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The variability of female reproductive ageing

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The delay in childbearing is an important societal change contributing to an increasing incidence of subfertility. The prevailing concept of female reproductive ageing assumes that the decline of both quantity and quality of the oocyte/follicle pool determines an age-dependent loss of female fertility. There is an apparent discrepancy between the ability to maintain a regular ovulatory cycle pattern and the several years earlier cessation of female fertility. This latter is largely explained by an age-related increase of meiotic non-disjunction leading to chromosomal aneuploidy and early pregnancy loss, such that most embryos from women ⩾40 years old are chromosomally abnormal and rarely develop further. The final stage of reproductive ageing—the occurrence of menopause—shows a huge variation between women. Age at last birth in natural fertility populations, which marks the end of female fertility, shows an identically wide variation as age at menopause, but occurs on average 10 years earlier. Given the high heritability for age at menopause, the variation in both age of menopause and last birth are probably under genetic control by the same set of genes. Some of those genes must carry heritable variants which modulate the rate of ovarian ageing and give rise to the wide age variations for the various phases of reproductive ageing.

Keywords: age at last birth/age at menopause/delayed childbearing/genetic control/reproductive ageing

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Introduction

Increasing female educational levels and participation in the labour force are important trends in emancipation of women currently taking place in most Western countries (Blossfeld, 1995; United Nations, 1996). Part of this pattern of societal adjustment involves extraordinary changes in reproductive behaviour such as consciously choosing a life without children, delays in childbearing, rapid decreases in the number of children per couple and the reproductive sequelae of an increasing incidence of divorce and remarriage (Johnson *et al.*, 1987; te Velde, 1991; Blossfeld, 1995). All these demographic trends gained momentum from the end of the 1960s. An essential prerequisite for this so-called 'second demographic transition' (van de Kaa, 1987, 1993) was the introduction of reliable and well-tolerated forms of contraception, which led to a radical change in reproductive behaviour (Leridon, 1998). For the first time in history, the link between sexuality and reproduction was broken. Having children became no longer the unavoidable biological destiny of a woman, but an issue for careful consideration, involving choices of planned postponement, deciding to remain childless and, if more than one child was desired, spacing their birth order. Currently, increasing numbers of couples remain in doubt as to whether or not they want children (van Luijn, 1996). The key to this doubt not only lies in the difficulty of combining further education or a profession with having children, but also in the threatened loss of personal freedom (van Balen *et al.*, 1995b; van Luijn, 1996). Increasing numbers of women decide at a young age not to have children, but change their mind at a later age (van Luijn, 1996). Others deliberately delay childbearing to a period in life when having children seems more compatible with their chosen lifestyle. In The Netherlands, the mean age at which women deliver their first child has risen from 24.6 years in 1970 to 29.1 years in 1999. At present, taking all children into account, women more often deliver a child when above the age of 30 years than below. Although, the trend of delay is strongest in The Netherlands, similar patterns are present in most Western-style societies (Council of Europe, 2000).

Figure 1 gives the maternal age-related birth frequency curves for three representative periods in The Netherlands, namely 1965–1969, 1975–1979 and 1995–1999. Interestingly, comparison of the curves for the periods 1965–1969 and 1975–1979 shows that

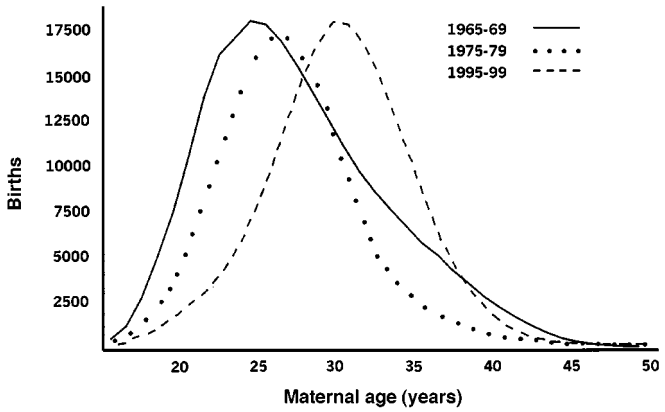

Figure 1. Relationship of birth frequency and maternal age for three time periods in The Netherlands showing significant increases in maternal age during the last 30 years. Each curve comprises ±900 000 births. Distributions compiled from data from The Netherlands Central Bureau for Statistics.

the first large-scale decision women took after their newly found freedom was to have fewer children, especially later in life. Only after 1979 did the current general trend of delaying childbearing become obvious. This resulted in an increase in the proportion of births occurring at age ⩾35 years from 6% in the 1975–1979 period to 18% in the 1995–1999 period. This trend will continue: demographic extrapolations from The Netherlands Central Bureau for Statistics (www.cbs.nl) suggest that in the period 2005–2009, the proportion of pregnancies occurring at age ⩾35 years will increase to >25%.

Both the large-scale use of effective contraception and the growing popularity of assisted reproductive technology have given the impression that female fertility can be manipulated according to wish, and at any stage of life. With regards to the ability to stop fertility temporarily, this impression is justified; however, with regards to the resumption of fertility at what appears to be a convenient point in their life, this is sometimes an illusion for which couples may have to pay the price of subfertility or even infertility. As we will discuss below, it is the progressive increase of reproductive ageing due to a delay in childbearing which significantly contributes to the growing incidence of unwanted subfertility, and that some couples have a genetically determined higher predisposition to experience such infertility.

Age-related decline of female fertility

In most contemporary Western countries social behaviour and effective contraception play an important role in determining if and when a couple has children. Therefore, female fertility (defined here as the biological capacity of a woman to reproduce) and its age-related decline can best be studied in so-called natural fertility populations, in which birth control is hardly ever practised. In such populations it is found that fertility declines with age in a manner that is universal throughout the human species (Leridon, 1977; Bongaarts, 1982; Spira, 1988; Wood, 1989). Although decreasing coital frequency certainly plays a part, the female age-related contribution to this decline appears to be more important, especially after age 30 years (Weinstein *et al.*, 1993). Male factors probably only start to play a significant role after age 40–45 years (Anderson, 1975; Schwartz *et al.*, 1983; Spira *et al.*, 1985; Johnson, 1986; Curtis *et al.*, 1997). At such ages—the average male is usually a few years older than his female partner—female fertility is already seriously compromised.

The age-related decline of female fertility consists of two components: first, the decreased monthly probability of conception and second, the increased probability that a pregnancy will terminate sooner or later after conception or implantation (embryo loss, pregnancy loss, fetal loss, spontaneous abortion). The monthly probability of conception probably does not decline until the age of 40 years (Holman *et al.*, 2000). Various studies demonstrate that early pregnancy loss often occurs and strongly influences female fertility and its age-related decline (Miller *et al.*, 1980; Edmonds *et al.*, 1982; Whittaker *et al.*, 1983; Wilcox *et al.*, 1988; Boklage, 1990; Hakim *et al.*, 1995; Holman, 1996; Holman *et al.*, 2000). Very early, unnoticed pregnancy loss probably occurs more often than most women and clinicians realise. As early as 1964, Bishop suggested that early pregnancy loss should be regarded as an unavoidable and necessary mechanism to eliminate 'unfit genotypes' (Bishop, 1964) (see section on chromosomal aneuploidy).

In contemporary populations of women in the USA and The Netherlands who actively tried to conceive, the age-related decline of fertility was obvious (Hendershot, 1984; van Balen *et al.*, 1995a). Data derived from the National Survey of Family Growth probably provide the best insight into this problem. The survey was based on interviews with a large representative USA sample of all marital statuses, varying between 15–44 years of age. The results of the last census performed in 1995 demonstrate that the proportion of women who tried but did not succeed in conceiving their first child within 1 year, increased from 6% in the 15–24 years age group to >30% in the 35–44 years age group (Abma *et al.*, 1997). Results from studies in presumptively fertile women treated with donor sperm because of their husband's sterility, have the advantage of eliminating possible confounding effects due to differences in coital frequency or to male reproductive ageing. In general, these studies confirmed the National Survey results and showed that the probability of achieving a pregnancy within 1 year was significantly higher in women <30 years than in women >35 years (Schwartz and Mayaux, 1982; Shenfield *et al.*, 1993). In a similar study, the monthly probability of conception leading to live birth was found to remain optimal until age 31 years and to progressively decrease thereafter (van Noord-Zaadstra *et al.*, 1991). At age 38 years, it had dropped to one-quarter of that in women <30 years. This figure is in line with the mean age of 40–41 years for the birth of the last child as found in natural fertility populations. This age can be regarded as the mean age at which female fertility comes to an end and sterility starts. It appears to be remarkably similar in natural fertility populations all over the world, including contemporary ones (Leridon, 1977; Knodel, 1978; Bongaarts, 1982; Trussell and Wilson, 1985; Menken *et al.*, 1986; Wood, 1989). At that age, most women have seemingly normal and regular cycles with a biphasic basal body temperature (Treloar, 1981) reflecting progesterone production during the luteal phase. Surprisingly, although the menopause is traditionally regarded as the age at which sterility ensues, it occurs ~10 years later at a mean age of 50–51 years. The presumed temporal relationship

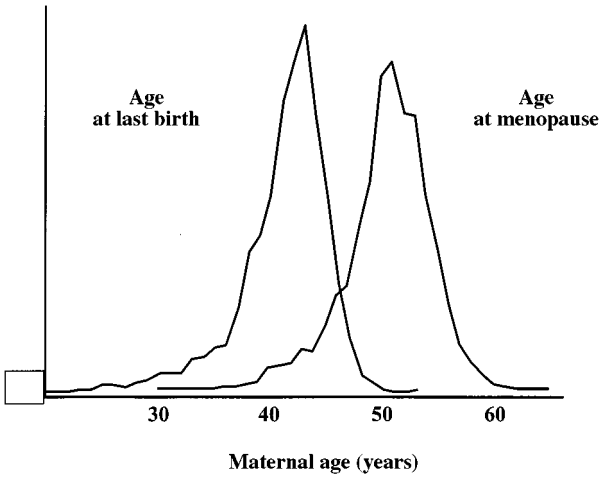

Figure 2. Comparison of distributions for age at last birth and age at menopause. Note identical distributions with a 10 year shift between them. The distributions are derived from independent populations: the age at last birth distribution was compiled from data from a 19th century French Canadian natural fertility population (Bouchard et al., 1989); the age at menopause distribution was derived from a large contemporary Netherlands cohort exhibiting natural menopause (van Noord et al., 1997). Each curve is normalized for number of observations (~1100, age at last birth; ~5500, age at menopause). Both curves exhibit a left side skewness suggesting an overlap of the main peaks with groups of women exhibiting even earlier infertility and menopause and corresponding to premature ovarian failure (POF).

between ages at last birth and at natural menopause is depicted in Figure 2, using a 19th century Canadian natural fertility population (mean age at last birth 40.6 years) and a contemporary population (mean age at menopause 51 years). The almost equal distributions of both populations suggest that not only mean ages differ by ~10 years, but that individual women also experience a 10 year difference between the age at which they become sterile and their age at menopause.

Apart from the decreasing monthly fertility rate, there are other manifestations of age-related decrease of fertility. The first endocrine sign of reproductive ageing is a rise in FSH levels at age 35–40 years (te Velde *et al.*, 1997). In the same age range, menstrual cycles tend to become shorter (Treloar *et al.*, 1967). Moreover, the risk of giving birth to a child with Down's syndrome and other numerical chromosome abnormalities rises exponentially with maternal age (Hecht and Hook, 1996) and becomes a significant personal hazard in the mid- to late thirties. In addition to chronological age, elevated FSH appears to be a further risk indicator for a Down's conception (Nasseri *et al.*, 1999; van Montfrans *et al.*, 1999).

The same general picture also emerges from IVF; both the implantation rate per embryo (van Kooij *et al.*, 1996) and the probability of pregnancy and live birth sharply decrease from age 37–38 years onwards (FIVNAT, 1993). In a British study based on >35 000 IVF cycles, female age was by far the most important determinant of success (Templeton *et al.*, 1996); live birth rates per embryo transfer dropped from 24% for women <30 years to 8% in women aged 40–44 years and 3.5% in women ≥45 years old. Since most women with irregular blood loss, menopausal complaints or other symptoms of reproductive ageing are likely to be counselled against using IVF, the latter percentages are probably an over-estimation of IVF success in women >40 years.

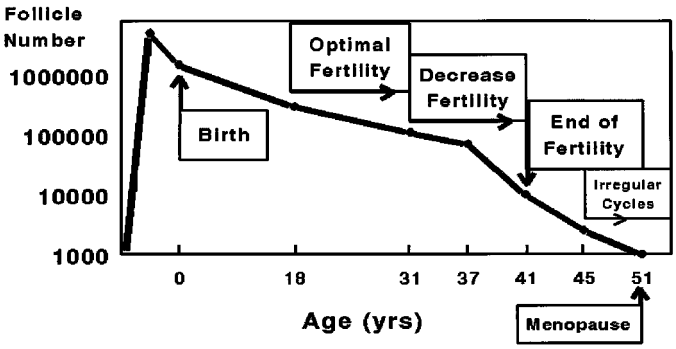

Figure 3. The declining oocyte/follicle pool according to Faddy et al. (1992) and the corresponding reproductive events.

Concepts of reproductive ageing

The prevailing concept of human reproductive ageing assumes that the age-dependent loss of female fertility is dictated by the decline of both the quantity and quality of the oocyte/follicle pool. During fetal life the ovaries are endowed with the entire stock of follicles (oocytes surrounded by ovarian granulosa cells), which has to serve a woman's reproductive needs for the rest of her life. Thereafter, numbers of follicles decline exponentially, with a marked increase in the rate of disappearance from age 37–38 years onwards. From the millions present before birth, only ~300 000 follicles are left at the beginning of puberty and subsequently hundreds vanish every month, including during periods when no ovulations occur, such as pregnancy, breast-feeding and use of oral contraceptives. Below a critical number of some thousands, reached at a mean age of 45–46 years, the menstrual bleeding pattern becomes irregular (Richardson *et al.*, 1987) and when the menopause is reached at a mean age of 51 years, the supply is reduced to a thousand or less follicles, a number insufficient to sustain the cyclic hormonal process necessary for menstruation (Faddy *et al.*, 1992).

The essence of the ovarian concept is that after a period of optimal fertility from age ~18–30 years, oocyte quality decreases in parallel to the progressive loss of follicle numbers. The rate of follicle disappearance has been extensively studied by various reproductive biologists (Block, 1952, 1953; Baker, 1963; Gosden, 1985; Gougeon and Chainy, 1987; Richardson *et al.*, 1987; Faddy *et al.*, 1992; Gougeon *et al.*, 1994; Gougeon, 1996; Gosden and Finch, 2000). In Figure 3 their work is schematically summarized according to Faddy *et al*. and related to the important reproductive events in a woman's life, as advanced in the previous section (Faddy *et al*., 1992).

Although the ovarian concept of reproductive ageing is highly plausible, a definitive proof is difficult to provide because follicle number and morphological status can only be reliably assessed following removal of the entire ovary. Furthermore, the relationship between morphology and quality is ill defined. However, the concept is strongly supported by classical experiments in CBA mice showing that an enforced 50% reduction of the follicle pool by unilateral ovariectomy resulted in an earlier onset of infertility, cycle irregularity and oocyte aneuploidy (Brook *et al.*, 1984; Eichenlaub-Ritter *et al.*, 1988). Although the reproductive consequences of unilateral ovariectomy have not been extensively studied in humans (te Velde, 1993), the results appear to be

generally similar to the mouse; it is thought to lead to an earlier age at menopause (Hardy and Kuh, 1999), a rise of FSH levels (Khalifa *et al.*, 1992), poorer results after IVF (Khalifa *et al.*, 1992) and possibly an increased incidence of trisomy 21 (Freeman *et al.*, 2000).

Does the endometrium play a role in human reproductive ageing as it does in some laboratory animals? For example, old mice receiving oocytes from young donors retained a poor reproductive performance (Harman and Talbert, 1970). The remarkably good results of IVF with oocytes of young donors in (pre) menopausal women, argues strongly in favour of the ovarian concept and against the endometrium playing an important role. (Sauer *et al.*, 1993; Cohen *et al.*, 1999).

In the so-called neuro-endocrine concept, reproductive ageing is regarded as a dysregulation of the GnRH pulse generator in the hypothalamus due to a progressive lack of neuro-chemical control from other brain centres, resulting in changes in the normal GnRH pulse pattern (Wise *et al.*, 1996). The first sign of this transition is the early rise of FSH leading to acceleration of follicle depletion. In analogy with the situation in rats (Wise *et al.*, 1999) an additional role for the hypothalamus in the human cannot be ruled out. For example, the ovarian concept does not explain why climacteric complaints, such as hot flushes, may already be present in women who still have regular cycles and normal estrogen levels (Oldenhave *et al.*, 1993; te Velde, 2000). It was demonstrated that menopausal flush episodes are associated with the pulsatile release of LH, which reflects increased GnRH pulse activity (Casper *et al.*, 1979). Since the GnRH pulse generator and the thermoregulatory centre are both functionally and physically connected to each other, a common stimulus related to the ageing process of the hypothalamus or higher brain centres may well be responsible for their increased activity. The observation that changes in pulse patterns of LH start to occur in regularly cycling women from the age of ~39 years onwards, is also in line with an additional role of the hypothalamus (Matt *et al.*, 1998). However, most of the signs attributed to the hypothalamus occur well after the initiation of age-related decline of fertility. With regard to the close temporal relationship between changes in the ovarian follicle pool and age-related fertility decline, the dominant pacemaker in reproductive ageing must be the ovary.

Chromosomal aneuploidy is the major cause of the age-related decline of oocyte quality

There is a well-established relationship between increasing maternal age with an increasing incidence of Down's syndrome (Cuckle *et al.*, 1987; Hecht and Hook, 1996). Indeed, postponing childbearing to ages >35 years considerably increases the incidence of Down's syndrome (trisomy 21), and this explains why its incidence has not decreased in spite of extensive prenatal screening in The Netherlands (Wortelboer *et al.*, 2000) and the USA (Olsen *et al.*, 1996). However, the live born incidence of trisomy 21 must constitute only a small fraction of the presumed incidence at conception (Pflueger, 1999). Chromosome studies on spontaneous abortions show that at least 80% of trisomy 21 embryos spontaneously abort (Davison and Burn, 1990). Apart from trisomy 21, whole chromosome trisomies in live-borns are only known for chromosome 13 and 18, although in material from spontaneous abortions, trisomies have been observed for all chromosomes in varying frequencies and the incidence of spontaneous abortion also demonstrates an age-related increase (Creasy *et al.*, 1976; Warburton *et al.*, 1986; Fritz *et al.*, 2001). Monosomy X (Turner's syndrome) is the only monosomy found in live-borns and, astonishingly, also in spontaneous abortions. However, studies in the mouse (Martin-DeLeon and Boice, 1983; Magnuson *et al.*, 1985) and hamster (Sonta *et al.*, 1984) on fetal loss associated with chromosomal abnormalities clearly show that, in accordance with theoretical expectation, monosomies are involved in conception at an equal frequency to trisomies. Apparently, autosomal monosomies are lost at a much earlier stage of pregnancy and never come to term (Boué *et al.*, 1985). In the human, with the exception of XO embryos, monosomic embryos are probably lost too early even to be recognized. The inevitable conclusion is that the frequency of chromosomal abnormalities at conception is much higher than can be deduced from frequencies in either live-borns or spontaneous abortions (Davison and Burn, 1990). Furthermore, the frequency of chromosome abnormalities at conception can be expected to rise rapidly with increasing maternal age, such that the majority of embryos are chromosomally abnormal in women approaching 40 years old. This has been confirmed by investigations into the chromosome status of IVF embryos, which have demonstrated that the majority of embryos derived from women >37 years old are chromosomally abnormal (Gianaroli *et al.*, 1999; Wells and Delhanty, 2000) and contain all possible combinations of both monosomies and trisomies.

Mathematical models which define the age-related decline of female fertility in natural, non-contraceptive populations have been developed (Holman, 1996; Holman *et al.*, 2000). Backward extrapolation in time suggests that most pregnancy loss occurs so early that it is not yet detectable at the time of the expected menstruation and thus remains unnoticed. Furthermore, their analyses indicate that the probability of early pregnancy loss is already ~50% for a 20-year-old woman and increases to 96% by age 40 years. It is highly probable that chromosomal aneuploidies are the major cause of this pregnancy loss. This phenomenon also explains one of the great mysteries of female reproductive ageing, namely why fertility ceases some 10 years before commencement of the menopause (Figures 2 and 3). Between the ages of 40 and 50 years, there are still sufficient follicles left to sustain normal follicle development and menstruation, but too high a frequency of early pregnancy loss from chromosome abnormalities to allow successful continuation of pregnancy. In accordance with this explanation, we conclude that the increased incidence of trisomy 21 and spontaneous abortions in older women are 'tip of the iceberg' manifestations of the age-dependent loss of female fertility caused by increasing rates of aneuploidy in oocytes.

Meiotic non-disjunction is the major mechanism which largely explains the occurrence of the majority of aneuploidy in early embryos. By direct examination of mouse oocytes it was shown (Henderson and Edwards, 1968) that a reduced meiotic crossing-over in aged females was associated with a failure of homologous chromosomes to segregate normally into daughter cells at the first meiotic division. In their 'production-line' hypothesis, Henderson and Edwards assumed that, (i) the first oocytes to differentiate during fetal development have a higher meiotic chromosome recombination frequency than those which differentiate later and (ii) oocytes are going to ovulate in the order of their

differentiation according to the maxim first in–first out, last in–last out (Henderson and Edwards, 1968). However, this hypothesis does not explain why the order of oocyte differentiation induces the postulated differences in recombination frequency. Oocytes become arrested in prophase of the first meiotic division during fetal life at the so-called dictyate phase, after recombination has already occurred. The dictyate oocytes remain in this phase for 10–50 years and resume meiosis in individual oocytes at the moment of ovulation. All existing data point to oocytes becoming more error prone to chromosome non-disjunction as time goes by.

In 1997 a two-hit model of non-disjunction was proposed (Lamb *et al.*, 1997) in which the first hit is a reduced frequency and pattern of recombination in a fraction of oocytes right from the start, and the second hit is associated with an increased frequency of non-disjunction due to the effects of ageing. The two-hit model appears to be compatible with all relevant ideas on the effect of age, such as the accumulation of damage by oxidative stress (Tarín, 1995), an increasingly compromised micro-circulation around the leading follicle causing reduced oxygen levels in the follicular fluid (Gaulden, 1992; Van Blerkom *et al.*, 1997) or a progressively defective granulosa cell function (Warburton, 1989). Moreover, other models which have been created to account for age-related non-disjunction are also compatible with the 2-hit model. The 'limited oocyte pool' model proposes that, as the size of the selectable follicle pool decreases, it becomes less likely that a follicle will appear just at the optimal time in the intercycle FSH window (Warburton, 1989). Moreover, post-ovulatory ageing of oocytes due to delayed fertilization may also lead to a higher frequency of non-disjunction and early pregnancy loss (Guerrero and Rojas, 1975; Bomsel-Helmreich, 1976; Wilcox *et al.*, 1998). The risk of delayed fertilization increases with decreasing coital frequency, as a couple grows older (Simpson *et al.*, 1988). Although meiotic non-disjunction is undoubtedly the single most important consequence of oocyte ageing, a recent observation on the chromosome composition of individual blastomeres from entire IVF embryos from older women showed a high frequency of chromosome mosaicism, demonstrating that mitotic non-disjunction in early embryonic development is also associated with oocyte ageing (Wells and Delhanty, 2000).

What particular aspects of ovarian ageing may be relevant to inducing meiotic chromosome non-disjunction? Two studies in which the morphology of the meiotic spindles in human oocytes at the second meiotic division (metaphase; MII) was compared between young and old women provide valuable insights (Battaglia *et al.*, 1996; Volarcik *et al.*, 1998). Oocytes from young women formed regular bipolar spindles during the MII division, with the chromosomes being tightly arranged on the spindle equator, whilst the meiotic spindles of oocytes from older women were much more diffuse, frequently showed lack of bipolarity and the chromosomes were irregularly and loosely attached to the spindle at many different locations. These observations strongly suggest that the process of subsequent chromosome division and segregation will be under much poorer control in the oocytes of older than younger women. The reason that meiotic MII divisions were studied and not those of the more much more relevant metaphase I (MI)—most meiotic non-disjunctions occur at MI—is that MI spindle formations are rarely encountered in human preparations due to the high speed with which oocytes carry out the MI division. However, occasional MI spindle observations suggest that age changes in spindle morphology are similar in both MI and MII meiotic divisions of oocytes of the same woman (Volarcik *et al.*, 1998). Observations that centromeres undergo a putative premature division at MI in oocytes of older women (Angell, 1997) suggest an increasing failure to maintain appropriate centromere function during meiotic MI as part of the ageing process. Extensive studies in yeast have demonstrated that a highly conserved member of a family of cohesin proteins, known as Rec8, normally prevents the centromere from prematurely dividing at meiotic MI and promotes its division at MII (Watanabe and Nurse, 1999). Rec8-negative mutants in both yeast (Buonomo *et al.*, 2000) and *Caenorhabditis elegans* (Pasierbek *et al.*, 2001) exhibit high levels of first meiotic non-disjunction largely attributable to chromatid separation at MI in place of MII. Rec8 is known to be also conserved in humans with an as yet undefined meiotic function (Parisi *et al.*, 1999). It will be extremely important to determine whether and to what extent cohesin metabolism becomes dysregulated in ageing oocytes.

Oocyte/follicle development and growth

At an early stage of embryonic development germ cells separate from somatic cells. Some 1000–2000 migrate to the gonadal ridge where they start to multiply rapidly by mitotic division (Byskov, 1986). At 16–20 weeks gestation the ovaries are estimated to contain ~7×10^6 oogonia (Baker, 1963). At about that time they are transformed into primary oocytes by entering the prophase of the first meiotic division; this phase involves the critical sequence of DNA replication, homologous chromosome pairing, chromosome recombination (chiasma forming), diakinesis and lastly diplotene, commonly referred to as the dictyate phase in mammalian oocytes. At this stage the oocyte is arrested and surrounded by one layer of flat granulosa cells, the combination of which is called the primordial follicle. In humans, oocytes may stay in this 'resting' phase for many decades and only resume meiosis just prior to being released at ovulation. The stages of follicle growth from the primordial phase to full maturation and ovulation are depicted in Figure 4, which is based on the work of Gougeon (Gougeon, 1996). Although some increase in follicle growth is obtained by an increase of oocyte volume during maturation, the major increases arise by rapid proliferation of granulosa cells. The entire process from the initiation of follicle growth until ovulation takes at least half a year (Gougeon, 1996). However, the vast majority of follicles will go into atresia either by apoptosis or necrosis at some stage of development. The role of apoptosis is firmly established in follicle development. (Hsueh *et al.*, 1994; Yuan and Giudice, 1997) In resting follicles atresia is possibly caused by a process of necrosis (de Bruin *et al.*, 2001). Furthermore, the results of a recent study suggest that atresia in the human fetus mainly occurs through apoptosis of oocytes, while in adult life it is primarily determined by apoptosis of granulosa cells (Vaskivuo *et al.*, 2001). Only some follicles will reach the selectable phase (antral follicles 2–5 mm in diameter) and from there go into atresia, unless 'rescued' by the intercycle FSH rise, through which they are permitted to develop further (Fauser and Van Heusden, 1997). From this cohort of selectable

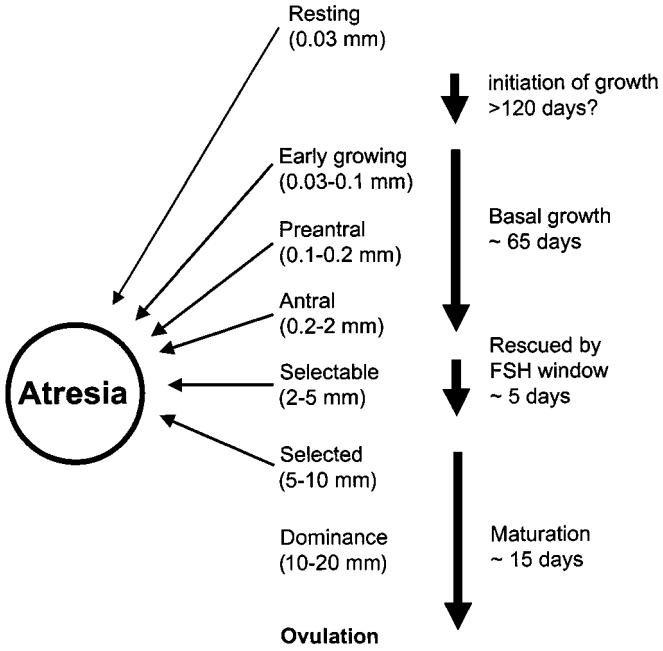

Figure 4. The various phases of follicle growth and development with the corresponding follicle diameters in parentheses. Adapted from Gougeon (Gougeon 1996).

follicles (Figure 3), which can be visualized by sonography during the early follicular phase, the follicle is selected which will become dominant. The mid-cycle LH peak is the trigger for ovulation and resumption of the first meiotic division. From the millions of oocytes present before birth, this ultimate destination is only reserved for some hundreds at the most.

In addition to direct atresia of resting follicles, initiation of growth and the inevitable loss at some stage thereafter is an important mechanism for the depletion of the resting follicle pool (Gougeon *et al.*, 1994; Faddy and Gosden, 1995). Why after a long state of quiescence some follicles leave the resting phase and start growing remains a mystery. However, various regulators of early follicular growth have been described in transgenic mouse models including growth differentiation factor-9 (Dong *et al.*, 1996; Elvin *et al.*, 1999; Vitt *et al.*, 2000), anti-Müllerian hormone (Durlinger *et al.*, 1999), BAX1 and BCL2 (Perez *et al.*, 1999) and the Wilm's tumour gene (Chun *et al.*, 1999). Although these factors play either a stimulatory or inhibiting role in the initiation of follicle growth, little is known about their exact function, mutual co-operation and why individual follicles become sensitive to their action at some stage during their life history.

The important role of FSH in rescuing selectable follicles, leading to selection of the dominant follicle and sustaining its further maturation until ovulation, is well established (Fauser and van Heusden, 1997). Whether FSH also plays a role in the early phases of follicle development is less certain. Experimental findings and observations in human clearly show that follicles are able to develop to the selectable stage without the presence of gonadotrophins. For example, follicles of 2–5 mm diameter are present from infancy to menopause, including during periods when FSH levels are (almost) zero (Sungurtekin *et al.*, 1995). However, there is some evidence indicating that FSH may facilitate the initiation of follicular growth and stimulate early follicular development (te Velde *et al.*, 1998) in complex co-operation with the aforementioned growth regulators. The stimulating effect of FSH is also suggested by the association between accelerated depletion of the resting follicle pool, once gonadotrophins levels start to rise (Faddy *et al.*, 1992) and by the earlier age at menopause in women with a familial trait of dizygotic twinning (Soberson *et al.*, 1996). Higher concentrations of FSH in such women may explain both a higher rate of poly-ovulation and an earlier depletion of the follicle store (Martin *et al.*, 1991).

Using a mathematical model, Faddy and Gosden provided estimates of the daily number of follicles entering the growth phase (Faddy and Gosden, 1995). They predicted a dramatic drop from ~45 follicles in the age group 25–30 years to about six follicles in the age group 38–45 years. The question of how many of these will reach the selectable stage has been addressed (Scheffer *et al.*, 1999). The authors found that the number of sonographically detectable follicles (diameter $\geqslant$2 mm) in the early follicular phase also decreases, but less steeply. Apparently, relatively more follicles survive at older ages, as has been found by others (Krarup *et al.*, 1969; Gougeon and Chainy, 1987; Hirshfield, 1994). The role of rising FSH levels in this process is in line with in-vitro experiments demonstrating that FSH is a major survival factor in early follicle development (Chun *et al.*, 1996). The findings also suggest that the criteria for determining the number and selection of follicles which are removed from the pool by atresia become less stringent with increasing age. Assuming that follicle atresia plays a role in determining the overall quality of follicles which reach the final stages of development, this implies a reduced quality control of follicles in older women.

Hormonal and sonographic changes related to reproductive ageing

The earliest endocrine sign associated with reproductive ageing is a rise in FSH in the early follicular phase from age 35–40 years onwards (Sherman *et al.*, 1976; Reyes *et al.*, 1977; Musey *et al.*, 1987; Lee *et al.*, 1988; Fitzgerald *et al.*, 1994; Klein *et al.*, 1996b; van Zonneveld *et al.*, 2001). At such ages, numbers of resting follicles have dropped to about one-tenth of the number present at puberty (Faddy *et al.*, 1992). Also, if a statistically significant elevation of early follicular FSH in groups of older women is present, a large overlap with levels in young women exists (Schipper *et al.*, 1998; van Zonneveld *et al.*, 2001). This observation is in line with the threshold concept of Brown, which proposes that women have an individual FSH level, above which follicles are stimulated to further growth (Brown, 1978). This threshold may demonstrate a considerable, but still physiological variation, which is possibly related to the recently described functional polymorphisms in the FSH receptor gene (Perez Mayorga *et al.*, 2000). Thus, the significance of single or multiple FSH determinations with the aim of gaining insight into a woman's reproductive age is limited. Only very high FSH levels have a clear significance, namely the presence of an already almost exhausted ovarian follicle pool (Levi *et al.*, 2001). However, it is possible that long-term measurement of FSH

levels in individual women may reveal significant changes at earlier stages of ovarian ageing. In studies with a standard transversal (non-longitudinal) design, such subtle individual changes cannot be recognized.

It is assumed that the FSH rise is caused by the feedback of FSH-modulating proteins from the ovary, including inhibin-A, inhibin-B, activin-A and follistatin. Small antral follicles predominantly secrete inhibin-B in the early follicular phase, while inhibin-A is produced by the dominant follicle and the corpus luteum (Groome *et al.*, 1996; Illingworth *et al.*, 1996). It has been demonstrated that decreasing levels of inhibin-B are clearly associated with the FSH rise in the early follicular phase. Therefore, most investigators assume that decreasing levels of inhibin-B reflect a compromised function of the antral follicle pool (Klein *et al.*, 1996a; Burger *et al.*, 1998; Danforth *et al.*, 1998; Reame *et al.*, 1998; Welt *et al.*, 1999). In a large group of normal fertile women of different ages, we were able to confirm a highly significant inverse relationship between the levels of early follicular FSH and inhibin-B (Scheffer, 2000). However, <20% of the large variation of FSH was explicable by inhibin-B levels. Reame and colleagues proposed that the FSH rise may be secondary, not only to a deficiency of inhibin-B, but also to increased levels of activin-A (Reame *et al.*, 1998). However, the fact that circulating follistatin (the carrier/binding protein of activin) is available in large excess and completely blocks the biological function of activin-A casts considerable doubt on this proposal (McConnell *et al.*, 1998).

LH levels also rise with age, but much later than FSH (Ahmed Ebbiary *et al.*, 1994). The available data on serum estradiol and progesterone levels generally do not show appreciable changes with increasing age, as long as the regular menstrual pattern is maintained (Sherman *et al.*, 1976; Reyes *et al.*, 1977; Musey *et al.*, 1987; Fitzgerald *et al.*, 1994; Klein *et al.*, 1996b). The age-related decrease in levels of circulating growth hormone and insulin-like growth factor, appears to be a phenomenon of general ageing not specifically related to ovarian ageing (Weltman *et al.*, 1994). We conclude that in spite of a statistically significant age-related trend in the levels of particularly FSH and inhibin-B, the large overlap between values of older and younger women obscures their potential value for ovarian ageing prediction at an individual level. Moreover, the large variation in these hormone levels between women cannot be directly related to their fertility status.

With regard to age-related sonographic changes, a highly positive correlation of ~0.7 was found between the number of sonographically detectable follicles and chronological age in a large group of regularly cycling volunteers with proven normal fertility (Scheffer *et al.*, 1999). In such women, chronological age was assumed to approximate reproductive age much more closely than in subfertile women. However, in spite of a much higher correlation with chronological age than established for any of the hormones described, there was still a wide variation in the number of observed antral follicles between consecutive cycles within and between individuals of the same age. Nevertheless, assessment of the number of antral follicles is possibly useful in predicting a poor ovarian response in women undergoing IVF treatment (Bancsi *et al.*, 2002). The development of the dominant follicle, the subsequent ovulation and the appearance of the endometrium as detected by sonographic examination, remain remarkably normal in older women who still have regular menstrual cycles (van Zonneveld *et al.*, 2001). Until the very late stages of reproductive ageing most endocrine and sonographic cycle characteristics remain more or less normal. Only at a mean age of ~46 years, when the resting follicle pool is almost exhausted, do normal cycle characteristics disappear. At this age the size of the antral follicle cohort arriving in the intercycle FSH window apparently becomes too small to safeguard normal selection and development of a dominant follicle.

Both endocrine and sonographic changes can be considered to reflect granulosa cell function of the antral follicle pool, while oocyte quality determines a woman's fertility. Given the discrepancy in average age at which both come to an end, changes in oocyte quality and granulosa cell function seem different processes, the latter continuing to function adequately much longer than the former. However, it becomes increasingly evident that there are complex interactions between oocytes and granulosa cells (Eppig, 1992; Dong *et al.*, 1996; Vanderhyden, 1996; Picton *et al.*, 1998; Sadraie *et al.*, 2000) which are also reflected by changes in the content of follicular fluid (Stouffer *et al.*, 1993; Klein *et al.*, 1996a, 2000; Van Blerkom *et al.*, 1997; Manau *et al.*, 2000). Age-related changes leading to an increased probability of meiotic non-disjunction (e.g. accumulation of oxidative stress) may well be mediated by subtle disturbances of granulosa cell function. More robust functions of the follicular apparatus, including secretion of inhibin-B, estradiol and progesterone, and the ability to ovulate, remain undisturbed until the very late stages of ovarian ageing. Prior to that time, hormonal changes and sonographic findings provide limited information about the process of ovarian ageing in individual women.

Age at menopause as a key event of reproductive ageing

Menopause is the final reproductive event and marks the definite exhaustion of the primordial follicle pool (Figure 3). It is the only event in the cascade of changes involved in ovarian ageing whose commencement can be unambiguously determined in individual women. Determination of the ages and periods of the preceding events are retrospective abstractions from large groups of women and relate to mean ages. The mean age of menopause is 50–51 years in all Western countries, with a huge variation from 40–60 years (Treloar, 1981). If reproductive ageing is generally determined by a gradual decline of the quantity and quality of the oocyte/follicle pool, all the preceding reproductive events are likely to show similar age variations as menopause, such that the commencement of menopause at an earlier age is associated with an earlier induction of subfertility, sterility and transition to cycle irregularity and vice versa. This temporal relationship is shown in Figure 5. Curve 4 depicts the Gaussian distribution of age at menopause in a cumulative fashion and is based on data by Treloar (Treloar, 1981). Curve 3 shows the variation in the age of transition from cycle regularity to irregularity, which has been shown to be related to a reduction in the numbers of follicles left in the follicle pool to below ~10 000 (Richardson *et al.*, 1987). In an independent study we confirmed that the difference between the commencement of cycle irregularity and age at menopause appeared to always be 6–7 years, irrespective of whether the individual concerned exhibited early, mid- or late menopause (den Tonkelaar *et al.*, 1998). Curve 2 depicts variation in the age

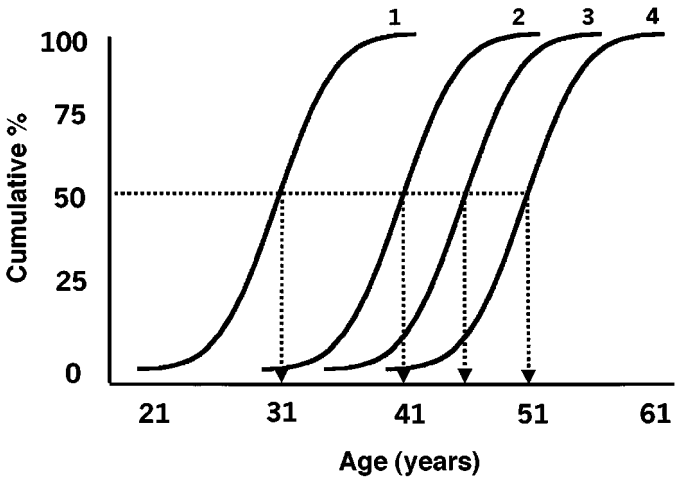

Figure 5. The age variations of the various stages of reproductive ageing depicted in a cumulative fashion. Curve 1: variation in age at the beginning of subfertility (mean age 31 years). Curve 2: variation in age at the beginning of sterility (mean age 41 years). Curve 3: variation in age at the transition from cycle regularity to irregularity (mean age 46 years). Curve 4: variation in age of menopause (mean age 51 years). See text for details on origin of data.

of becoming sterile, which appears to show the same age distribution as age at menopause (see Figure 2). The age variation of the first crucial reproductive event—the beginning of subfertility, shown in curve 1 of Figure 5—cannot be corroborated by scientific data, because markers defining the subtle difference between normal fertility and the start of subfertility do not exist. However, it is highly plausible that the start of fertility decline, just as with the subsequent stages, also depends on the size of the oocyte pool remaining at that stage. Because of the wide age variation in all phases of ovarian ageing within the population, it is not the chronological age of a woman, but the period of time that must elapse before menopause occurs, that defines her reproductive phase.

If age at menopause is related to the time sequence of the preceding reproductive events (see Figures 3 and 5), the same factors that determine age at menopause are expected to also determine all reproductive events. Various factors related to lifestyle, environment, fertility and inheritance have been implicated, of which smoking is the most extensively studied. The results of the latter studies are in line with all known aspects of ovarian ageing. Cigarette smoking has been associated with an earlier age at menopause (Jick and Porter, 1977; Weinberg *et al.*, 1989; Torgerson *et al.*, 1994; Cramer *et al.*, 1995c; van Noord *et al.*, 1997; Gold *et al.*, 2001), decreases in the size of the ovarian follicle pool (Westhoff *et al.*, 2000), reduction in the spontaneous fertility rate (Howe *et al.*, 1985; Hull *et al.*, 2000), success rate of IVF treatment (Pattinson *et al.*, 1991; Van Voorhis *et al.*, 1996; El-Nemr *et al.*, 1998), increases in FSH concentrations (Cooper *et al.*, 1995) and incidence of trisomy 21 (Yang *et al.*, 1999). A recent publication provides some insight into how cigarette smoking may influence the course of ovarian ageing (Matikainen *et al.*, 2001). It was found that polycyclic aromatic hydrocarbons, which are extremely bio-reactive ingredients of cigarette smoke, up-regulate the *Bax* pro-apoptosis gene resulting in enhanced follicle damage and premature ovarian failure (POF) in treated mice. With regards to food consumption, extreme malnutrition leads to a much earlier menopause (Gray, 1976), but also in less extreme circumstances diet may play a role (Baird *et al.*, 1988). There are probably other, more cryptic lifestyle factors, because higher social economic class appears to be a relatively strong predictor of later menopause (Kaufert *et al.*, 1987; Weinberg *et al.*, 1989; Torgerson *et al.*, 1994; van Noord *et al.*, 1997). The effect of oral contraceptives is controversial in that some studies found a delaying effect (van Keep *et al.*, 1979; Stanford *et al.*, 1987), while others found no effect (Brambilla and McKinlay, 1989; Cramer *et al.*, 1995b; Bromberger *et al.*, 1997). Most studies failed to exclude women who used oral contraceptives or hormone replacement therapy for menopausal complaints, leading to a spurious assessment of when menopause actually occurred. Correcting for this effect, we demonstrated that high dose oral contraceptives have a weak, but statistically significant effect of reducing the age at menopause (de Vries *et al.*, 2001). In contrast, a delaying effect of oral contraceptives on reproductive ageing was suggested by the observation that older women, who had used oral contraceptives for a long time, had a decreased incidence of abortions (Ford and MacCormac, 1995). Factors related to fecundity include parity and cycle characteristics. Parity appears to be linked with menopausal age in that nulliparous women have an earlier menopause than multiparous women (Kaufert *et al.*, 1987; Stanford *et al.*, 1987; Torgerson *et al.*, 1994; van Noord *et al.*, 1997) and that increasing parity is associated with later menopause (Kaufert *et al.*, 1987; Soberson *et al.*, 1996). With regard to cycle characteristics at younger ages, women with relatively short or long menstrual cycles have menopause respectively earlier or later (Whelan *et al.*, 1990; Dahlgren *et al.*, 1992; Cramer *et al.*, 1995b).

To what extent do lifestyle and fecundity-related factors explain the large variation of age at menopause? In a large cohort of women, we evaluated the uni- and multivariate relationships between many lifestyle and fecundity-related factors and age at menopause (van Noord *et al.*, 1997). The results were generally in line with those of others, as mentioned. Although the effect of some factors was highly significant because of the large sample sizes involved, their combined contribution to total variation in age of menopause was limited and explained <5% of the variation. The potential significance of inheritance was first demonstrated by finding a positive correlation between mothers and daughters in age of menopause (Torgerson *et al.*, 1994) and between sisters with POF (Cramer *et al.*, 1995a). In a study of ~180 sister pairs, the heritability for age at menopause appeared to be high, indicating that ~85% of the phenotype variation was genetically determined (de Bruin *et al.*, 2001). A significant heritability for age at menopause was also demonstrated in twin studies (Snieder *et al.*, 1998; Treloar *et al.*, 1998). These findings imply a strong genetic involvement in the process of follicle depletion leading to menopause and suggest that the enormous variation of age at menopause is predominantly caused by genetic variation in the genes involved in the ovarian ageing process.

Various conflicting reports in the literature suggest that, in addition to age at menopause, reproductive ageing can be either positively or negatively associated with age at death (Snowdon *et al.*, 1989; Kirkwood and Rose, 1991; Perls *et al.*, 1997; Cooper and Sandler, 1998; Westendorp and Kirkwood, 1998; Cooper *et al.*, 2000). If either association turns out to be true, this would

imply that some of the genes concerned with general ageing are likely to be also involved in ovarian ageing.

Genetic variation in reproductive ageing

Given the ovarian concept of reproductive ageing as advanced in the previous sections and the high heritability of age at menopause, genes which determine the size of the ovarian oocyte/follicle pool during fetal life, and the rate of its decline thereafter, must be the same genes that are responsible for the variations in age at menopause and the commencement of subfertility and infertility. However, the observed age variations in menopause and the preceding reproductive phases are not just an expression of involvement of certain genes, but reflect differences in gene expression between individuals caused by genetic variants (polymorphisms) within the genes concerned. It is the inheritance of particular genetic variants within the genes and not the genes themselves that give rise to the wide variation in the population and high correlations seen within families for age of menopause (high heritability).

A distinction has to be made between POF, which has been arbitrarily defined as the occurrence of menopause before the age of 40 years (Coulam *et al.*, 1986), and normal ovarian failure in which menopause occurs at ⩾40 years. POF and normal ovarian failure are probably two separate conditions, although the age of 40 years is arbitrary and the age distribution of both conditions is likely to overlap (Tibiletti *et al.*, 1999). POF appears to be heterogeneous, with various causes including deletions of the X-chromosome (Devi and Benn, 1999; Davis *et al.*, 2000), fragile-X carriership (Allingham-Hawkins *et al.*, 1999; Marozzi *et al.*, 2000; Hundscheid *et al.*, 2001), FSH receptor mutations (Aittomaki *et al.*, 1996) and mutations in the *FOXL2* gene (Crisponi *et al.*, 2001). Turner's syndrome (XO-monosomy), in which the process of follicular depletion is so rapid that no follicles are left at puberty and no menarche occurs, can be considered an extreme early form of POF. Familial transmission is observed in up to 30% of POF cases (Tibiletti *et al.*, 1999; Vegetti *et al.*, 2000). Phenotype distinction within families generally occurs in a bimodal fashion, indicating a Mendelian segregation and involvement of mutations in single genes.

Normal ovarian failure leading to natural menopause occurs in the majority of the population, albeit with a wide age variation. The high heritability and lack of distinction between separate phenotype classes within families indicates that the trait variation is determined by several genes interacting with environmental factors to result in what is known as multifactorial inheritance. The advent of the Human Genome Project and the availability of molecular tools and DNA sequence information stored in public databases, now makes it possible to map and identify the genes determining so-called complex genetic traits, including the age of menopause, and to determine which DNA sequence changes in such genes contribute to the phenotypic variation of the trait. Essentially, the pattern of inheritance of arbitrarily selected segments of the total variation of the trait concerned is compared with that of several hundred highly polymorphic genetic markers spread all over the human genome in a large number of small family samples (sib-pair analysis). How many genes, which genes and which sequence variants contribute to the trait variation is the critical information to be determined. The exciting aspect is that it is not necessary to make *a priori* assumptions on which genes are involved. By this approach it is possible that genes will be recognized whose association with ovarian ageing and age at menopause is entirely novel. A complimentary approach is searching for associations with candidate genes previously described to play a possible role in ovarian ageing. An example of this is the estrogen receptor gene (*ESR1*) for which a non-coding polymorphism was shown to be associated with reduction in the age of menopause (Weel *et al.*, 1999). Other possible candidates include genes observed to be involved in general ageing, including *APOE* in humans (Skoog *et al.*, 1998) and *SOD1* in experimental animals (Parkes *et al.*, 1998), genes known to be involved in apoptosis, such as *BAX1* and *BCL2* (Perez *et al.*, 1999) and genes involved in POF as described previously. In the light of the important role of chromosome non-disjunction in inducing female infertility, genes possibly involved in inducing spindle instability and chromosome non-disjunction should be added to this list.

Final remarks and future challenges

The delay in childbearing is an important societal change, which results from the trend of growing female emancipation. Unfortunately, it also contributes to the growing incidence of subfertility. Clinicians involved in reproductive medicine should be made aware of the consequences of these changes for their daily work. Appropriate information about the sequelae of delay on female fertility should be provided to all women considering having children later in life (DeCherney and Berkowitz, 1982). Moreover, such women should also know that the ability of assisted reproductive technology to overcome age-related subfertility is grossly over-estimated, not only in the lay press but also in medical circles.

As a consequence of the original observations of Block, Gougeon, Gosden and others on follicle/oocyte growth and development, reproductive ageing has been largely viewed as a product of hormonal interactions of the hypothalamic–pituitary–ovarian axis, which provide the driving force for all changes in ovarian physiology. However, it becomes increasingly obvious that the robust functions of the follicular apparatus and the ability to ovulate remain relatively undisturbed until a mean age of 45–46 years, at which stage most women have already been sterile for several years. It has been argued that the long-term stability of circulating hormone levels is a mechanism to compensate for the dramatic fall in the size of the follicle pool (Soules *et al.*, 2000). The several years earlier cessation of female fertility is largely explained by the age-related increase of meiotic non-disjunction leading to chromosomal aneuploidy and early embryo loss. Which specific age-related mechanisms cause an increased frequency of meiotic non-disjunction remains a matter of speculation. Although accumulation of genetic damage through oxidative stress has been proposed as a likely mechanism, it is also possible that specific ageing effects in the meiotic apparatus play a significant role. The complex process of meiosis evolved nearly 1 billion years ago and has remained essentially unchanged throughout the plant and animal kingdoms. Many of the genes concerned have been highly conserved and probably play an identical or very similar role in human meiosis to that in the meiosis of yeast and *C. elegans*. However, by comparison with

practically all other organisms, there is a unique aspect of meiosis in human females, namely the requirement for individual oocytes to remain meiotically competent for up to 40 years. It is not surprising that human oocytes frequently fail in their task of delivering a balanced haploid set of chromosomes to the next generation, particularly in older women, since they are operating on a design principle created and tested in reproductively short-lived organisms. The only other female mammals with a remotely comparable length of fertility are whales and elephants, for which there is no information available on the causes of cessation of fertility. However, mutational studies in model organisms, including yeast, *C. elegans* and Drosophila, will provide vital clues into which meiotic genes are particularly prone to the sort of ageing changes observed in humans.

The huge variation in the rate of follicle depletion is another key issue of reproductive ageing. Given the high heritability found for the age at menopause, this variation must be under genetic control by an as yet unknown number of genes. According to the genetic concept advanced in this review, several of those genes are likely to carry genetic variants, which largely determine the wide age differences for the various phases of reproductive ageing. It is logical to assume that we will only derive well-based solutions for predicting the course of reproductive ageing when we have a better understanding of the nature of the underlying genetic variability. The rapid developments in genomic and proteomic technology have opened the way to study reproductive ageing at a level which will permit us to distinguish which factors are involved in cause and effect. Such developments may lead us back to hormone metabolism and provide new insights from a different direction. However, to reach these goals there will have to be some radical changes in the way in which infertility research studies are conducted in the future. The emphasis will have to move away from a clinically driven setting to one in which molecular biologists make use of the enormous battery of genomic and proteomic techniques currently available in order to study various selected fundamental aspects of the ovarian ageing process. The main role of infertility physicians will be to interest molecular and cell biologists in the clinical relevance and scientific importance of carrying out such research.

The initial clinical goal of applying the information derived from such studies will be to construct DNA fingerprints that identify which women have a heritable predisposition to either early or late infertility. This would permit women to make a more informed choice on the possible reproductive consequences of delaying childbearing. In the long run, such information may also be used to design drugs, which may, in addition to their contraceptive action, delay the process of follicle depletion.

To a large extent, female emancipation has been an attempt to close the gap of social and economic inequalities between males and females. In this process, the male implicitly has been the standard for the female. Unique female biological features, such as the ability to become pregnant, deliver a child, breastfeed and take care of babies, have been ignored in this discussion. These essential female characteristics are often regarded as inconvenient and counterproductive in our efficiency-driven society. However, such social intolerance is short-sighted; in the long run society as a whole will benefit if women get wholehearted support to combine reproduction and work as a matter of course. Effective emancipation, both of women and men, will emphasize the positive aspects of male/female differences. In a society which not only tolerates but also welcomes these differences, delays in childbearing until ages when women are less fertile may no longer be necessary.

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