proved to be normal, this would indeed widen options for fertility preservation and treatment of ovarian ageing.

As exciting as these new findings are, caution needs to be exercised in evaluating the immediate significance of the work to our understanding of the *in vivo* biology of ovarian function, as well as the ultimate relevance of this work to reproductive health in women. The successful purification and characterization of what appear to be *bona fide* OSCs from adult ovarian tissue in mice and humans, while important, does not immediately equate to proof that these cells serve a contributory role in determining the size of the post-natal follicle pool or the timing of ovarian failure under normal physiological conditions. Even if activity of the OSCs *in vivo* can be demonstrated and it is accepted they function to sustain the adult follicle pool by partially offsetting the high rate of follicle loss through atresia, it remains a fact that natural menopause happens approximately halfway or so through a woman's chronological lifespan. Nonetheless, these cells will remain an important target for future clinical applications. Studies such as these move us closer towards the application of stem cell-based regenerative medicine and the possibility that this may 1 day become a safe and effective strategy to control the timing of age-related ovarian failure and menopause when it might be clinically desirable to do so.

Conclusion

Ageing has incontrovertible effects on female reproductive function. This is most obvious for the ovary, and our understanding of the mechanisms by which oocyte quantity and quality decline with age is improving. It is also becoming clear that other reproductive organs, including the uterus, are detrimentally affected by age, and this may underlie the increased prevalence of late pregnancy complications in older women. These changes are in addition to age-related comorbidities, which together may have synergistic effects on reducing the probability of a successful pregnancy outcome. At present, given the detrimental impact of ageing on reproductive outcomes, increasing public awareness and societal support for having a family at an earlier age are required. Although our understanding of ovarian ageing has improved dramatically and, in particular, the potential early identification of individuals at greatest risk may allow us to redefine the concept of family planning, therapeutic interventions at present remain limited. The potential existence of stem cells capable of being used to restore the primordial follicle, and thereby oocyte pool, raises the intriguing possibility of novel *in vivo* and *in vitro* strategies to reduce the inexorable decline in female reproductive potential.

Authors' roles

S.M.N., E.T. and R.A.A. all contributed substantially to the design of the review. The literature review was repeated by each author, with

the data for their section used. S.M.N. and R.A.A. amalgamated the sections, with all authors editing and approving the final draft.

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Conflict of interest

None declared.

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