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Follicle dynamics and anovulation in polycystic ovary syndrome

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BACKGROUND: Polycystic ovary syndrome (PCOS) is the commonest cause of anovulatory infertility and menstrual cycle abnormalities, but the factors responsible for failure to select a dominant follicle remain unclear. METHOD: Source is authors' own studies and search of the relevant literature. RESULTS: Arrest of antral follicle growth is associated with an abnormal endocrine environment involving hypersecretion of luteinizing hormone and insulin (and perhaps hyperandrogenism). The net effect is secondary suppression of FSH, which leads to inhibition of maturation of otherwise healthy follicles in the cohort. There is, however, emerging evidence for an intrinsic abnormality of folliculogenesis in PCOS that affects the very earliest, gonadotrophin independent, stages of follicle development. There is an increased density of small pre-antral follicles and an increased proportion of early growing follicles. These abnormalities in anovulatory PCOS are further defined by abnormal granulosa cell proliferation and disparate growth of oocyte and surrounding granulosa cells. This suggests that the normal 'dialogue' between oocyte and granulosa cells in these early growing follicles is altered. There is evidence that abnormal, local (follicle-to-follicle) signalling of anti-Müllerian hormone may play a part in disordered folliculogenesis, but it is plausible that other local regulators that have been implicated in normal and abnormal pre-antral follicle development—such as insulin-like growth factors and sex steroids—have a role in aberrant folliculogenesis in PCOS. CONCLUSIONS: Significant abnormalities in the very earliest stages of folliculogenesis may be the root cause of anovulation in PCOS.

Keywords: mathematical modelling; statistics; assisted reproduction

Introduction

Polycystic ovary syndrome (PCOS) is the commonest endocrine disorder in women of reproductive age. In its classic form—a combination of oligo/anovulation and hyperandrogenism—it is estimated to affect >5% of the female population (Franks, 1995; Knochenhauer *et al*., 1998; Asuncion *et al*., 2000; Azziz *et al*., 2004; Ehrmann, 2005). It is the major cause of anovulatory infertility, menstrual disturbances and hirsutism. PCOS is also associated with a metabolic disturbance, central to which is peripheral insulin resistance and compensatory hyperinsulinaemia (Dunaif, 1997; Ehrmann, 2005). These metabolic abnormalities have implications both for reproductive function and for long-term health.

Although the most commonly recognized clinical manifestation of PCOS is the combination of symptoms of anovulation with those of androgen excess, it has become increasingly apparent that the spectrum of presentation of women with PCOS can be extended to embrace, on the one hand, women with anovulatory cycles (or amenorrhoea) who do not have clinical evidence of androgen excess (but who frequently have elevated serum concentrations of androgens) and, on the other, women with hirsutism who have regular, ovulatory cycles (Adams *et al*., 1986; Conway *et al*., 1989; Franks, 1989, 1995; Rotterdam, 2004a). These clinical categories are not necessarily rigid; it is not uncommon for subjects with polycystic ovaries and androgen excess to experience a mixture of ovulatory and anovulatory cycles (Baird *et al*., 1977; Yen, 1980; Franks, 1995) or, indeed, to change from a predominately anovulatory pattern to a mainly ovulatory picture (or vice versa) as may be seen, e.g. with changes in body weight (see below).

The recognition of the wider range of symptomatic presentation of women with polycystic ovaries is the main motivation that prompted a re-evaluation of the diagnostic criteria for PCOS at a joint meeting between the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) in Rotterdam in May 2003. The resulting Rotterdam consensus statement presented revised criteria for diagnosis, the merits of which have been extensively debated elsewhere (Rotterdam, 2004a, b; Azziz, 2006; Azziz *et al*., 2006; Franks, 2006). Nevertheless, there is evidence that the resulting phenotypic subgroups do indeed represent varying manifestations of the same syndrome, but there are also interesting differences between the subgroups (Dewailly *et al*., 2006; Welt *et al*., 2006; Barber *et al*., 2007; Norman *et al*., 2007) that may

shed some light on the mechanism of anovulation, as will be discussed later in this article.

This review is focused on the key features and possible mechanisms of abnormal folliculogenesis and anovulation in PCOS. We will not include a discussion of the clinical management of anovulation, which has been the subject of several recent reviews. Importantly, the proceedings of the recent 2nd ESHRE/ASRM consensus conference on PCOS, on the subject of induction of ovulation, are to appear shortly in both *Human Reproduction* and *Fertility and Sterility* (ESHRE/ASRM, 2008a, b).

Methods

For this review, we drew on published and unpublished data from our own laboratory, an 'in-house' library of relevant publications and a PubMed search which included the terms 'follicle development in PCOS' and 'anovulation in PCOS'.

Endocrine and metabolic features of anovulatory women with PCOS

The typical endocrine abnormalities of anovulatory women with PCOS are raised serum concentrations of androgens and luteinizing hormone (LH), with normal, or slightly suppressed serum FSH levels (Yen, 1980; Franks, 1995). Interestingly, elevated circulating LH and androgens are not specific to women with anovulation; they are also observed in hirsute women with polycystic ovaries and regular cycles (Adams *et al*., 1986; Franks, 1989, 1995), although LH concentrations tend to be higher in anovulatory cycles, due to failure of the negative feedback signal that is normally exerted by cyclical changes in estradiol and, in particular, progesterone. But there is an important difference in the metabolic profile of anovulatory compared with ovulatory women with PCOS and hyperandrogenism: insulin resistance and hyperinsulinaemia are features of anovulatory but not ovulatory subjects (Dunaif *et al*., 1987; Conway *et al*., 1989; Robinson *et al*., 1993; Barber *et al*., 2007). The association between anovulation and hyperinsulinaemia raises the question of a causal link between elevated insulin levels (and/or insulin resistance) and impaired ovulation. As discussed below, there is evidence that hyperinsulinaemia may indeed contribute to the mechanism of antral follicle dysfunction and anovulation in PCOS.

Obesity has a significant impact on the clinical and endocrine presentation of women with PCOS (Conway *et al*., 1989; Kiddy *et al*., 1990). Overweight women with PCOS are more likely to be anovulatory and to have symptoms of androgen excess. Furthermore, obesity affects reproductive outcome in response to induction of ovulation (Balen *et al*., 2006; Legro *et al*., 2007). Conversely, diet and lifestyle intervention, resulting in reduced calorie intake and a weight reduction of as little as 5–10%, is a successful strategy in terms of restoring ovulation and fertility (Kiddy *et al*., 1992; Clark *et al*., 1995, 1998; Homan *et al*., 2007). The mechanism of the effects of obesity on reproductive function is complex, but hyperinsulinaemia and/or insulin resistance appear to play an important part. There is a disadvantageous interaction between PCOS and obesity in that although obesity is associated with reduced insulin sensitivity in normal women, obese women with PCOS are relatively more insulin resistant than BMI-matched subjects with normal ovaries (Dunaif *et al*., 1987; Robinson *et al*., 1992; Franks, 1995; Dunaif, 1997; Ehrmann, 2005).

It is unlikely, however, that hyperinsulinaemia is the only significant factor to explain anovulation. Lean women with oligo/anovulation and hyperandrogenism often have normal insulin secretion and sensitivity (but are more likely to show hypersecretion of LH than obese women with PCOS) (Conway *et al*., 1989; Taylor *et al*., 1997). The Rotterdam criteria for diagnosis have introduced a 'new' category of subjects with PCOS, those with anovulation and polycystic ovaries who have neither clinical nor biochemical evidence of androgen excess (Dewailly *et al*., 2006; Welt *et al*., 2006; Barber *et al*., 2007; Norman *et al*., 2007). This subgroup is one that has attracted particular attention from those who are sceptical about the validity of the Rotterdam criteria (Azziz, 2006; Azziz *et al*., 2006), although, in our view, there is ample evidence to suggest that those women with polycystic ovaries and either estrogen-replete amenorrhoea or oligomenorrhoea are part of the same spectrum as those with anovulation and hyperandrogenism (Franks, 2006; Welt *et al*., 2006; Barber *et al*., 2007). Nevertheless, there is a striking difference in the metabolic profile between those with and without hyperandrogenism; the latter group have normal insulin levels and do not have reduced insulin sensitivity (Dewailly *et al*., 2006; Welt *et al*., 2006; Barber *et al*., 2007; Norman *et al*., 2007) (Fig. 1). Although the group with androgen excess are more obese than those without, the difference in insulin sensitivity remains significant after adjustment for BMI (Barber *et al*., 2007). In summary, endocrine and metabolic factors appear to have an influence on the development of anovulation in women with PCOS, but these factors do not exclude the possibility of an intrinsic abnormality of folliculogenesis in PCOS.

Oocyte quality and fertility in PCOS

Before discussing the characteristics of follicle development in PCOS and the relation to anovulation it is important to consider whether factors other than anovulation contribute to subfertility in women with PCOS. Broadly speaking, there is little evidence that either endometrial dysfunction or impaired oocyte function has a significant bearing on fertility. The most persuasive

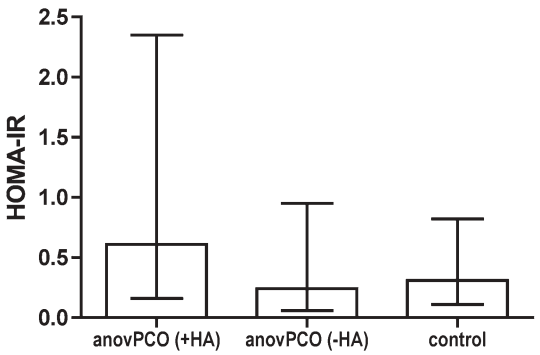

Figure 1: Insulin resistance, as measured by HOMA-IR, in subgroups of women with PCOS and controls subjects.
The subgroups were anovulatory women with hyperandrogenaemia (anovPCO (+HA)) or anovulatory women with normal androgens (anovPCO (−HA)). Values are geometric mean ± SD. HOMA-IR was higher in anovPCO (+HA) than controls ($P < 0.0001$) but similar to controls in women without hyperandrogenaemia (Data from Barber *et al*., 2007).

support for this statement is that appropriate treatment to restore ovulation in anovulatory women with PCOS typically leads to normal cumulative conception rates [see consensus statement from the ESHRE/ASRM meeting, Thessaloniki, February, 2007 (ESHRE/ASRM, 2008a, b)].

Nevertheless, endometrial 'abnormalities' in women with polycystic ovaries have been noted, including increased expression of androgen receptors (Apparao *et al*., 2002), and it remains possible that this has a small effect on fertility. A number of studies have investigated oocyte quality in women with polycystic ovaries undergoing assisted reproductive techniques. Although one study (using *in vitro*-matured oocytes) found that the rates of fertilization and normal embryo development were lower in women with PCOS than in those with regular cycles (Barnes *et al*., 1996), the weight of evidence suggests that the developmental potential of oocytes derived from women with PCOS is normal (Hardy *et al*., 1995; Mikkelsen and Lindenberg, 2001; Heijnen *et al*., 2006; Legro *et al*., 2007). As discussed in the next section, antral follicles in women with PCOS are heterogeneous and, in anovulatory subjects, include a proportion that has undergone premature arrest. The oocytes in such follicles are unlikely to have normal developmental potential but there remain sufficient 'healthy' follicles to provide normal oocytes after superovulation.

Abnormal antral follicle development and function

The characteristic morphological feature of polycystic ovaries in anovulatory women is accumulation of antral follicles in the range of 2–8 mm in diameter (Franks *et al*., 2000). In other words, there is an apparent failure to select a dominant follicle and it is assumed that the larger antral follicles (5–8 mm) have been arrested in development. The simple explanation for this phenomenon is that serum concentrations of FSH, while rarely frankly low, are suppressed below the 'threshold' level required during the early follicular phase to stimulate normal follicle maturation (Hillier, 1994). Indeed, increasing serum FSH (by treating with anti-estrogens or exogenous FSH) is the mainstay of successful induction of ovulation. The key question, however, is what is the reason for the suppression of FSH? Considerable insight into the mechanisms underlying abnormal follicle function has been obtained by systematic, predominately *in vitro* studies of the characteristics of antral follicles derived from polycystic ovaries.

Theca

The most consistent biochemical abnormality in women with PCOS is hypersecretion of androgens (Franks, 1991) and there is now evidence that hyperandrogenism reflects an intrinsic abnormality of theca cell function. Primary cultures of theca cells isolated from polycystic ovaries (whether from anovulatory or ovulatory subjects) demonstrate greatly increased production of androgens (Gilling-Smith *et al*., 1994). These studies also showed that the production of intermediate steroids in the androgen biosynthetic pathway was increased, suggesting a global up-regulation of steroidogenic enzymes. Although it can be argued that such abnormalities could reflect the abnormal endocrine environment from which the cells were derived (e.g. higher than normal levels of LH and/or insulin), it is significant that a similar overall increase in steroidogenesis has been noted in theca cells that have undergone several passages in culture (Nelson *et al*., 1999, 2001). Thus, theca cells from polycystic ovaries are characterized by constitutively increased activity of steroidogenic enzymes but this does not, of course, necessarily mean that such a phenotype represents the primary aetiological abnormality in PCOS. Many questions remain about the factors that control formation and activity of the theca cell layer during normal pre-antral folliculogenesis, let alone those involved in abnormal thecal development. Data presented later in this review suggest that the root of abnormal function of antral follicles may lie in the earliest stages of pre-antral follicle development.

Granulosa

Whereas there is little difference in the characteristics of theca cells between follicles obtained from ovulatory compared with anovulatory women with polycystic ovaries, there are major differences according to ovulatory status in granulosa cell steroidogenesis. These characteristics have been described in detail in primary publications and in previous reviews (Franks *et al*., 2000), but it is useful to summarize the findings here. The background to these studies was, at the time, the surprising observation that granulosa cells cultured from follicles derived from anovulatory women with PCOS were hyper-responsive to FSH in terms of estradiol production (Erickson *et al*., 1992; Mason *et al*., 1994) (Fig. 2). This seemed counter-intuitive given that impaired maturation of antral follicles is associated with suppressed activity of the enzyme $P450_{arom}$ (aromatase). Although reduced aromatase activity can readily be explained by the suboptimal level of circulating FSH (rather than an intrinsic abnormality of granulosa cells), the exaggerated response to exogenous FSH [which has since been confirmed in *in vivo* studies (Wachs *et al*., 2006)] is harder to fathom. The answer to this paradox appears to lie in the marked heterogeneity of antral follicles in anovulatory PCOS.

In subsequent experiments in our laboratory, granulosa cells were aspirated from individual follicles taken from unstimulated ovaries and cultured in the presence of FSH or LH. Granulosa cells from small to medium-sized antral follicles (1–9 mm in diameter) taken from women with ovulatory cycles and normal ovaries showed, as expected, a variable response to FSH and no

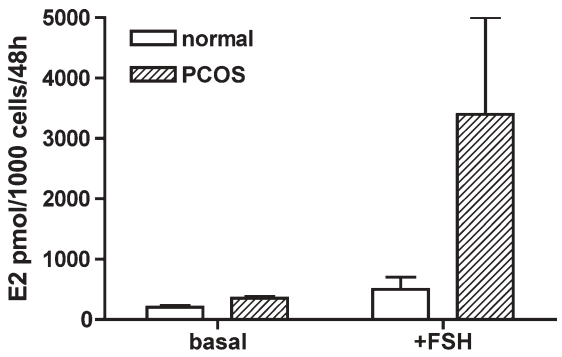

Figure 2: Estradiol response to FSH (mean ± SEM) in granulosa cells taken from follicles from ovaries of women with anovulatory PCOS or control subjects with normal ovaries and regular cycles.
The response to FSH was significantly greater ($P = 0.03$) in granulosa cells from PCOS compared with cells taken from normal ovaries (adapted from Mason *et al*., 1994).

response to LH in terms of production of estradiol and progesterone (Willis *et al*., 1998). Only granulosa cells taken from a larger (13 mm), dominant follicle, responded to LH. Cells derived from a similar spectrum of follicles from women with polycystic ovaries and regular cycles showed a very similar pattern, but there was a striking difference in the steroidogenic response of cells obtained from polycystic ovaries in women with chronic anovulation. The most important features were increased responsiveness, in terms of estradiol (and progesterone) production to FSH [echoing the results of previous studies (Erickson *et al*., 1992; Mason *et al*., 1994)] and inappropriate (premature) responsiveness to LH in some follicles as small as 2–4 mm. In addition there were high basal levels of these steroids with an exaggerated response to FSH and LH in a proportion of medium-sized antral follicles. Overall, there was much more heterogeneity of responsiveness among follicles from anovulatory women with PCOS than in the other two groups. In particular, small antral follicles (2–9 mm) from normal ovaries, and polycystic ovaries from ovulatory subjects were consistently unresponsive to LH but there was a much more variable response in follicles from polycystic ovaries in anovulatory women; some were inappropriately responsive to LH while others behaved as in normal ovaries (Willis *et al*., 1998).The implication of the premature responsiveness of granulosa cells in a proportion of small antral follicles is that inappropriate acquisition of LH receptors in granulosa cells (which in the normal follicular phase is confined to the dominant follicle) is likely to result in terminal differentiation in the granulosa layer (Hillier, 2001) and thence premature arrest of follicle growth.

We have proposed that hyperinsulinaemia and/or hypersecretion of LH may contribute to the aberrant response of granulosa cells to LH and to the consequent arrest of follicle growth (Robinson *et al*., 1993; Willis *et al*., 1996; Franks *et al*., 2000). In a separate series of studies we demonstrated that pre-incubation of normal granulosa cells in the presence of insulin synergistically amplified the subsequent steroidogenic response to LH (Willis *et al*., 1996). It might be argued that higher than normal circulating concentrations of insulin would have a reduced impact in a state of peripheral insulin resistance but there is evidence that in classical insulin target tissues such as muscle, insulin resistance in PCOS is a post-receptor abnormality and is signalling pathway-specific (Dunaif *et al*., 1992, 1995; Corbould *et al*., 2005, 2006). It is likely that this phenomenon also applies to insulin action in the ovary. Results of studies of insulin effects in granulosa-lutein cells from women with anovulatory PCOS indicate that whereas glucose uptake and metabolism is impaired (Lin *et al*., 1997; Fedorcsak *et al*., 2000; Rice *et al*., 2005), insulin-stimulated steroidogenesis (progesterone production) is intact and similar to the response in cells from normal ovaries (Rice *et al*., 2005).

The abnormal endocrine environment may therefore contribute to advancement of follicle maturation and premature arrest of development in a proportion of antral follicles, but this does not fully explain why healthy follicles within the cohort are unable to advance towards ovulation. The key to this 'suspension' of normal follicle growth is the suppression of FSH, which can be reversed by judicious administration of low-dose FSH resulting in the growth and subsequent ovulation of a healthy dominant follicle (White *et al*., 1996; Homburg and Howles, 1999; Franks *et al*., 2000). Thus, anovulation in PCOS is characterized by increased responsiveness of some follicles to FSH and LH, multiple follicle development, but arrested growth of antral follicles associated with suppression of serum FSH. On the face of it, the link between these phenomena and, in particular, the suppression of FSH is not easy to explain. Because of the negative non-linear feedback via the pituitary, the outcome of the interaction between a group of follicles during the selection phase is impossible to predict by intuition alone. In order to understand the integrated effect of these various factors, we therefore turned to mathematical modelling of follicle dynamics.

Modelling follicle dynamics in PCOS

Several models have been utilized to better understand unifollicular development and ovulation in the human cycle. The most extensively studied, and probably the best-known model has been developed by Lacker *et al*. (1987a, b). This is based on a simple feedback loop between ovary and pituitary whereby FSH stimulates follicle growth and estradiol production and as the level of estradiol in the circulation rises, it exerts negative feedback which leads to regression of all but the most responsive follicle.

Lacker's model tracks the maturity of a cohort of follicles as a function of time through the selection phase (Fig. 3). To initialize the model, we assign two properties to each follicle: (i) its initial maturity as it enters the terminal phase, and (ii) its sensitivity to gonadotrophins for any given level of maturity. The sensitivity of a follicle is thus a function of its maturity, rather than just a single number. Note that even a 'low sensitivity' follicle will respond strongly to gonadotrophins once it is mature enough.

Lacker allows the initial maturity to vary between follicles, but assumes that all follicles have the same sensitivity function. He shows that in such a case it is possible to get robust selection of the ovulating follicle. Thus heterogeneity in follicle sensitivity to gonadotrophins is not necessary to reproduce a normal pattern of ovulation. By changing the follicle sensitivity it is possible to obtain ovulation of more than one follicle, corresponding to other species.

Lacker's model can also exhibit a situation where a group of follicles arrest at a large size. It is even possible to choose sensitivity functions such that different choices of initial maturities lead either to arrest, or to ovulation. However, it can be mathematically proved that in such a model the number of follicles that can ovulate is always larger than the number that can arrest. Hence, if we initialized the model to exhibit the arrest of, say five follicles, minor variations in maturity or sensitivity would lead to cycles with at least six follicles ovulating. This is not consistent with human PCOS where one individual can show periods of arrest of a number of follicles interspersed with cycles where a single follicle ovulates.

The only way that one obtain a model that can both exhibit ovulation of a single follicle (Fig. 3A) and arrest of a number of follicles (Fig. 3B) is to have a population of follicles with a mixture of different sensitivities (Chavez-Ross *et al*., 1997). Simulation, supported by mathematical theorems, then shows that if at the start of the cycle we have one or more sufficiently mature 'low sensitivity' follicles, then one of these will dominate (Follicle 1 in Fig. 3A), suppressing all others which regress by atresia. This corresponds to a normal ovulatory cycles. On the other hand, if one or more follicles with higher sensitivity are

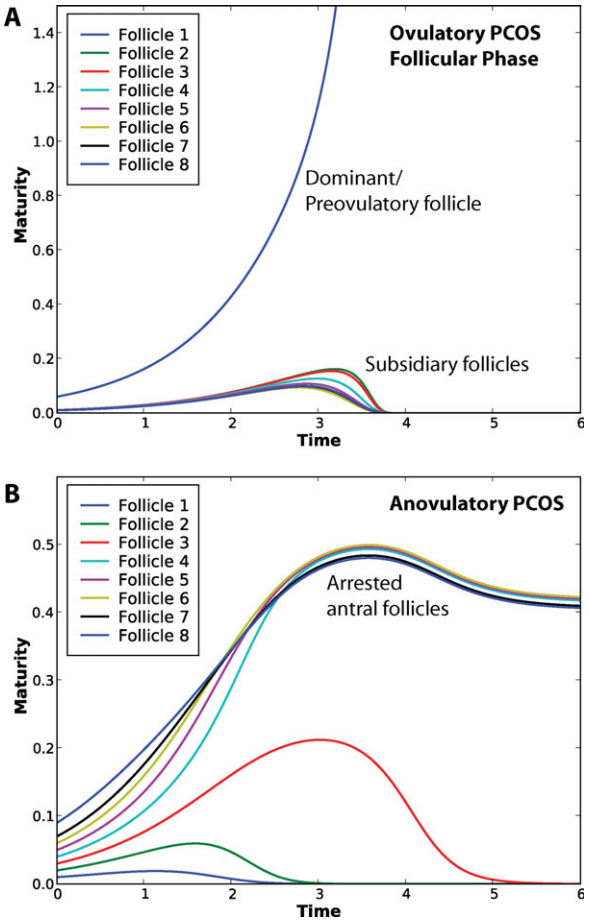

Figure 3: Mathematical modelling of follicle maturation in PCOS.
Two simulations of a cohort of eight follicles in the follicular phase, showing how the outcome depends on a subtle balance between the initial maturities and sensitivities of the follicles at the start of the phase. Each given follicle has the same sensitivity in both (**A**) and (**B**), but the initial maturity is different. In (A) Follicle 1 is more mature than the others, but has relatively low sensitivity to gonadotrophins. It develops normally and ovulates, suppressing the other follicles that die by atresia. The same qualitative outcome would be observed if there were several low sensitivity follicles of similar maturity; only one of these would go on to ovulate. In contrast, in (B) Follicles 4–8, which are high sensitivity follicles, are more mature at the start of the cycle. This leads to an arrested state where these five follicles can persist at a large size indefinitely. The remaining follicles die by atresia as before. Both time and maturity are measured in arbitrary units (adapted from Chavez-Ross *et al*., 1997).

relatively more mature at the start of the selection phase (Follicles 4–8 in Fig. 3B), then anovulatory arrest will result. This is because they too quickly reach a size where their estradiol output suppresses the other follicles from maturing. We emphasize that the only difference between Fig. 3A and B is the initial maturity of each follicle; all other properties of the model are identical. The precise outcome of each cycle depends subtly on the balance between maturity and sensitivity of the follicles at the start. If all follicles are 'low sensitivity' then normal ovulatory cycles as in Fig. 3A will always result, if the majority of follicles are 'high sensitivity' then arrest will usually occur as in Fig. 3B, and if there is a mixture in the ovary we may have periods of arrest interspersed with normal cycles.

From this analysis, we can suggest the following conclusions about the nature of PCOS (Chavez-Ross *et al*., 1997): (i) the primary cause of follicular dysfunction is at the level of the ovary, rather than the pituitary, and is characterized by variable responsiveness of follicles to gonadotrophins; (ii) arrested follicles have different properties from 'healthy' follicles (i.e. those that have the potential to ovulate). This is important because it implies that these properties may have already been determined during the pre-antral, gonadotrophin-independent stages of follicle development (see below); (iii) the abnormal response to gonadotrophins is best characterized by enhanced relative sensitivity to FSH (and LH) in a subpopulation of follicles (which of course is consistent with the observations from our experimental data) and (iv) those follicles that are hyper-responsive to gonadotrophins prematurely reach a level of maturity where they produce a sufficiently high concentration of circulating estradiol to suppress FSH to a level that is too low to encourage further development of healthy follicles in the cohort. Again, this conclusion is supported by observational data: in women with anovulatory PCOS, serum estradiol levels are slightly but significantly higher, and FSH lower, than in the normal early follicular phase. In summary, mathematical modelling provides a plausible pathway of interaction of follicular and endocrine factors that contribute to anovulation in PCOS but, as suggested in point (ii), the model raises the question of abnormal pre-antral follicle development. The mechanism by which abnormal early follicle development predisposes to later endocrine effects remains to be determined but one possible explanation is precocious acquisition of LH receptors in the granulosa layer.

Abnormal pre-antral folliculogenesis in PCOS

The gonadotrophin-dependent antral stages of follicle development represent only the latter few weeks in the life of a follicle. Progression of follicle development from the primordial (resting) stage to the late pre-antral stage in the human ovary probably takes several months (Gougeon, 1996; Hardy *et al*., 2000). Little is known about the factors that control initiation and progression of follicle growth in these early stages, but it is clear that local (paracrine and autocrine) factors are important and that gonadotrophins play little or no part (Gougeon, 1996). FSH receptors can be identified in granulosa cells as early as the primary stage of follicle growth (Oktay *et al*., 1997) [and there is evidence that FSH can enhance follicle development even at this stage (Wright *et al*., 1999)], but follicles are able to grow as far as the large pre-antral (or early antral) stage in the complete absence of bioactive FSH (Matthews *et al*., 1993). Data from studies of early follicle development in the rodent, domestic species and in primate ovaries suggest that growth factors of the transforming growth factor-β (TGFβ) superfamily (Elvin *et al*., 2000; Pangas and Matzuk, 2004), insulin-like growth factors (IGFs) (Monget and Bondy, 2000) and sex steroids (Vendola *et al*., 1998; Skinner, 2005) may all be implicated in initiation and maintenance of follicle growth. It is also clear that a 'dialogue' between oocyte and surrounding granulosa cells is a key element in normal follicle development (Eppig, 1991; Matzuk *et al*., 2002). Nevertheless, the critical factor (or combination of factors) involved remains to be clearly elucidated. In short, little is known about the regulation of pre-antral folliculogenesis in the normal human ovary, let alone in PCOS.

Our own recent studies of pre-antral follicle development in normal and polycystic ovaries were initially stimulated by a classic histological study published 25 years ago by Hughesdon (1982), a distinguished ovarian pathologist. He noted that, in histological ovarian sections taken from a series of women with normal and polycystic ovaries, there was not only an increase in the mean number of antral follicles per section in PCOS compared with normal ovaries, but that the number of primary and secondary follicles was also significantly higher in polycystic ovaries. These data clearly support the hypothesis that abnormalities of follicle development in PCOS may have their root in the early stages of growth. We had developed, in our laboratory, a method for studying pre-antral follicle development in small ovarian biopsies (Hovatta *et al*., 1999; Wright *et al*., 1999), obtained (with ethical approval and informed consent) from women undergoing routine laparoscopy. Tissue was fixed and prepared for histological examination either immediately after removal or after culture of pieces of tissue for up to 15 days.

In the first study of early folliculogenesis in PCOS, we compared the morphological characteristics of tissue fixed immediately after removal from subjects with (i) normal ovaries and regular cycles (ii) polycystic ovaries and regular cycles and (iii) polycystic ovaries and oligomenorrhoea or amenorrhoea (Webber *et al*., 2003). The key findings are illustrated in Fig. 4. The density of pre-antral follicles (including primordial to multilayered preantal follicles) was significantly increased in tissue from anovulatory women with PCOS, mainly due to an increase in the primary follicle pool. The follicle density in biopsies taken from ovulatory subjects with polycystic ovaries was intermediate between the other two groups. A subsequent publication from the group of Dr Gregory Erickson confirmed the increased accumulation (what they termed 'stockpiling') of primary follicles in polycystic ovaries (Maciel *et al*., 2004) and data from both studies are consistent with Hughesdon's earlier findings. An additional and novel finding in our study was the observation that the proportion of follicles that initiated growth was significantly higher (and the proportion of primordial follicles reciprocally lower) in polycystic ovary tissue (both from anovulatory and ovulatory women) compared with biopsies from normal ovaries. Thus, there is an important difference between normal and polycystic ovaries in the dynamics of early follicle development.

The key questions arising from these data are (i) what is the cause of the increased density of small pre-antral follicles and (ii) what is the cause of the increased proportion of growing follicles in polycystic ovaries?

More small pre-antral follicles in polycystic ovaries

The increased density of pre-antral follicles could be explained by a higher initial population of primordial follicles in the polycystic ovary which, in turn, may result from one or more of three mechanisms: (i) more primordial germ cells entering the ovary during fetal life, (ii) more cell divisions (of germ cells or granulosa cells) which could lead to (iii) more follicles being formed (Webber *et al*., 2003) (Fig. 5). Another explanation for the larger 'stock' of small pre-antral follicles and the longevity of this stock is that there is less follicle loss by atresia in polycystic ovaries (Fig. 5).

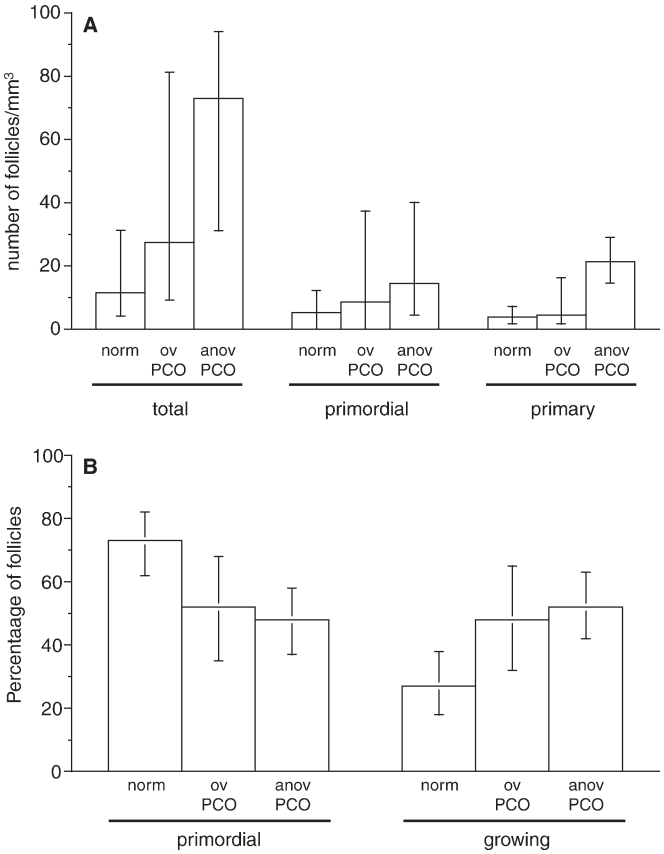

Figure 4: (**A**) density of pre-antral follicles (median, 95% CIs) in cortical biopsies from 22 women with normal ovaries, 14 ovulatory women with polycystic ovaries and 15 anovulatory women with polycystic ovaries.
Total pre-antral follicle density ($P = 0.009$) primary follicle density ($P = 0.004$) were greater in biopsies from anovulatory women with polycystic ovaries than in tissue from normal ovaries. (**B**) proportions (95% CIs) of total healthy pre-antral follicles at primordial and primary stages. Both ovulatory ($P = 0.03$) and anovulatory PCO groups ($P = 0.001$) differed significantly from normal. (from Webber *et al*., 2003).

We have used the human ovarian tissue slice culture system to study the growth and survival of pre-antral follicles in normal and polycystic ovaries (Webber *et al*., 2007). By culturing intact follicles within surrounding stroma, this system allows maintenance of normal interactions between oocyte, granulosa cells and thecal/stromal cells. Ovarian tissue was cultured for up to 15 days, then fixed in Bouin's solution, embedded in paraffin wax, serially sectioned and stained with haematoxylin and eosin. Follicles and enclosed oocytes were counted, measured and assessed for stage of development and health (i.e. categorized as either healthy or atretic). The proportion of atretic follicles was similar in biopsies from normal and polycystic ovaries in tissue fixed on the day of removal (Day 0), but during culture the proportion of atretic follicles was significantly lower in PCOS tissue (Webber *et al*., 2007) (Fig. 6). The reduced rate of follicle loss (or increased survival of follicles) in polycystic ovaries may therefore be an important contributory factor in the establishment of a higher density of pre-antral follicles in PCOS. Of course, the possible factor (or factors) responsible for the reduced rate of atresia in follicles from polycystic ovaries remains to be determined.

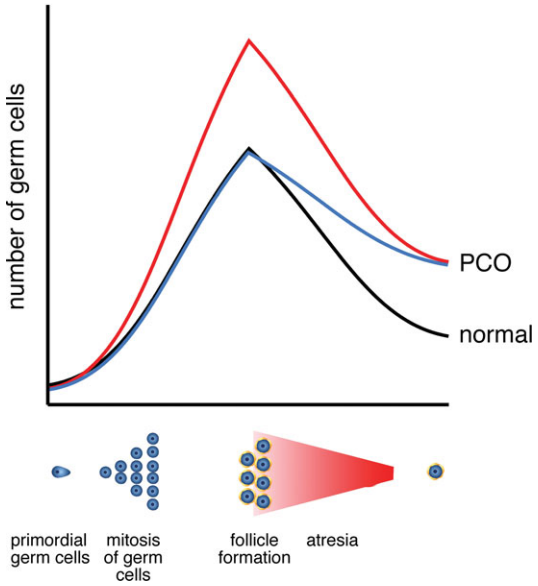

Figure 5: Fate of germ cells in the human fetal ovary and proposed mechanisms to explain increased density of small pre-antral follicles in the polycystic ovary.
There may be a higher initial population of primordial follicles (red line) or a slower rate of loss by atresia (blue line) (or both) (from Webber *et al*., 2003).

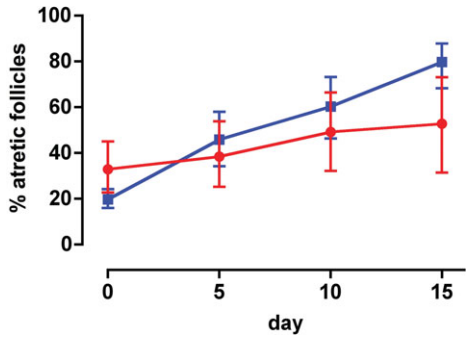

Figure 6: Increased survival in culture of preantral follicles from polycystic ovaries.
Reduced proportion of atretic pre-antral follicles during culture of ovarian tissue for anovulatory women with PCOS (red) compared to tissue from women with normal ovaries (blue) (from Webber *et al*., 2007).

An increased proportion of growing follicles in PCOS—a role for AMH?

Just as the search for candidate molecules that contribute to the increased density of small pre-antral follicles continues, so too does the quest for likely candidates that are responsible for the higher than normal proportion of early growing follicles in polycystic ovaries. One such candidate is anti-Müllerian hormone (AMH) which, in addition to its role during male sexual development, appears also to have an important role in the ovary. Expression of AMH, and the AMH-specific type 2 receptor in the ovary coincides with follicle growth (Ueno *et al*., 1989; Durlinger *et al*., 2002a, b), and there is good evidence that it is a key factor in regulating entry of follicles into the growing pool. Culturing mouse ovaries in the presence of AMH reduces the number of growing follicles (Durlinger *et al*., 2002a, b). In mice lacking the AMH gene, there is a reduction in the proportion of primordial follicles and a reciprocal increase in the proportion of growing follicles (Durlinger *et al*., 1999). These findings prompted us to explore the possibility that deficiency of AMH in small pre-antral follicles in polycystic ovaries contributes to disordered early folliculogenesis.

Using immunohistochemistry, with a highly specific antibody to AMH, we studied the expression of AMH protein in sections of ovary obtained from archived human ovarian tissue (Stubbs *et al*., 2005). Ovaries were carefully classified as belonging to one of three groups, according to histological, clinical, ultrasonographic and biochemical data: normal ovaries (with history of regular cycles and evidence of ovulation), polycystic ovaries from women with regular cycles and polycystic ovaries from women with anovulatory cycles or amenorrhoea. AMH was localized in granulosa cells of follicles of all sizes (including small pre-antral follicles) but a significantly smaller proportion of primordial and transitional (early growing) follicles from polycystic ovaries derived from anovulatory women expressed AMH compared with the pattern of expression in women with either normal or polycystic ovaries who had a history of regular, ovulatory cycles (Fig. 7). Extrapolating from studies in the AMH null mouse (Durlinger *et al*., 1999), we therefore proposed that such a deficiency of AMH in early growing follicles would result in an inappropriate increase in the proportion of follicles entering the growing phase. In other words, the observed reduction in AMH expression in small pre-antral follicles could cause, or at least contribute to the cause of, disordered folliculogenesis and anovulation.

These data may seem paradoxical given that a number of studies have shown that serum AMH concentrations are significantly higher in women with polycystic ovaries than in appropriate controls. However, the most abundant source of circulating AMH is small and medium-sized antral follicles and since this population of follicles is characteristically increased in polycystic ovaries and AMH production therefore significantly higher in polycystic than normal ovaries (refs). It is unlikely that any deficiency in the local production of AMH from small pre-antral follicles would be reflected in circulating AMH concentrations. In the mouse, exogenous AMH inhibits FSH-mediated follicle growth

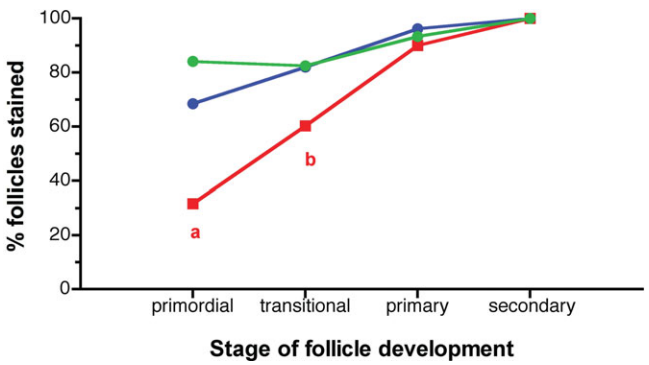

Figure 7: Proportion (%) of pre-antral follicles staining positive for AMH by immunohistochemistry in sections of ovarian tissue from normal ovaries (green), polycystic ovaries from ovulatory women (blue) and from anovulatory women (red).
The proportions of AMH positive primordial and transitional stages in anovulatory PCO were significantly lower (a: $p = 0.0015$; b: $p = 0.0017$) compared with the proportion in tissue from ovulatory subjects with either normal or polycystic ovaries (adapted from Stubbs *et al*., 2005).

(Durlinger *et al*., 2001). It is therefore conceivable that excess AMH produced by large pre-antral and small antral follicles plays a part in the aetiology of anovulation in PCOS but, as yet, there are no parallel data in human studies to support that hypothesis. It is unlikely, however, that the circulating high concentrations associated with PCOS have any significant impact on the relatively avascular cortical area in which is localized the vast majority of small pre-antral follicles. Nevertheless, the role of AMH as a paracrine signal in early follicle development in the human ovary remains open to question. One study found that mRNA for the (specific) type 2 AMH receptor was detectable in only a small proportion of small pre-antral follicles (Rice *et al*., 2007). Clearly, further studies are needed to elucidate the effects of AMH in pre-antral human follicles.

Granulosa cell proliferation in pre-antral follicles from normal and polycystic ovaries

A key question invited by our observations of pre-antral follicle dynamics in polycystic ovaries is: does the increased proportion of small growing follicles in PCOS reflect increased granulosa cell division compared with normal ovaries? To address this question, we used a marker of cell division that has been used to identify proliferating cells in cancer studies, namely mini-chromosome maintenance protein-2 (MCM-2) (Madine *et al*., 2000; Scott *et al*., 2003; Laskey, 2005). MCM-2 binds to DNA and 'licenses' DNA to replicate but ensures only one DNA replication per cycle. It persists throughout the cell cycle but is not present in quiescent (G0) or differentiated cells. We therefore considered this to be a good marker of proliferation in granulosa cells of early human pre-antral follicles. Once again, we used the archived human ovarian tissue to which we refer in the previous section, and performed systematic immunohistochemical analysis, using a specific antibody to human MCM-2 (Stubbs *et al*., 2007).

We were interested to see that even primordial follicles showed a small proportion of MCM-2 positive granulosa cells, suggesting that, contrary to current dogma, primordial follicles are not completely quiescent and that there may be a low level of cell division even in the resting pool of follicles. However, at each stage of pre-antral follicle development, from primordial up to and including the primary stage, there was a higher proportion of MCM-2 positive follicles in tissue from anovulatory women with polycystic ovaries than in either of the two ovulatory groups (Fig. 8). Oocytes were significantly larger and there were more granulosa cells per follicle in transitional and primary follicles in the anovulatory PCOS group. Although the diameter of the oocyte was greater in these early pre-antral stages in follicles from anovulatory women with polycystic ovaries, there was a disparity in the relationship between oocyte growth and granulosa cell proliferation. The increase in the number of granulosa cells was disproportionately greater than the increase in oocyte diameter in the follicles from anovulatory polycystic ovaries, emphasizing the aberrant dynamics of early follicle development (Fig. 9).

Candidate molecules in aberrant folliculogenesis

The finding of clear evidence of accelerated granulosa cell proliferation during the initial stages of follicle development obviously raises the question, once again, of which molecules are responsible for in the abnormal regulation of early

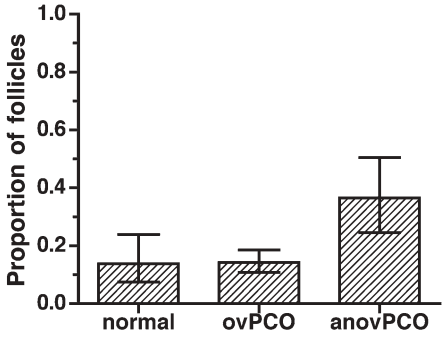

Figure 8: Proportions (95% CIs) of MCM-2 positive follicles in sections of normal and polycystic ovaries.
A higher proportion of follicles were MCM-2 positive in polycystic ovaries from women with anovulation (anovPCO) than in tissue from either normal ovaries ($P = 0.013$) or polycystic ovaries from women with regular cycles (ovPCO, $P = 0.0006$) (from Stubbs *et al*., 2007).

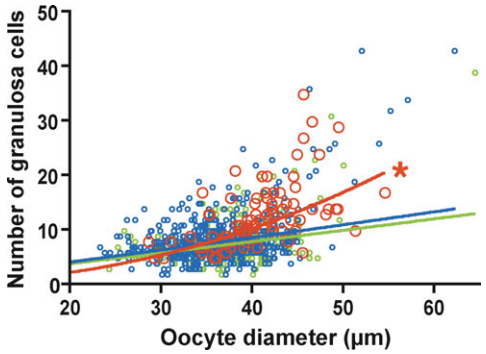

Figure 9: Relationship between oocyte diameter and granulosa cell number in follicles from polycystic ovaries from women with anovulation (red), polycystic ovaries from women with regular cycles (blue) and in normal ovaries (green). The lines are non-linear fits; the curvature of the anovPCO line is significantly different ($P = 0.0004$) from the ovPCO and normal lines (from Stubbs *et al*., 2007).

folliculogenesis. As yet we have no clear answers to this question but there are data to suggest that both IGFs (El-Roeiy *et al*., 1994; Voutilainen *et al*., 1996; Vendola *et al*., 1998, 1999), and androgens may have a role. Studies of folliculogenesis in the Rhesus monkey ovary have indicated that androgens can stimulate initiation of follicle development and that this effect may be mediated by increased expression of the type 1 IGF receptor (Vendola *et al*., 1998, 1999). IGF-1 has been reported to stimulate growth of human pre-antral follicles in tissue culture (Louhio *et al*., 2000), and preliminary data from our own laboratory support these findings. Prenatal exposure to androgens is associated with aberrant folliculogenesis in the ovaries of offspring in both the Rhesus monkey and the sheep (Eisner *et al*., 2002; Steckler *et al*., 2005; Abbott *et al*., 2006; Dumesic *et al*., 2007; Forsdike *et al*., 2007), although recent data suggest that this action may not be a direct effect of testosterone but rather by metabolism to estradiol (Steckler *et al*., 2007). If androgens are implicated in aberrant folliculogenesis in the ovary, the critical question is: what is the source of excess androgen? It is most likely to be produced by theca cells of the ovary but since theca formation is a key element of normal follicle development, it is unclear whether excess production of androgen by theca

(an intrinsic abnormality in the polycystic ovary, as discussed above) is the primary abnormality or whether it is a function of abnormal pre-antral folliculogenesis.

Some groups have turned to gene expression profiling of tissue from normal and polycystic ovaries to provide insight into the factors responsible for abnormal follicular function. As might be expected, the results highlighted several interesting metabolic pathways that were abnormally regulated in tissue from polycystic ovaries. In microarray studies of tissue (including theca, stroma, granulosa and oocytes) from women with normal and polycystic ovaries (as well as ovaries from testosterone-treated female to male transsexuals), dysregulated genes included those involved in Wnt signalling and in extracellular matrix formation (Jansen *et al*., 2004). Importantly, they noted that there was considerable overlap between the expression profiles of polycystic ovaries and those obtained after high-dose androgen treatment. In expression profiling of theca cell lines derived from polycystic ovaries, increased expression of genes encoding molecules involved in all-*trans* retinoic acid synthesis and in the transcription factor GATA-6 were among those differentially expressed when compared with cells from normal ovaries (Wood *et al*., 2003). In a very recently published study, microarray analysis of oocytes, obtained at the time of oocyte collection for *in vitro* fertilization, revealed differential expression of genes involved in chromosome segregation (Wood *et al*., 2007). Interestingly, many of the differentially expressed genes had putative androgen receptor binding elements, again suggesting that many of the differences between polycystic and normal ovaries in gene expression reflect 'downstream' effects of exposure to excess androgen.

Finally, some mention should be made about the search for candidate genes in PCOS and how that might provide clues to the nature of the factors involved in abnormal follicular function. To date, the results of candidate gene studies have proved largely disappointing (Franks and McCarthy, 2004; Nam Menke and Strauss, 2007; Urbanek, 2007). Recently, however, a locus on chromosome 19p has been reported to be associated with PCOS (Urbanek *et al*., 2005). Intriguingly, fine mapping of this locus puts it in the region of the gene coding for fibrillin 3 (FBN3) (Stewart *et al*., 2006). Fibrillins are extracellular matrix molecules primarily involved in tissue architecture (Isogai *et al*., 2003), but they are now known to have another role as regulators of TGFβ growth factors (Hubmacher *et al*., 2006) and the possible relevance to early follicle development in the ovary should not be overlooked. Nevertheless, these results, fascinating though they might be, remain to be confirmed in other large genetic studies and, at present, the link between FBN3 and ovarian abnormalities in PCOS can best be described as informed speculation.

Summary

Abnormalities of follicle development in polycystic ovaries are most obviously manifest in the later, antral stages of the life of the follicle, and we have provided an explanation that links the abnormal endocrine environment and arrested antral follicle development with the mechanism of anovulation. It is clear, however, that abnormal follicle development is not confined to the gonadotrophin-dependent antral stages. There are significant abnormalities of the very earliest stages of folliculogenesis which are most marked in polycystic ovaries from women with a history of oligo- or anovulation, suggesting that such abnormalities are at the root of (or at least highly relevant to) the aetiology of anovulation. The cause of these early abnormalities is not yet clear but the unanswered questions are an undeniably strong stimulus for further research in this intriguing area of reproductive biology and endocrinology.

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