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ORIGINAL ARTICLE *Reproductive endocrinology*

Insulin resistance and free androgen index correlate with the outcome of controlled ovarian hyperstimulation in non-PCOS women undergoing IVF

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BACKGROUND: The dual effects of insulin and androgen on the ovary act to promote early folliculogenesis. In the context of polycystic ovarian syndrome (PCOS), the presence of hyperinsulinaemia, resulting from increased insulin resistance (IR), and hyperandrogenaemia lead to the appearance of multiple antral follicles and frequently a multi-follicular response to gonadotrophin stimulation for assisted reproductive treatments (ARTs). The effect of IR and androgen status in women without PCOS on the follicular outcome of controlled ovarian hyperstimulation (COH) is not known.

METHODS: We assessed the IR [using the homeostasis model assessment (HOMA)] and androgen status of 49 women without PCOS undergoing an ART cycle. This was then related to the treatment cycle outcome.

RESULTS: We found a significant positive correlation between HOMA and BMI, and free androgen index (FAI) and testosterone. The FAI significantly positively correlated with total follicle count after COH. The total follicle count was significantly higher in those with a HOMA >2.5, and HOMA positively correlated with total follicle count in this group of IR women (HOMA > 2.5).

CONCLUSIONS: Our results suggest a positive correlation of HOMA-IR levels above a threshold level of 2.5 and a continuous positive correlation of free androgen (FAI) to total ovarian follicle count following COH in the non-PCOS patient.

Key words: insulin resistance / free androgen index / testosterone / ovarian stimulation / ovarian follicle count

Introduction

The ovarian response to controlled ovarian hyperstimulation (COH) with exogenous gonadotrophin during assisted reproductive treatment (ART) is often difficult to predict and dependent on multiple factors such as age (Engmann *et al*., 1999), body mass index (BMI) (Balen *et al*., 2006), ovarian reserve and endocrinopathy (Homburg, 2003; Mulders *et al*., 2003).

Metabolic factors such as androgen and insulin levels have also been suggested to have important roles in fertility and folliculogenesis within the ART setting. Studied in the context of polycystic ovarian syndrome (PCOS), hyperinsulinaemia, resulting from increased insulin resistance (IR), a key feature of the syndrome, is associated with a higher rate of anovulatory infertility (Robinson *et al*., 1993). However, in response to gonadotrophin stimulation for IVF, IR is frequently associated with a multi-follicular response, and this group of patients with IR are, in addition, more prone to ovarian hyperstimulation syndrome (OHSS) and ART cycle cancellation (Fulghesu *et al*., 1997; Dale *et al*., 1998).

The process of folliculogenesis is complex. Females are born with a fixed pool of primordial follicles, the majority of which remain in a resting pool, while only a fraction are selected to enter the growth process (Gougeon, 1986, 1996). The recruitment of primordial follicles and the transition to primary follicle are coordinated by both locally produced para- and autocrine growth factors and factors affecting atresia (Erickson and Shimasaki, 2000; Skinner, 2005).

Insulin receptors are present on ovarian stromal cells and the granulosa and theca cells of the developing follicles (Kezele *et al*., 2002; Phy *et al*., 2004). An *in vitro* study by Kezele *et al*. (2002) demonstrated that insulin increases the primordial to primary follicle transition (Kezele *et al*., 2002). In addition, insulin growth factors have been shown to increase the FSH responsiveness of granulosa cells when

follicles enter the gonadotrophin-dependent stages of folliculogenesis (Monget and Bondy, 2000). The increased levels of insulin and IGFs in women with PCOS therefore play a significant role in the disordered folliculogenesis typically seen (Franks *et al*., 2008).

The role of androgen in folliculogenesis has become clearer in recent years. In the primate ovary, androgens increase the recruitment of primordial follicles to the growing pool and also increase follicular IGF-1 and IGF-1 receptor expression up to the small antral follicle stage (Vendola *et al*., 1999a, b). Webber *et al*. (2003) demonstrated that polycystic ovaries are characterized histologically by higher numbers of growing primary and pre-antral follicles, along with a decrease in the density of primordial follicles (Webber *et al*., 2003, 2007), attributable to the hyperandrogenaemic environment.

Therefore, collectively, hyperinsulinaemia and hyperandrogenism in women with PCOS lead to an increased number of antral follicles and the characteristic polycystic ovarian morphology (Monget and Bondy, 2000). The literature is less clear, for both women with and without PCOS, on the quantitative relationship of IR and androgen levels to ovarian morphology, and whether those with highest levels demonstrate the greatest multi-follicular appearance.

Increased antral follicle numbers in women with PCOS also explains why they are particularly at risk of developing OHSS in response to exogenous gonadotrophins during IVF treatment (Brinsden *et al*., 1995). A recent meta-analysis revealed that metformin, an insulin lowering agent, reduces the incidence of OHSS in women with PCOS undergoing ART cycles (Costello *et al*., 2006). In addition, it has been demonstrated that co-therapy with metformin during ART reduces androgen levels in PCOS women and improves the treatment outcomes (Tang *et al*., 2006).

Despite the above, the evidence for the dual effects of insulin and androgen on folliculogenesis, specifically within the ART setting, in women without PCOS is limited. Therefore, we designed this prospective study to investigate the effect of IR and androgen levels on the outcome of an IVF cycle in ovulatory infertile women who did not have PCOS to determine if the normal continuum of IR and androgen levels are influential in the process and independent in their effect and if a threshold effect of either could be observed.

Materials and Methods

A total of 49 infertile women who were about to commence an IVF cycle were recruited to the study prior to cycle commencement. All women had a regular menstrual cycle and none had polycystic ovaries on ultrasound. Women with a diagnosis of PCOS based on the Rotterdam criteria (two out of the triad of oligo-amenorrhoea, biological or clinical hyperandrogenism and multi-follicular appearance of ovaries on transvaginal ultrasound scan; The Rotterdam ESHRE/ASRM—Sponsored PCOS Consensus Workshop Group, 2004) were excluded from the study.

Blood samples and measurements were undertaken after an overnight fast on Day 4 of the menstrual cycle preceding the commencement of pituitary down-regulation for IVF. Fasting venous blood was collected into serum gel and fluoride oxalate tubes. Samples were separated by cooled centrifugation at 2000*g* for 15 min at 4°C, and the aliquots stored at −20°C.

Serum testosterone and FSH were measured by direct immunoassay on an Architect analyser (Abbott Laboratories, Maidenhead, UK), and SHBG was measured by immunometric assay with fluorescence detection on a DPC Immulite 2000 analyser (Euro/DPC, Llanberis, UK) using the manufacturer's recommended protocol. The free androgen index (FAI) was obtained as the total testosterone × 100/SHBG.

Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyser (Euro/DPC). The analytical sensitivity of the insulin assay was 2 μU/ml, the coefficient of variation was 6%, and there was no stated cross-reactivity with proinsulin. Plasma glucose was measured using the Synchron LX20 analyser (Beckman-Coulter), using the manufacturer's recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 94.6 mg/dl (5.3 mmol/l) during the study period.

The IR was calculated using the homeostasis model assessment (HOMA) [HOMA-IR = (insulin × glucose)/22.5]. A HOMA value above 2.5 was used to indicate IR (Matthews *et al*., 1985). HOMA is a validated surrogate marker for IR and has good agreement with gold standard hyperinsulinaemic euglycaemic clamp (Bonora *et al*., 2000).

All participants underwent a standard long-protocol agonist IVF cycle. Briefly, patients underwent down-regulation with buserelin acetate before superovulation with a recombinant FSH preparation; the initial dose given was based on patient age and baseline serum FSH levels, and then titrated to USS monitored ovarian response. Total follicle count was assessed by transvaginal ultrasound, and oocyte retrieval was performed 34 h after human chorionic gonadotrophin administration. The treatment cycle was abandoned if the total follicle count was below 5, above 25 or if other signs/symptoms of ovarian hyperstimulation were observed. Abandoned cycles were not excluded from analysis.

Power and sample size for the study was based on the review by Birkett and Day (1994) that concluded that a minimum of 20 degrees of freedom was required to estimate effect size and variability. Hence, a minimum of 25 patients needed to be recruited to allow for dropouts and covariate adjustment.

Statistical analysis was carried out using SigmaStat, version 2. Linear regression analysis was used to assess the relationship between IR (HOMA) and androgen levels and the outcomes of IVF cycle. Mann–Whitney *U*-test was used to compare means as the biological data were not normally distributed. *P*-values are two-sided, and values <0.05 represent statistical significance. Data are reported as mean ± SD.

Results

The mean age at the start of the IVF/ICSI cycle was 33.2 ± 4.0 years (mean ± SD). The mean BMI was 25.7 ± 4.7 (mean ± SD) and the mean FSH (taken Day 4) was 5.8 ± 1.5 (mean ± SD). Table I shows the mean demographic and biochemical data and Table II shows the outcome of the stimulated ovarian cycle. An isolated elevated FAI was present in 10 patients (10 patients had an FAI over 8.5, of which five had FAI over 10). A total of six cycles (12%) were abandoned prior to oocyte retrieval, two for poor ovarian response and four for ovarian hyperstimulation. These were not excluded from the final analysis.

Linear regression analysis showed that HOMA had a significant positive correlation with BMI ($P = 0.012$, Fig. 1). HOMA also demonstrated a significant positive correlation with FAI ($P \leq 0.001$, Fig. 2) and testosterone ($P = 0.02$, Fig. 3) and showed a significant negative correlation with SHBG ($P = 0.01$, Fig. 4).

Multiple linear regression showed that the total follicle count had a significant positive correlation with FAI ($P = 0.022$, Fig. 5), after adjustment for BMI, age and total dose of stimulation drug used. However, there was no significant correlation between HOMA or fasting insulin and the total follicle count. No significant correlation was found between number of

Table I Mean demographic and biochemical data ($n = 49$)

	Mean data (± SD)
Age	33.2 ± 4.0
BMI	25.7 ± 4.7
FSH (mIU/ml)	5.8 ± 1.5
Fasting insulin (mIU/ml)	8.6 ± 7.1
Fasting glucose (mmol/L)	4.8 ± 0.3
HOMA-IR	1.9 ± 1.6
SHBG (nmol/L)	65.2 ± 38.6
Testosterone (nmol/L)	2.5 ± 1.4
Free androgen index	6.2 ± 6.1

Table II Mean values for outcome data of stimulated IVF cycle ($n = 49$)

	Mean data (± SD)
Follicle count	22.9 ± 12.9
Oocyte number collected	10.4 ± 4.7
Fertilization rate (%)	73.5 ± 21.1
Cleavage rate (%)	85.1 ± 19.9

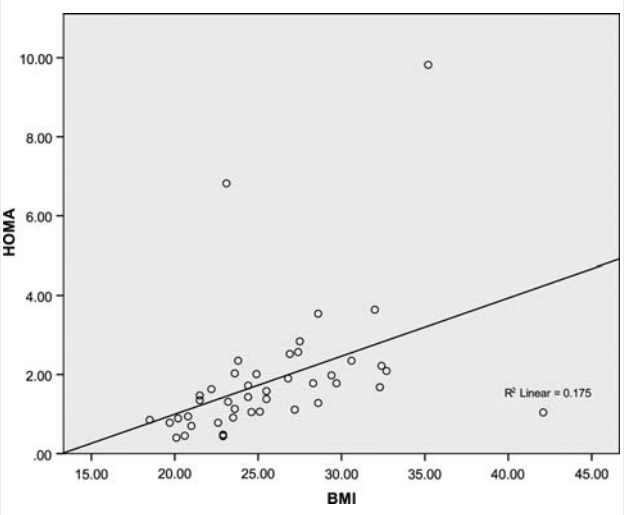

Figure 1 Regression line demonstrating positive correlation between BMI and HOMA ($P < 0.05$).

oocytes retrieved, fertilization or cleavage rates and fasting insulin, HOMA or androgen levels. There was no significant correlation between HOMA and the total dose of gonadotrophin used for stimulation, but there was a trend towards a negative correlation, with those with higher HOMA tending to require a lower dose though this did not reach significance.

Nine patients (18%) had a HOMA level indicating IR (>2.5). The total follicle number in the insulin resistant group (32.7 ± 10.0, mean ± SD) was significantly higher than the total follicle number in the group with HOMA < 2.5 (20.2 ± 12.7, mean ± SD) ($P = 0.006$, Fig. 6). The group with IR (HOMA > 2.5) showed a significant positive correlation between the HOMA level and total follicle count ($P = 0.047$, data not shown). This was not dependent on the follicle size. There was no correlation between HOMA and total follicle count for those with HOMA <2.5.

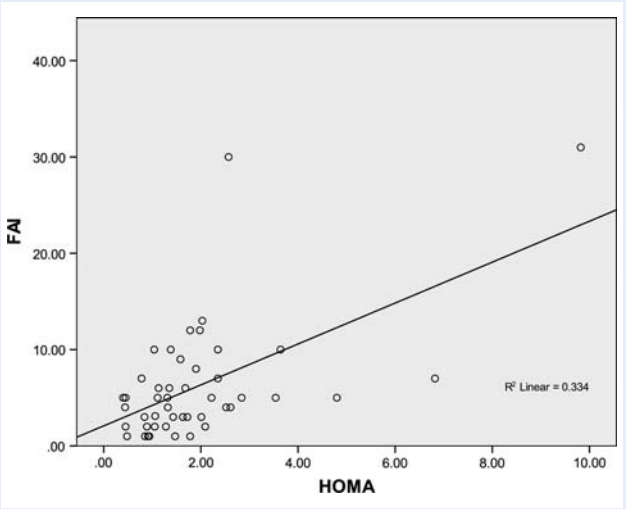

Figure 2 Regression line demonstrating positive correlation between FAI and HOMA ($P < 0.05$).

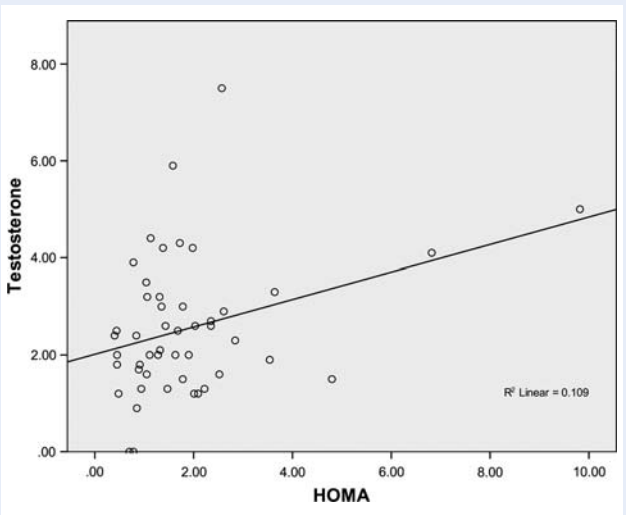

Figure 3 Regression line demonstrating positive correlation between testosterone and HOMA ($P < 0.05$).

Discussion

We have demonstrated that the level of IR in women without PCOS can have an impact on the outcome of a stimulated ovarian cycle for IVF and that, in addition, their androgen status also appears to have a

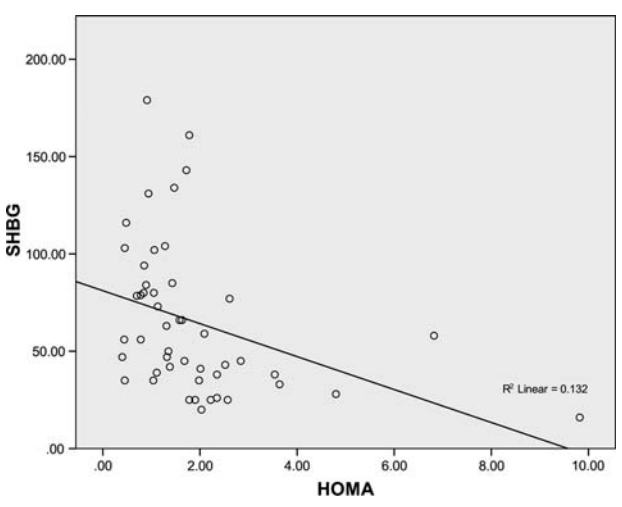

Figure 4 Regression line demonstrating negative correlation between SHBG and HOMA ($P < 0.05$).

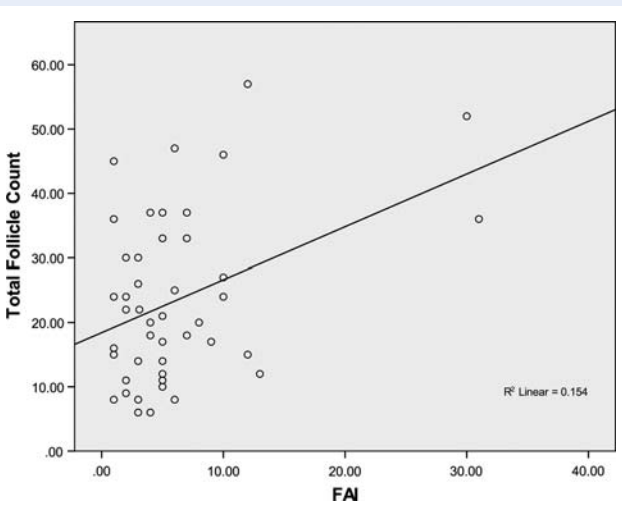

Figure 5 Regression line demonstrating positive correlation between total follicle count and FAI ($P < 0.05$).

bearing on the ovarian response. Previous evidence on the effects of insulin and androgen levels on ovarian response to COH for IVF in this non-PCOS group is limited.

This study found a greater total number of follicles at the end of ovarian stimulation for IVF in patients with greatest IR, suggesting that the relatively higher level of insulin stimulates follicle recruitment or development. In addition, there appeared to be a threshold effect as for those with HOMA >2.5, there was a positive correlation between degree of IR and total follicle count. We did not look at the rate of follicle development; however, the positive correlation remained significant after adjustment for total dose of stimulation drug used, which reflects the duration of stimulation. We found no association with the degree of IR and follicle size, although follicle

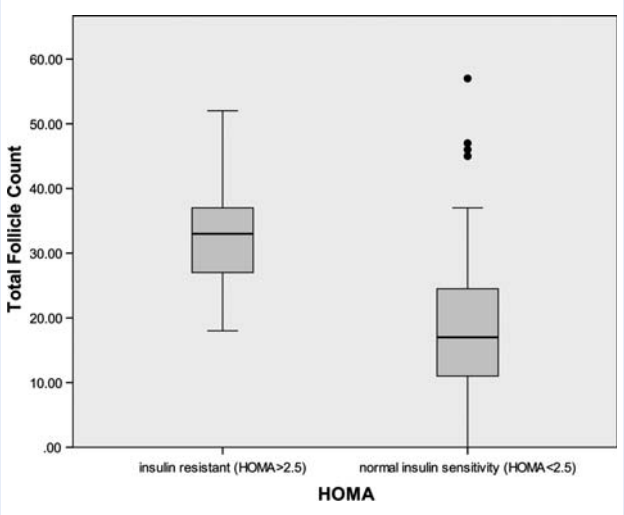

Figure 6 Box-whisker plot demonstrating a greater total follicle count after COH in women with a higher IR (HOMA > 2.5).

size may not reflect follicle selection, and it appears that the greatest effects of insulin occur at earlier stages of follicle development and selection rather than maturation (Maciel *et al*., 2004). This is in accord with Fleming *et al*. who reported that women with PCOS and IR compared with controls had a larger cohort of follicles recruited during the early stages of FSH stimulation for IVF (Fleming *et al*., 2006). There was no subsequent difference in the rate of follicle maturation and oocyte yield or fertilization rates. The authors suggest the use of insulin and/or androgen may improve follicular recruitment during IVF in those resistant to conventional approach IVF (Fleming *et al*., 2006).

This study also showed that the FAI was correlated with the total follicle count in a continuum and without a threshold effect. Androgens act directly through the androgen receptor and appear to be important in particular in promotion of early follicular growth (Weil *et al*., 1998); Hillier *et al*. (1997) found that androgen receptor expression is greatest at the pre-antral and antral stages of follicle development and decreases in the pre-ovulatory follicle. In addition, androgen has been demonstrated to have an indirect effect by promoting follicular expression of FSH (Weil *et al*., 1999), as well as increasing IGF-1 and IGF-1 receptor expression (Vendola *et al*., 1999a). Our study is in accord with the data of Fabregues *et al*. (2009) where pretreatment with transdermal testosterone successfully improved the outcome of stimulated IVF cycles in women with a previous poor response. The use of testosterone significantly increased the number of patients reaching the oocyte retrieval stage, compared with controls, in those with normal baseline FSH (Fabregues *et al*., 2009), suggesting that androgen improves FSH sensitivity.

In reality, insulin and androgen levels have a synergistic effect on the ovary and cannot be viewed in isolation as separate systems. Insulin acts to enhance LH responsiveness leading to greater steroidogenesis (Mason *et al*., 1994), and so androgen production. That hyperinsulinaemia drives excess androgen production in PCOS is well-documented. Therefore, it is not surprising to see similar effects in

this study; indeed a positive correlation is seen between FAI and HOMA. The negative correlation between SHBG and HOMA is also to be expected as SHBG has previously been demonstrated to be a reliable surrogate accurate marker of IR (Jayagopal *et al*., 2003).

An FAI of 8.5 is used as a cut off to delineate biochemical hyperandrogenaemia in the diagnosis of PCOS (Vermeulen *et al*., 1999). The level of FAI reflects the severity of hyperandrogenaemia and as such represents a continuous data set; those with higher FAI tend to have more severe symptoms and higher BMI. Our data demonstrate that FAI and BMI are also closely correlated in non-PCOS women and that the relationship continues below an FAI of 8.5 and within a normal BMI range. This results in a continuum of effect of androgen on ovarian response, even within those not classically demonstrated to have hyperandrogenaemia. This continuum of effect is also observed in the anticipated observation that IR increases with BMI, even within a normal range of BMI values.

We did not demonstrate a correlation between IR or androgen status and oocyte retrieval or fertilization rates. This may be because our sample size was too small to assess this effect. In PCOS, a high level of IR is detrimental to oocyte quality, fertilization rates and implantation rates (Dumesic and Abbott, 2008). However, studies have less consistently demonstrated this effect in non-PCOS patients. In our group, the level of IR is relatively low, thus the detrimental effect of hyperinsulinaemia is unlikely to be seen in this group. Also, PCO has many other factors which may be contributory. In fact, the predominant effect of androgen and insulin appears, from *in vitro* studies, to be on early follicular development (Hillier *et al*., 1997; Weil *et al*., 1998; Maciel *et al*., 2004). In their 'physiological' roles, these hormones may have less effect on oocyte development and maturation.

The effect of androgen status in this group may be masking an independent effect of IR. In multiple regression analysis, only FAI was associated with total follicle account, after adjustment for BMI, age and dose of stimulation drug used. However, the significant positive correlation between markers of androgen status (FAI, testosterone and SHBG) and HOMA supports the close relationship and likely similar effects on ovarian response. Again, our sample size may not be large enough to demonstrate individual effects. The study by Fleming *et al*. (2006) also did not demonstrate a difference between oocyte yield between those with IR and controls. In addition, Imani *et al*. (2000) found that FAI was the most prominent endocrine predictor of ovarian response after stimulation with clomiphene citrate.

In conclusion, our study suggests that there is a threshold effect of insulin below which no clinical effect on follicular number is detectable. In addition there appears to be a continuum effect of androgen with increasing levels related to an increased follicle number in women without PCOS. These data support the growing evidence that both factors can have a positive effect on short-term follicle development, in response to exogenous gonadotrophin stimulation that may be possible to exploit clinically. However, patients with a higher level of androgen and IR are more likely to develop OHSS, which is possibly not confined to those with PCOS. In particular, those with obesity may be at risk if they have an associated raised IR.

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