Follistatin and activin A serum concentrations in obese and non-obese patients with polycystic ovary syndrome

T.Eldar-Geva[1,2,5], I.M.Spitz[2], N.P.Groome[3], E.J.Margalioth[1] and R.Homburg[4]

[1]IVF Unit and [2]Institute for Hormone Research, Shaare-Zedek Medical Center, Ben-Gurion University, P.O. Box 3235, Jerusalem, Israel, [3]Oxford Brookes University, Oxford, UK and [4]Fertility Clinic, Lis Maternity Hospital, Tel Aviv University, Tel Aviv, Israel

[5]To whom correspondence should be addressed at: IVF Unit, Shaare-Zedek Medical Center, P.O. Box 3235, Jerusalem 91031, Israel. E-mail: gevat@szmc.org.il

BACKGROUND: Activin promotes ovarian follicular development, inhibits androgen production and increases FSH and insulin secretion. Follistatin, an activin-binding protein, neutralizes activin bioactivity. Therefore, a decrease in the ratio of activin/follistatin might encourage characteristic features of polycystic ovary syndrome (PCOS). We investigated whether women with PCOS showed disordered follistatin and/or activin serum concentrations. METHODS: The study group included 24 obese and 20 non-obese (body mass index $\geq$ and <27 kg/m^2 respectively) clomiphene-failure PCOS patients. The control group included 16 obese and 46 non-obese patients with normal ovulatory cycles. Blood samples were obtained from the patients on day 3–5 of a progesterone-induced or spontaneous cycle and were assayed for LH, FSH, testosterone, 17-hydroxy-progesterone, androstenedione, follistatin, activin A, fasting glucose and insulin. RESULTS: Follistatin concentrations were comparable between obese and non-obese PCOS patients (mean $\pm$ SE; 1171 ± 103 and 1045 ± 159 pg/ml respectively) and significantly higher than their respective controls (628 ± 61 and 592 ± 49 pg/ml, $P < 0.0001$ and $P < 0.02$ respectively). Activin A concentrations were comparable between the four groups (590 ± 35, 513 ± 74, 661 ± 87 and 595 ± 43 pg/ml in obese and non-obese PCOS and controls respectively). Stepwise regression analyses for relationships between follistatin or activin A levels and all other variables indicated that follistatin was significantly and independently positively affected by PCOS ($P < 0.0001$), age ($P < 0.02$), androstenedione ($P < 0.03$) and weight ($P < 0.05$). Activin A was significantly and independently negatively affected by PCOS ($P < 0.003$) and FSH ($P < 0.03$), and positively affected by weight ($P < 0.009$) and androstenedione ($P < 0.02$). CONCLUSIONS: Serum follistatin is increased in PCOS patients, regardless of obesity. PCOS is the most significant variable that relates to high follistatin and low activin A serum concentration. A high follistatin/activin ratio could well contribute to the pathophysiology of PCOS.

Key words: activin A/follistatin/obesity/PCOS

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder, characterized by typical ovarian morphology, follicular arrest, anovulation, hyperandrogenism and insulin resistance, which are not always fully associated (Yen, 1999). The aetiology of PCOS has not yet been fully elucidated, although the observation of familial aggregation (Legro *et al.*, 1998; Urbanek *et al.*, 1999) is consistent with a genetic basis for this disorder. Growth factors are heavily involved in the pathophysiology, either contributing to or as a consequence of the arrested development of follicles, abnormal steroidogenesis, and hyperinsulinaemia (Homburg, 1998).

Follistatin is a monomeric glycosylated polypeptide chain, which was initially identified in and isolated from follicular fluid on the basis of its inhibition of pituitary FSH secretion (Robertson *et al.*, 1987; Ueno *et al.*, 1987). Later it was well documented that follistatin exerts this and other activities by neutralizing activin bioactivity, being an activin-binding protein (Mather *et al.*, 1997). Both follistatin and activin are expressed in numerous tissues, including the gonads, pituitary, adrenal cortex, liver and pancreas. Activin, a member of the transforming growth factor-β superfamily, promotes ovarian follicular development, inhibits thecal cell androgen production, increases pituitary FSH secretion and modulates pancreatic β-cell insulin secretion (Shibata *et al.*, 1996; Mather *et al.*, 1997; Florio *et al.*, 2000). A decrease in concentration or functional activity of activin, as well as an increase in follistatin, might therefore encourage characteristic features of PCOS.

Some studies support the assumption that high follistatin gene expression might be linked to PCOS. In transgenic mice, overexpression of follistatin resulted in arrested ovarian folliculogenesis, with or without suppression of serum concentrations of FSH (Guo *et al.*, 1998). Urbanek *et al.* tested 37

candidate genes for linkage and association with PCOS or hyperandrogenism in data from 150 families (Urbanek *et al.*, 1999). The strongest evidence for linkage was with the follistatin gene. Although later studies failed to identify any mutation in the follistatin gene in PCOS (Liao *et al.*, 2000; Urbanek *et al.*, 2000), these observations led us to investigate whether women with PCOS showed disordered follistatin and/or activin serum concentrations.

Recently, Norman *et al.* published their findings that circulating follistatin concentrations are higher and activin concentrations are lower in PCOS (Norman *et al.*, 2001). However, the question of a correlation of these findings with body mass index (BMI) has been left open. Therefore, we compared follistatin and activin A serum concentrations in obese and non-obese patients with PCOS or normal ovarian function.

Materials and methods

Subjects

One hundred and six patients aged 21–40 years and undergoing treatment in the infertility clinic in Shaare-Zedek Medical Center between March and December 2000 were included in the study. The study group (with PCOS) included 44 patients with typical ultrasonic ovarian morphology ($\geq$10 follicles with a diameter of <9 mm) (Adams *et al.*, 1985) and chronic menstrual irregularity (oligo- or amenorrhoea). They had all failed to ovulate in response to a maximal dose of clomiphene citrate, 150 mg daily from day 5–9 of a spontaneous or progesterone-induced bleed (clomiphene-resistant PCOS). The control group included 62 patients with normal ovulatory cycles with a mean length of 27–32 days and no endocrine abnormalities, such as hyperprolactinaemia or abnormal thyroid function. Each group was further divided into obese and non-obese (BMI $\geq$ and <27 kg/m^2 respectively). Patients being treated with steroids, oral hypoglycaemic agents or insulin were not included. All patients were in good physical and mental health. The Shaare-Zedek Medical Center Research and Ethics Committee, Jerusalem, approved the study protocol.

Protocol

Blood samples were obtained from PCOS patients after 35–90 days of amenorrhoea with no hormonal treatment. After ruling out the possibility of pregnancy (β-human chorionic gonadotrophin <10 IU/l) or spontaneous ovulation (progesterone <15 nmol/l), medroxyprogesterone acetate (Aragest, Dexon, Hadera, Israel) 10 mg/day was administered for 10 days. Second blood samples were obtained on day 3–5 of the cycle. Blood samples were obtained from the control group patients on day 3–5 (early follicular) and 20–22 (mid-luteal, confirmed by progesterone >25 nmol/l) of the cycle.

The samples were centrifuged and sera were stored at –20°C. Samples from day 3–5 were assayed for LH, FSH, testosterone, oestradiol, 17-hydroxy-progesterone (17-OH-P) and androstenedione ($\triangle$4A). All samples were assayed for total follistatin and total activin A.

Overnight fasting glucose and insulin concentrations were determined without regard to cycle day. Insulin resistance (IR) was considered to be present if fasting glucose/insulin ratio was <4.5 (Legro *et al.*, 1998).

Methods

Serum concentrations of testosterone, oestradiol, $\triangle$4A and 17-OH-P were determined using the Coat-a-Count radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). FSH and LH levels were measured using the Abbott IMX system (Abbott Diagnostics, Abbott Park, IL, USA). Follistatin was measured using two site-specific enzyme-linked immunosorbent assays (ELISA) for follistatin 288 as reported previously (Evans *et al.*, 1998). The assay sensitivity was 19 pg/ml and the between assay variation was 15%. The cross reactivity with follistatin 315 was 9.9%. Activin A was measured using highly sensitive two-site ELISA (Serotec, Oxford, UK). Glucose was measured by the glucose oxidase technique with a Vitros 950 instrument (Johnson and Johnson, USA) and insulin was measured with an Axsim instrument (Abbott Diagnostics).

Statistics

Results are presented as mean $\pm$ SE, unless otherwise indicated. Student's *t*-test was used to test for differences between groups. Normality of distribution was found for all variables. Stepwise regression analyses were performed with follistatin and activin A concentrations as dependent variables and diagnosis (PCOS or control), age, weight, BMI, LH, FSH, testosterone, $\triangle$4A and insulin sensitivity (normal or insulin-resistant) as independent parameters. Statistics Package for Social Sciences was used for all statistical analyses. Statistical significance was defined as $P < 0.05$.

Results

Serum follistatin concentration on cycle day 3–5 in PCOS patients was significantly higher than in the controls (mean $\pm$ SE; 1100 $\pm$ 91 and 601 $\pm$ 39 pg/ml respectively, $P < 0.0001$). Weight (73.3 $\pm$ 2.7 and 66.3 $\pm$ 2.1 kg respectively) and BMI (27.3 $\pm$ 0.9 and 24.8 $\pm$ 0.7 kg/m^2) of the PCOS patients were also significantly higher ($P < 0.05$). Therefore, each group was further divided into obese and non-obese (BMI $\geq$ and <27 kg/m^2 respectively) and the four groups were compared.

Serum follistatin and activin A on cycle day 3–5 in obese and non-obese PCOS and control groups are shown in Figure 1. Follistatin levels were comparable between obese and non-obese PCOS patient groups (mean $\pm$ SE; 1171 $\pm$ 103 and 1045 $\pm$ 159 pg/ml respectively) and significantly higher than their respective controls (628 $\pm$ 61 and 592 $\pm$ 49 pg/ml, $P < 0.001$ and $P < 0.05$ respectively). Activin A concentrations were comparable between the four groups (590 $\pm$ 35, 513 $\pm$ 74, 661 $\pm$ 87 and 595 $\pm$ 43 pg/ml in obese and non-obese PCOS groups and their corresponding controls respectively). Follistatin to activin ratios were 2.4 fold higher in PCOS patients compared with the controls (2.17 $\pm$ 0.21 and 0.92 $\pm$ 0.10 respectively, $P < 0.0001$) (Figure 2).

Clinical data and cycle day 3–5 serum hormone concentrations (mean $\pm$ SE) in obese and non-obese PCOS and control patients are shown in Table I. LH concentrations were 3-fold higher ($P < 0.001$) in both obese and non-obese PCOS patients compared with their respective controls, in the face of comparable FSH concentrations. LH concentrations were 50–60% higher ($P < 0.05$) in non-obese than obese groups. Testosterone concentrations were 2.5 and 1.5 fold higher in non-obese ($P < 0.05$) and obese ($P = 0.05$) PCOS patients than in their respective controls. $\triangle$4A concentrations were double in obese PCOS patiects compared with their respective controls ($P < 0.001$) and 1.5 fold higher in non-obese patients compared with obese controls ($P < 0.05$). Serum oestradiol concentrations were comparable in all groups. Concentrations
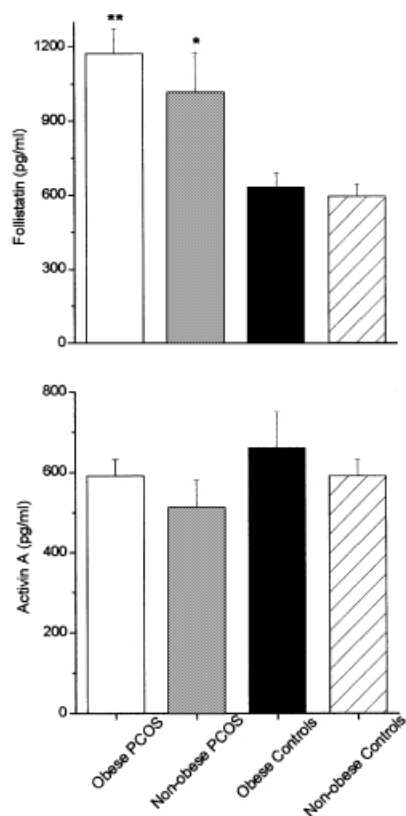
Figure 1. Serum follistatin and activin A in obese PCOS, non-obese PCOS, obese controls and non-obese controls (mean ± SE). *$P < 0.05$; **$P < 0.001$ versus corresponding controls.
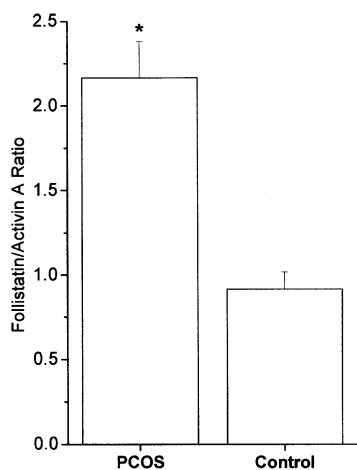
Figure 2. Serum follistatin/activin A ratio in PCOS and controls (mean ± SE). $P < 0.0001$.

of 17-OH-P were 2.3 and 1.4 times higher in obese ($P < 0.05$) and non-obese ($P < 0.05$) PCOS groups than in their respective controls.

In both PCOS groups, serum follistatin concentrations were similar after 35–90 days of amenorrhoea and on day 3–5 of progesterone-induced bleeding (1034 ± 151 and 1100 ± 91 pg/ml respectively). In the control group, serum follistatin concentrations were similar on cycle day 3–5 and 20–22 (605 ± 41 and 629 ± 46 pg/ml respectively). Serum activin A levels were also similar before and after progesterone-induced bleeding in the PCOS group (649 ± 80 and 593 ± 70 pg/ml

respectively) and on cycle day 3–5 and 20–22 in the control group (599 ± 35 and 666 ± 69 pg/ml respectively).

To determine which independent variables affect serum follistatin and activin A concentrations, stepwise linear regression analyses with follistatin and activin A as the dependent variables and the presence of PCOS and IR, age, weight, BMI, serum LH, FSH, T, △4A and 17-OH-P concentrations were performed. Eighty-eight patients, who had no missing data, were included in the regression analyses. Follistatin concentrations were significantly and independently positively affected by PCOS ($P < 0.0001$), age ($P < 0.02$), △4A levels ($P < 0.03$) and weight ($P < 0.05$). Activin A concentrations were significantly and independently negatively affected by PCOS ($P < 0.003$) and day 3–5 FSH concentrations ($P < 0.03$), and positively affected by weight ($P < 0.009$) and △4A levels ($P < 0.02$). IR correlated with follistatin concentrations in both PCOS and control groups ($r = 0.30$ and $r = 0.28$ respectively, $P < 0.05$). However, stepwise regression analysis showed that IR was not a statistically significant independent factor affecting follistatin concentrations; it also positively correlated with weight and BMI ($r = 0.34$ and $r = 0.31$ respectively, $P < 0.05$), and negatively correlated with LH ($r = –0.41$, $P < 0.01$) in the PCOS patients.

Discussion

Our study was instigated by recent reports that follistatin can encourage and activin can oppose many features of PCOS, such as increased ovarian androgen synthesis, decreased pituitary FSH, disturbed pancreatic insulin secretion and inhibition of follicular development. Our data indicated that the serum follistatin/activin ratio was 2.4 fold higher in PCOS patients compared with controls. Follistatin was increased by 80–90% in PCOS patients, independent of obesity. A negative influence of PCOS on activin A levels was found by stepwise regression analysis. Moreover, PCOS was the most significant variable that independently increased follistatin and decreased activin A serum levels when weighed against age, weight, BMI and hormonal status. All these results achieved high statistical significance ($P < 0.003$ to $P < 0.0001$).

Following the completion of our study, Norman *et al.* published their findings and quite independently discovered follistatin and activin concentrations in PCOS, very similar to our findings (Norman *et al.*, 2001). We have, in addition, answered the question which Norman *et al.* left open, i.e. a correlation of these findings with BMI. We have demonstrated that weight is not the explanatory factor.

It has been shown that virtually all circulating follistatin in women (Muttukrishna *et al.*, 1996; McConnell *et al.*, 1998) and girls (Foster *et al.*, 2000; Phillips *et al.*, 2000b) is activin-bound. In contrast, a substantial amount of free follistatin has been detected in the follicular fluid and pituitary. These observations are compatible with the hypothesis that the main biological role of circulating follistatin is to restrict activin bioavailability. Currently, there is no reliable method to measure free activin levels due to disruption of activin/follistatin complexes during the assay procedures. Using the same immunoassay described above, changes in the ratio between total follistatin and activin A were found in pregnant women with

Table I. Clinical data and day 3–5 serum hormone concentrations in obese and non-obese polycystic ovary syndrome (PCOS) and control patients (values are mean ± SE)

Characteristic	Obese PCOS (n = 24)	Non-obese PCOS (n = 20)	Obese controls (n = 16)	Non-obese controls (n = 46)
Age (years)	29.4 ± 1.0	27.3 ± 0.8[a]	30.5 ± 1.2	29.9 ± 0.6
Weight (kg)	86.4 ± 2.4[b]	57.5 ± 2.1	87.4 ± 3.9[b]	58.9 ± 1.4
BMI (kg/m^2)	32.0 ± 0.8[b]	21.7 ± 0.7	32.1 ± 1.2[b]	22.3 ± 0.4
LH (IU/l)	10.2 ± 1.5[c,d]	16.4 ± 2.1[c]	3.4 ± 0.3[d]	5.2 ± 0.3
FSH (IU/l)	5.1 ± 0.4	5.6 ± 0.4	5.3 ± 0.4	5.9 ± 0.4
Testosterone (nmol/l)	2.4 ± 0.2	4.3 ± 1.0[a]	1.6 ± 0.3	1.7 ± 0.1
△4A (nmol/l)	7.8 ± 0.8[c]	7.5 ± 1.5	3.9 ± 0.3[d]	5.7 ± 0.6
Oestradiol (pmol/l)	190 ± 25	237 ± 45	137 ± 19	168 ± 13
17-OH-P (nmol/l)	4.5 ± 1.0[a]	2.9 ± 0.3[a]	2.0 ± 0.3	2.1 ± 0.2

[a]$P < 0.05$, PCOS versus corresponding controls; [b]$P < 0.0001$, obese versus corresponding non-obese;
[c]$P < 0.001$, PCOS versus corresponding control; [d]$P < 0.05$, obese versus corresponding non-obese.
BMI = body mass index; △4A = androstenedione; 17-OH-P = 17-hydroxy-progesterone.

hypertensive disorders (D'Antona *et al.*, 2000), suggesting that unbound, biologically active activin A is increased in these disorders. Our results support the assumption that high follistatin levels accompanied by a low biologically active activin A level contribute to the pathophysiology of PCOS.

The source of serum follistatin and activin in women is unascertained. Both peptides are secreted from ovarian granulosa cells in response to FSH stimulation (Vale *et al.*, 1990). The concentrations of follistatin and activin A in follicular fluid exceed serum concentrations by as much as 100–200 and 30-fold respectively (Erickson *et al.*, 1995; Khoury *et al.*, 1995; Muttukrishna *et al.*, 1996; Evans *et al.*, 1998). However, follistatin concentrations remain stable with no consistent trend across the menstrual cycle (Demura *et al.*, 1993; Khoury *et al.*, 1995; Kettel *et al.*, 1996; Evans *et al.*, 1998). Furthermore, serum follistatin concentrations have been found to be similar in eugonadal women, women with hypothalamic amenorrhoea and women after oophorectomy (Khoury *et al.*, 1995), and did not change during puberty or after ovarian suppression with gonadotrophin-releasing hormone analogue administration (Kettel *et al.*, 1996). Other investigators found that serum total activin A concentration did not vary significantly across the peri-menopause in women (Muttukrishna *et al.*, 2000) or pubertal stages in girls (Foster *et al.*, 2000). However, follistatin concentrations in late puberty were less than those in early puberty (Phillips *et al.*, 2000b). Post-menopausal women had higher (Kettel *et al.*, 1996) or similar (Khoury *et al.*, 1995) follistatin levels than young cycling women. Variation in serum activin A during the normal menstrual cycle is much less than that observed for inhibins (Harada *et al.*, 1996; Knight *et al.*, 1996; Muttukrishna *et al.*, 1996). No changes in serum activin A were found in IVF patients after pituitary down-regulation and during FSH ovarian stimulation (Lockwood *et al.*, 1996). In the present study, we also found no differences in either follistatin or activin A levels between early follicular and mid-luteal phases in normal cycling women, or before and after progesterone administration in PCOS women. These data argue against the ovary as a significant source of circulating follistatin or activin A.

Interestingly, no differences in the levels of follistatin were found in follicular fluids from healthy, atretic or polycystic ovaries (Erickson *et al.*, 1995). There was no difference in follicular fluid activin A concentration between PCOS and size-matched follicles from normally cycling women (Magoffin and Jakimiuk, 1998). Lambert-Messerlian *et al.* found increased levels of follicular fluid follistatin concentrations with follicular volume in normal cycling IVF patients, but not in PCOS patients (Lambert-Messerlian *et al.*, 1997). It is difficult to explain why follistatin mRNA was entirely absent from sections of polycystic ovaries examined by in-situ hybridization compared with normal ovaries (Roberts *et al.*, 1994). Together, our finding and the above reports implicate an extra-gonadal origin of the high circulating follistatin in PCOS.

Nevertheless, both follistatin and activin have multiple sites of production and multiple inducers. For example, changes in activin/follistatin have been found in atherosclerosis (Kozaki and Ouchi, 1998), inflammatory response (Jones *et al.*, 2000), septicaemia (Michel *et al.*, 1998) and neoplasia (Danila *et al.*, 2000). The exclusion of patients treated with anticoagulants such as heparin is also important, since heparin administration can induce an increase in circulating levels of both activin A and follistatin (Phillips *et al.*, 2000a). An increased synthesis or secretion of follistatin from the liver, adrenal glands, pancreas, endothelial cells or another organ or tissue in PCOS patients could be speculated as a source of the high serum follistatin concentrations in this syndrome. In fact, follistatin mRNA levels in cultured fibroblasts from PCOS and control women hardly differ (Urbanek *et al.*, 2000). However, as with insulin resistance in PCOS, there might be tissue specificity and fibroblasts are not the main physiological source of serum follistatin.

In 1999 a large group of investigators reported strong evidence for a link between the follistatin gene and PCOS (Urbanek *et al.*, 1999). However, in a later expanded study of 249 families, the evidence for linkage between PCOS and the single rare mutation found was weak (Urbanek *et al.*, 2000). Furthermore, no mutations could be found in the entire coding region of the follistatin gene in 64 Chinese patients with PCOS (Liao *et al.*, 2000). However, the whole process of follistatin mRNA and protein synthesis, processing, binding and secretion is very complex. The existence of multiple follistatin isoforms with different bioactivities may provide another mechanism

for the control of follistatin activity through alterations in post-transcriptional processing (Esch *et al.*, 1987).

In summary, we found that the serum follistatin/activin ratio was increased in PCOS patients compared with controls. Serum follistatin was increased in PCOS patients regardless of obesity. Using stepwise regression, we showed that PCOS was the most significant variable that increased follistatin and decreased activin A serum levels when weighed against age, weight, BMI and hormonal status. We postulate that by neutralizing the bioactivity of activin, high serum follistatin levels could well contribute to the pathophysiology of PCOS. While the measurement of follistatin and activin will probably not be relevant for the diagnosis of PCOS, their role in the patho-physiology of this condition and the possibility of developing therapeutic alternatives make further investigation imperative.

Acknowledgements

References

Adams, J., Franks, S., Polson, D.W. *et al.* (1985) Multifollicular ovaries: clinical and endocrine features and response to pulsatile gonadotropin-releasing hormone. *Lancet*, **ii**, 1375–1379.

Danila, D.C., Inder, W.J., Zhang, X. *et al.* (2000) Activin effects on neoplastic proliferation of human pituitary tumors. *J. Clin. Endocrinol. Metab.*, **85**, 1009–1015.

D'Antona, D., Reis, F.M., Benedetto, C. *et al.* (2000) Increased maternal serum activin A but not follistatin levels in pregnant women with hypertensive disorders. *J. Endocrinol.*, **165**, 157–162.

Demura, R., Suzuki, T., Tajima, S. *et al.* (1993) Human plasma free activin and inhibin levels during the menstrual cycle. *J. Clin. Endocrinol. Metab.*, **76**, 1080–1082.

Erickson, G.F., Chung, D.G., Sit, A. *et al.* (1995) Follistatin concentrations in follicular fluid of normal and polycystic ovaries. *Hum. Reprod.*, **10**, 2120-2124.

Esch, F.S., Shimasaki, S., Mercado, M. *et al.* (1987) Structural characterization of follistatin: a novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad. *Mol. Endocrinol.*, **1**, 849–855.

Evans, L.W., Muttukrishna, S., Groome, N.P. *et al.* (1998) Development, validation and application of an ultra-sensitive two-site enzyme immunoassay for human follistatin. *J. Endocrinol.*, **156**, 275–282.

Florio, P., Luisi, S., Marchetti, P. *et al.* (2000) Activin A stimulates insulin secretion in cultured human pancreatic islets. *J. Endocrinol. Invest.*, **23**, 231–234.

Foster, C.M., Phillips, D.J., Wyman, T. *et al.* (2000) Changes in serum inhibin, activin and follistatin concentrations during puberty in girls. *Hum. Reprod.*, **15**, 1052–1057.

Guo, Q., Kumar, T.R., Woodruff, T. *et al.* (1998) Overexpression of mouse follistatin causes reproductive defects in transgenic mice. *Mol. Endocrinol.*, **12**, 96–106.

Harada, K., Shintani, Y., Sakamoto, Y. *et al.* (1996) Serum immunoreactive activin A levels in normal subjects and patients with various diseases. *J. Clin. Endocrinol. Metab.*, **81**, 2125–2130.

Homburg, R. (1998) Involvement of growth factors in the pathophysiology of polycystic ovary syndrome. *Gynecol. Endocrinol.*, **12**, 391–397.

Jones, K.L., Brauman, J.N., Groome, N.P. *et al.* (2000) Activin A release into the circulation is an early event in systemic inflammation and precedes the release of follistatin. *Endocrinology*, **141**, 1905–1908.

Kettel, L.M., DePaolo, L.V., Morales, A.J. *et al.* (1996) Circulating levels of follistatin from puberty to menopause. *Fertil. Steril.*, **65**, 472–476.

Khoury, R.H., Wang, Q.F., Crowley, W.F. Jr *et al.* (1995) Serum follistatin levels in women: evidence against an endocrine function of ovarian follistatin. *J. Clin. Endocrinol. Metab.*, **80**, 1361–1368.

Knight, P.G., Muttukrishna, S. and Groome, N.P. (1996) Development and application of a two-site enzyme immunoassay for the determination of 'total' activin-A concentrations in serum and follicular fluid. *J. Endocrinol.*, **148**, 267–279.

Kozaki, K. and Ouchi, Y. (1998) Activin/follistatin and atherosclerosis—a review. *J. Atheroscler. Thromb.*, **5**, 36–40.

Lambert-Messerlian, G., Taylor, A., Leykin, L. *et al.* (1997) Characterization of intrafollicular steroid hormones, inhibin, and follistatin in women with and without polycystic ovarian syndrome following gonadotropin hyperstimulation. *Biol. Reprod.*, **57**, 1211–1216.

Legro, R.S., Spielman, R., Urbanek, M. *et al.* (1998) Phenotype and genotype in polycystic ovary syndrome. *Recent. Prog. Horm. Res.*, **53**, 217–256.

Liao, W.X., Roy, A.C. and Ng, S.C. (2000) Preliminary investigation of follistatin gene mutations in women with polycystic ovary syndrome. *Mol. Hum. Reprod.*, **6**., 587–590.

Lockwood, G.M., Muttukrishna, S., Groome, N.P. *et al.* (1996) Circulating inhibins and activin A during GnRH-analogue down-regulation and ovarian hyperstimulation with recombinant FSH for in-vitro fertilization–embryo transfer. *Clin. Endocrinol. (Oxf.)*, **45**, 741–748.

Magoffin, D.A. and Jakimiuk, A.J. (1998) Inhibin A, inhibin B and activin A concentrations in follicular fluid from women with polycystic ovary syndrome. *Hum. Reprod.*, **13**, 2693–2698.

Mather, J.P., Moore, A. and Li, R.H. (1997) Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. *Proc. Soc. Exp. Biol. Med.*, **215**, 209–222.

McConnell, D.S., Wang, Q., Sluss, P.M. *et al.* (1998) A two-site chemiluminescent assay for activin-free follistatin reveals that most follistatin circulating in men and normal cycling women is in an activin-bound state. *J. Clin. Endocrinol. Metab.*, **83**, 851–858.

Michel, U., Shintani, Y. and Nau, R. (1998) Serum follistatin concentrations are increased in patients with septicaemia. *Clin. Endocrinol. (Oxf.)*, **48**, 413–417.

Muttukrishna, S., Fowler, P.A., George, L. *et al.* (1996) Changes in peripheral serum levels of total activin A during the human menstrual cycle and pregnancy. *J. Clin. Endocrinol. Metab.*, **81**, 3328–3334.

Muttukrishna, S., Child, T., Lockwood, G.M. *et al.* (2000) Serum concentrations of dimeric inhibins, activin A, gonadotrophins and ovarian steroids during the menstrual cycle in older women. *Hum. Reprod.*, **15**, 549–556.

Norman, R.J., Milner, C.R., Groome, N.P. and Robertson, D.M. (2001) Circulating follistatin concentrations are higher and activin concentrations are lower in polycystic ovarian syndrome. *Hum. Reprod.*, **16**, 668–672.

Phillips, D.J., Jones, K.L., McGaw, D.J. *et al.* (2000a) Release of activin and follistatin during cardiovascular procedures is largely due to heparin administration. *J. Clin. Endocrinol. Metab.*, **85**, 2411–2415.

Phillips, K.P., Leveille, M.C., Claman, P. *et al.* (2000b) Changes in serum inhibin, activin and follistatin concentrations during puberty in girls. *Hum. Reprod.*, **15**, 1052–1057.

Roberts, V.J., Barth, S., el-Roeiy, A. *et al.* (1994) Expression of inhibin/activin system messenger ribonucleic acids and proteins in ovarian follicles from women with polycystic ovarian syndrome. *J. Clin. Endocrinol. Metab.*, **79**, 1434–1439.

Robertson, D.M., Klein, R., de Vos, F.L. *et al.* (1987) The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochem. Biophys. Res. Commun.*, **149**, 744–749.

Shibata, H., Kanzaki, M., Takeuchi, T. *et al.* (1996) Two distinct signaling pathways activated by activin A in glucose-responsive pancreatic beta-cell lines. *J. Mol. Endocrinol.*, **16**, 249–258.

Ueno, N., Ling, N., Ying, S.Y. *et al.* (1987) Isolation and partial characterization of follistatin: a single-chain Mr **35** 000 monomeric protein that inhibits the release of follicle-stimulating hormone. *Proc. Natl Acad. Sci. USA*, **84**, 8282–8286.

Urbanek, M., Legro, R.S., Driscoll, D.A. *et al.* (1999) Thirty-seven candidate genes for polycystic ovary syndrome: strongest evidence for linkage is with follistatin. *Proc. Natl Acad. Sci. USA*, **96**, 8573–8578.

Urbanek, M., Wu, X., Vickery, K.R. *et al.* (2000) Allelic variants of the follistatin gene in polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.*, **85**, 4455–4461.

Vale, W., Hsueh, A.J., Rivier, C. *et al.* (1990) The inhibin/activin family of hormones and growth factors. In Sporn, M.B. and Roberts, A.B. (eds) *Peptide Growth Factors and Their Receptors II.* Springer-Vertag, New York, pp. 211–248.

Yen, S.S.C. (1999) Polycystic Ovary Syndrome (Hyperandrogenic Chronic Anovulation). In Yen, S.S.C., Jaffe, R.B. and Barbieri, R.L (eds) *Reproductive Endocrinology*, 4th edition, W.B. Saunders Company, Philadelphia, Pennsylvania USA, pp. 436–478.