Effect of clomiphene citrate treatment on the Sertoli cells of dysmetabolic obese men with low testosterone levels

DR CARLA PELUSI (Orcid ID : 0000-0002-6390-2146)

DR NICOLA BARTOLOMEO (Orcid ID : 0000-0002-8540-4615)

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C. Pelusi[1], F. Fanelli[1], M. Baccini[1], V.Triggiani[2], N. Bartolomeo [3], M.D. Carbone[4], G. De Pergola[5], G. Di Dalmazi[1], U. Pagotto [1], R .Pasquali[1] and V. A. Giagulli[6]

Affiliation:

[1] Endocrinology Unit and Center for Applied Biomedical Research, Department of Medical & Surgical Sciences (DIMEC), Alma Mater Studiorum University of Bologna, S. Orsola-Malpighi Hospital, Bologna, Italy; [2]Interdisciplinary Department of Medicine, Internal Medicine, Geriatrics, Endocrinology and Rare Diseases, University of Bari, Bari, Italy; [3] Department of Biomedical Science and Human Oncology, University of Bari, Bari, Italy;[4]Institute of Clinical and Hormonal Research, Foggia, Italy; [4] Nutrition Outpatient Clinic, Clinical Oncology Unit, University of Bari, Bari, Italy; [6] Outpatients Clinic of Endocrinology and Metabolic Disease, Conversano Hospital, Bari, Italy.

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Correspondence: Dr VA Giagulli Outpatients Clinic of Endocrinology and Metabolic Disease, Conversano Hospital, Bari, Italy. E-mail vitogiagulli@alice.it; Telephone number +390804091368

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Abstract

Background: Clomiphene citrate (CC) has been shown to restore the hypothalamic pituitary-gonadal (HPG) axis by increasing testosterone (T) levels to physiological levels in patients with dysmetabolic conditions such as obesity, metabolic syndrome and type 2 diabetes mellitus (T2DM). However, the data are unclear regarding the effects on Sertoli cell (SC) function.

Aim: To study SC function by assessing Inhibin B (IB) and Anti-Mullerian Hormone (AMH) levels at baseline and after 3 month of CC treatment.

Materialsand methods: This is an ancillary study of a cross-over, randomised, double-blind, placebo-controlled trial performed to evaluate androgen response to CC treatment in dysmetabolic obese subjects with low T levels treated with metformin. We evaluated SC function by assessing IB and AMH levels at baseline and after three months of each treatment in ten dysmetabolic obese subjects with low T levels. In all subjects,the influence of the clinical characteristics, metabolic and hormonal baseline parameters on SC and Leydig (LC) function, evaluated respectively with AMH, IB, FSH and T levels, were tested.

Results: No significant changes were observed for IB and AMH concentrations after each treatment period. Whereas T and estradiol (E2) levels were shown to be significantly higher in the CC plus metformin phase (CC/Met) only. No clinical, metabolic or hormonal parameters showed significant effects on serum AMH at baseline or after treatments. However, baseline T, dihydrotestosterone (DHT) andE2 positively affected IB levels during CC/Met therapy (P=0.003, P=0.038 and P=0.049 respectively). Baseline leptin, and FSH had a negative (P=0,031) and positive (P=0.048) respectively role on T levels during CC/Met, as they were statistically significant compared to the placebo period (Plac/Met).

Conclusion: Unlike the LC activity, CC was unable to influence SC function, as shown by the lack of IB and AMH serum modifications, thus suggesting an intrinsic non-reversible defect of SC cells in patients with dysmetabolic conditions.

Introduction

A recent study carried out on a large number of adult and elderly European men (40-79 years) found that secondary hypogonadism [low serum testosterone (T) with low/normal luteinizing hormone (LH) levels] is the most prevalent hypogonadism condition (12%) followed by a compensated form (10%) (normal serum T levels with high LH levels). On the other hand, only 2% of subjects were affected by primitive hypogonadism characterized by low plasma T with significantly high LH levels (1). Taking into consideration the relationship between plasma T and LH levels, primitive hypogonadism (hypergonadotropic hypogonadism) is characterized by a serious damage of the testes. Instead, the compensated hypogonadism might be due to a defect of Sertoli cells (SCs) and a partial dysfunction of Leydig cells (LCs). Importantly, these clinical conditions are more frequent in adult men with idiopathic infertility suffering from severe oligozoospermia or even azoospermia (2-3). Within the forms of secondary hypogonadism, such as defects at the hypothalamus-pituitary levels due to genetic abnormalities (i.e.Kallmann Syndrome) or post-pubertal infiltrative or tumoral diseases (4), those defined as functional are the most frequent in adult or elderly men. These functional forms are related to acquired chronic dysmetabolic conditions such as obesity, metabolic syndrome and type 2 diabetes mellitus (T2DM), (5,6.).These clinical conditions, also defined as dysmetabolic hypogonadotropic hypogonadism (DHH) (7),are characterized by an intact hypothalamus-pituitary-testis (HPT) axis but low T levels and inappropriately low-normal gonadotropin concentrations. In these cases, the damage is considered to be reversible, as the T deficit is susceptible to improvement especially in dysmetabolic patients after weight lost (8), with SERM administration, in particular clomiphene citrate (CC) (9) or antioestrogens (10) by reactivating the HPT axis. However, to date, there is the unequivocal evidence regarding SC function in men with dysmetabolic conditions (11-13). Recently, we performed a randomized controlled study (RCT) study on obese DHH patients with low T showing that CC at low doses (25 mg / day) significantly increased LH secretion and in turn, elicited T response from LCs, reinforcing the theory that these are reversible forms of hypogonadism (9). We therefore decided to analyse the functional state of SC by measuring the serum levels of Inhibin (IB) and Anti Mullerian Hormone (AMH) in 10 subjects from the same population and treatment strategy. The aim was to examine whether: i) there is a basal dysfunctional state of SC that can be reversed by CC treatment as shown for LC; ii) the clinical

characteristics and/or the metabolic and hormonal parameters characterizing these patients could play a key role in co-regulating LC and SC function.

Materials and Methods

Patients, study design and methods

Our study of males patients (aged 35-55 years) was a cross-over, double-blind, placebo-controlled RCT carried out in two Italian centers, the Unit Endocrinology Unit of the S. Orsola-Malpighi Hospital, Bologna, and the Endocrinology and Metabolic Outpatient Clinic of Conversano Hospital, Bari (9), The study consisted of two intervention periods (period A and B) of three months each, with a wash-out period of six weeks. Participants were randomized in order receive either CC (25 mg/day) (Serofene, Merck Serono, Rome, Italy) plus Metformin (CC/Met) or Placebo plus Met (Plac/Met) in period A, and then moved to the other treatment in period B. Metformin (Glucophage 1000, Bruno Farmaceutici, Rome, Italy), was administered at 2 g/daily. The main inclusive criteria for the study were the presence of obesity (body mass index, BMI $\geq$ 30 kg/m^2) and low T levels ($\leq$3 ng/ml or 10.4 nmol/L). The cut off of T was arbitrary, but in accordance with the American (6) and European (14) guidelines for hypogonadism. The measurement of T was performed twice in the morning with two methods: electrochemiluminescence immunoassay (ECLIA) and LC-MS/MS (9).As mentioned in our previous work (9), given the extreme difficulty in selecting the patients for the study, we included patients with a slightly lower BMI compared to the one requested in the protocol. In this sub-study, two out of 10 had a BMI equal to 29.6 and 29.7 kg/m^2 respectively.

All these patients had had a new diagnosis of impaired glucose tolerance (IGT) or type 2 diabetes mellitus (T2DM) (naïve patients) (ADA, 2010) (9). All subjects were asked to follow a 1,600 kcal standardized low calorie diet. All prepubertal or postpubertal causes of hypogonadism such as the genetic (karyotype) and/or organic (MRI scan) were ruled out, as were diabetic subjects already on anti-diabetic treatment or on medications interfering with glucose metabolism.

In 10 dysmetabolic obese patients with low T (6 with T2DM and 4 with IGT), from the same population as our previous study (9), we assessed IB and AMH levels. As previously described clinical, metabolic and hormonal parameters were evaluated. In addition, at the beginning of the study all patients underwent a genotype characterization by analyzing androgen receptor CAG

(AR-CAG) polymorphism (3,15). All participants had fathered a child before the enrollment in the study, and one reported that he was suffering from secondary infertility.

Although no specific symptoms were requested for inclusion in the study, we asked the participants to fill in the international index for erectile function (IIEF) questionnaires (16).
This study protocol was approved by the Ethics Committees of the S. Orsola-Malpighi Hospital of Bologna (protocol number UOE/012011) and of the Local Health District of ASL Bari (protocol number 778) (9).

Assessments and measurements

Serum AMH and IB were quantified by enzyme-coupled immunosorbent assay (ELISA). In particular, the AMH analysis was performed according to the procedure introduced in 2013 (Beckman Coulter, MN, USA 2013).The assessment range without dilution was 0.08- 23 ng/ml (0.5–160 pmol/L), while the intra-assay and inter-assay variation coefficients (CV%) were 5% and 7%, respectively. Our normal range obtained in 40 adult non-obese men (24-40 years) who had fathered children within last 2 years was 1.6-13.6 ng/ml (11.5-98 pmol/L). Circulating IB was analysed by Gen II ELISA (Beckman Counter, California, 2009) kit. The measuring range obtained without dilution was 20-325 pg/ml, while the intra-assay and inter-assay CV% were 7% and 9%, respectively. Our in house reference range obtained from a different population of 40 adult fertile non-obese men aged 24-40 years, was 35-305 pg/ml or ng/L. These values are in line with other studies that used the same kit(12, 17). As reported in our previous study (9), serum steroid hormones were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS)(9) using reference intervals in healthy adult men by Fanelli et al (18). Gonadotropins and sex hormone binding globulin (SHBG) were measured at the Central Laboratory of S.Orsola Malpighi Hospital (Bologna, Italy) by Modular Analytics E 170 (Roche Diagnostics, Mannhein, Germany) and by Immulite 2000 (Siemens Healthcare Diagnostics, Deerfield, IL) respectively, as previously described (19)

Statistical analysis

The Shapiro-Wilks test was applied to test scalar variables for the normal distribution. Variables showing a significantly different distribution from the normality were transformed using an

appropriate function, which enabled all transformed variables to be presented as normally distributed. Data were expressed as the mean and standard deviation (SD).

A mixed effects model for the analysis of repeated measures in cross-over studies (20) was applied to analyze the treatment effect on clinical characteristics, metabolic parameters and hormonal values.

We also determined the effects of the two treatments by comparing post- vs pre-treatment values and the efficacy of CC/Met by comparing the means between treatments (CC/Met vs Plac/Met). The cross-over effect of the treatments was also evaluated by comparing the subgroup of patients receiving CC/Met in period A and the Plac/Met in period B with the subgroup of patients receiving Plac/Met in period A and then CC/Met in period B.

A mixed effects model with interaction terms was also applied to estimate the effects of the clinical, metabolic and hormonal parameters at baseline on the IB, AMH, FSH and T, in each group onboth treatment regimens.

All tests were two-tailed, and *p*-values of less than 0.05 were considered statistically significant.

The relationship between AR-CAG triple repeat number and hormone blood levels was tested by means of the Pearson correlation coefficient.

In the power analysis for the crossover design, we assumed no carryover effect and no period effect. A sample size of 10 was required to detect a difference in the IB of 30 pg/ml (50 placebo vs. 80 treated with clomiphene, and a standard deviation of 15 and 30 respectively) with 82% power and 5% significance (two-sided test).

Statistical and power analysis were performed using SAS vers 9.4 for PC.

Results

Population characteristics.

Baseline clinical, metabolic and hormonal parameters of the subjects are shown in Table 1. In accordance with the inclusion criteria, all subjects were obese with low T levels and dysmetabolic (4 with IGT and 6 with naive T2DM). All participants had baseline normal circulating gonadotropins levels.

IB and AMH concentrations were within the kit reference range, but according to our in-house reference range, towards the lower limit.

AR-CAG repeats length polymorphism of all subjects did not correlate with any hormonal parameters: T (r=0.17 p=0.659); Free Testosterone (Free-T, r=0.53 p=0.138); DHT (r=-0.29 p=0.444); E2(r=0.55 p=0.199); Free Estradiol (Free-E2, r=0.48 p=0.270); SHBG (r=-0.21 p=0.590); LH (r=-0.13 p=0.729); FSH (r=-0.26 p=0.499); AMH (r=-0.44 p=0.231); IB (r=0.44 p=0.240).

Lastly, the IIEF mean score of the population at baseline was 22.7. Half of the subjects presented with middle/moderate erectile dysfunction, according to the IIEF score. In particular, in 5/6 T2DM obese men, the IIFE5 score ranged from6 to 25, while the score of the rest of the group (1 with T2DM and all 4 IGT men) ranged from 26 to29 (data not shown).

The effects of treatment and the influence of clinical, hormonal and metabolic parameters on Sertoli and Leydig cell function during both CC/Met and Plac/Met treatment periods

Regarding SC function, we evaluated the response of AMH and IB to treatments. Neither hormones showed any significant change over the course of each treatment period (AMH: 32.9±17.1 to 35.0±17.9pmol/L during CC treatment, p=0.06; and 32.1±18.6 to 34.3±21.4pmol/L during the placebo treatment, p=0.09; IB: 43.1±19.4 to 58.1±32.5 ng/L during CC, p=0.15; and 63.6±37 to 54.3±16.9 ng/L during placebo, p=0.65; see Figure 1) and between treatments (AMH p=0.871 and IB p=0.102). However, as shown in our previous work (9), T, E2 and their free-hormone fraction increased significantly during the CC/Met treatment period, but not during the Plac/Met phase ($p<0.001$; see Table 2). On the other hand, in this subgroup of patients, no significant modification in any clinical and metabolic parameters was observed at any time during the study protocol (Table 2).

In addition, we evaluated the role of all clinical, metabolic and hormonal parameters on both SC (IB, AMH and FSH) and LC markers (T levels).

As shown in Table 3, baseline T levels had a significant positive impact on IB serum concentration during CC/Met (estimate: 21.769; P=0.045) and negative impact during Plac/Met treatment (estimate:-29.118; P=0.012), thus showing a significant difference after comparing the two-treatment period (P=0.003). On the other hand, baseline DHT and E2 showed a modest positive influence on serum IB during CC/Met and a negative influence during Plac/Met treatment, which were statistically significant only when comparing the two treatment phases (P

=0.038 and 0.049, respectively). The remaining hormones, the clinical and biochemical parameters had no effects on IB levels.

Conversely, neither clinical characteristics nor metabolic and hormonal parameters showed significantly relevant effects on AMH in either the treatment arms (data not shown).

We also evaluated the impact of the each variable on FSH, as another known marker of SC function. Baseline leptin (estimate:-3.132, P= 0.047), E2 (estimate:-0.315, P=0.041) and AMH levels (estimate:-1.171, P=0.047) showed a negative impact on FSH levels during CC/Met treatment only. These influences were not observed during Plac/Met and they were not statistically different when comparing the two treatment phases.

Finally, T levels were shown to be negatively impacted by the baseline weight (estimate: -0.030, P=0.032), waist circumference (estimate: -0.038,P=0.04), leptin (estimate: -0,789, P=0.014), E2 (estimate: -0.079, P=0.004) and FE2 (estimate: -4.194, P= 0.021) and positively impacted by FSH (estimate: 0.464, P=0.021) during CC/Met treatment, but no effects were revealed during the Plac/Met phase. Only leptin and FSH impacts on T showed significance when comparing the two treatment phases (P=0.031, P=0.048 respectively).

Discussion

Several studies have recently reported the negative role of chronic metabolic diseases on male HPT axis, characterized by lower serum LH and T levels compared with healthy aged-matched control men (7,21,22). These clinical conditions have been defined as DHH because of the theoretically reversible damage at the hypothalamus-pituitary-gonadal level (5,6). Indeed, lifestyle changes (i.e the diet) (8) or both SERM (i.e. CC) (9) and anti-estrogen therapies (10) can improve T levels in men with DHH. However, there is little and inconsistent evidence regarding gonadal function, particularly in relation to SC activity in men with DHH. It is commonly accepted that SC carefully orchestrates both the development and function of all testes cells (23,24). In particular, SC promotes nutritional and physical support for the development of germ cells (25), and directly affects LC activity, regardless of the subject's age (26). In addition, recent data have shown that at least 10% of men from couples with primary infertility are affected by metabolic syndrome (11,12) and about 15% might be suffering from prediabetes (13),whose prevalence is surprisingly higher than that assessed in the general population of adult Italian males(27).

Interestingly, circulating IB and AMH levels have been described to be lower in patients with metabolic disturbances than in infertile men without metabolic syndrome or/and prediabetes. This suggests the negative impact of dysmetabolic conditions on SC function, besides the well-known effect on LC (11-13).

Based on this evidence, we decided to measure serum IB and AMH levels in a RCT study of obese dysmetabolic patients with low T levels, at baseline and in some patients after treatment, in order to evaluate SC function. As previously shown, CC therapy in these patients enhanced LC activity through the increase in LH and, in turn, T levels (9), therefore emphasizing the reversible function of these types of cells. In contrast, a lower range of baseline serum IB and AMH levels was shown in this study in the population of obese adult men together with unchanged IB and AMH concentrations by all treatments, suggesting the intrinsic damage of SC function in metabolic disorders (28).This hypothesis is supported by the known positive effect of FSH in stimulating IB and AMH secretion, which during CC treatment was more increased (29,30). However, opposite results on plasma IB and AMH levels during CC cannot be excluded, as T has the opposite effect on IB and AMH secretion (29,31), Meanwhile, the rise of E2 levels after CC treatment should be considered for their facilitating role in SCs proliferation (32). In contrast, during CC treatment a parallel rise in endogenous levels of E2 and T (both about 40%) was found without altering the T/E2 ratio (pre-treatment: O.16±0.05 and post-treatment 0.15±0.05, p=0.374) which supports the lack of damage of testicular steroidogenesis in our patients likely due to a dysfunctional hypothalamic-pituitary testis axis(9).

Overall, our data may point the possibility of damage to SCs in obese dysmetabolic men, thus supporting the hypothesis of a reduced number of SCs in obese compared to normal weight subjects (33). Similar data were found in a rat model with type 2 diabetes mellitus showing testicular hypotrophy, histologically backed up by the seminiferous tubules that were reduced in dimension (34).

In addition, based on these results and, in particular, taking into consideration the clinical characteristics and the indices that have been proved to be predisposing factors for the development of functional secondary hypogonadism in adult men (35), we evaluated whether any of these parameters had a facilitating or inhibiting role in secreting IB, AMH, FSH and T by SC and LC, respectively, during CC or Plac therapy arms in addition to Met. Regarding serum IB, we found that higher basal levels of DHT, E2 and T promoted IB secretion during CC/Met but not

during Plac/Met treatment. These results are in agreement with both previous preclinical and clinical studies. Indeed, in SC rat culture, DHT and E2 were modulated the glucose metabolism, influencing the key enzymes of the glycolytic process (i.e. lactate dehydrogenase), the protein expression levels of glucose membrane transporters (GLUT1 and GLUT3) as well as the specific proton/monocarboxylate transporter 4, whose overall effect is to produce energy by SC and thereby to provide available lactate to germ cells (36). In addition, in a group of patients, neither LH nor FSH, respectively, increased serum IB after three months of each treatment. In contrast, gonadotropin therapies, continuously conducted for 24 months, led to a rise in plasma IB levels in the range assessed in adult fertile subjects, stressing the essential T role in promoting IB secretion by SC (31).

All clinical indices, such as weight and waist circumference, insulin and especially BMI and HOMA-IR, although not statistically significant, seemed to favour lower IB plasma levels during CC/Met therapy. The dysmetabolic conditions such as obesity, prediabetes, and overt T2DM could therefore damage SC function, although CC therapy reactivated LC steroidogenesis, thus improving the hypothalamic-pituitary-testis axis (9).

These results strengthen previously compelling evidence indicating that obesity, metabolic syndrome and type 2 diabetes mellitus cause a severe damage to SC, irrespective of the fertility condition (11-13,15,28) and of the age of the male (37). Furthermore, the testes impairment has been associated with the individual components of metabolic syndrome (38).

Considering the effects of clinical signs, and the metabolic and hormonal indices including IB and AMH in the secretion of T by LC during CC/Met treatment, the clinical characteristics (weight and waist circumference), leptin, E2 and FE2 may negatively affect T secretion by LC. In fact, leptin and E2 may be significantly higher in obese men and are notoriously steroidogenic-inhibiting factors of T secretion by LC (39,40). Conversely, in subjects on CC/Met treatment the statistically significantly higher levels of FSH highlighted how it promotes T secretion by LC. Interestingly, this result confirms the relationship between LC activity and the well-known FSH paracrine-signalling role of SC reported in an "*in vitro*" study (41). In addition, basal circulating LH showed a negative, but not statistically significant effect, on T levels during both treatment regimens. This is surprising since LH is the main co-regulator of T secretion by LC. However, the above results mirror, at least partially, the suppression of HPT axis in men with dysmetabolic disorders, who present with inappropriately low LH levels, notwithstanding the low plasma T levels (the so-called uncompensated hypogonadtropic hypogonadism) (7).

Finally, although our study consisted of a small number of patients, AR-CAG repeat numbers were in the range of healthy fertile men(3), and did not correlate with any clinical features, metabolic and hormonal parameters. This indicates that they did not play a key role in modifying serum IB, AMH and T levels under either of the treatments arms.

Although this study is limited by the small number of patients analysed and the short period of treatment, the crossover design used, the inclusion and exclusion criteria applied and the absence of carry-over effects, all made the results reasonably conclusive. In addition, steroid hormones were measured by LC-MS/MS, which is the currently recommended technique for steroids.

In conclusion, although CC treatment displays a promoting role in gonadotropin secretion and LC activity in men with dysmetabolic conditions and T deficit, it has no similar effect on SC function, as shown by the lack of increased IB and AMH levels. Therefore, although dysmetabolic conditions such as obesity and metabolic syndrome negatively affect SC and LC function, only the SC function seems to be damaged. These results, however, are limited by the lack of a concomitant evaluation of semen analysis, which would have further corroborated our hypothesis. Finally, the different LC and SC responses to CC-induced gonadotropins secretion in our population, may suggest a mixed origin (primary and secondary) of low T levels usually observed in these patients (28).

However, to confirm our results it would be advisable to further assess the pathogenic role of dysmetabolic conditions on SC function in men, by studying a larger numbers of patients and for a longer period.

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Author's contributions: VAG, CP and RP conceived and designed the study. VAG, CP, FF and MDC carried out the experiment, while NB conducted the statistical analysis. VAG, CP, FF, MDC,GDP, VT, GDD, RP and UP were involved in the analysis and interpretation. All the authors have read and approved the final version of the manuscript, agreed with the order of prevention of the authors.

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Table 1.Baseline clinical, genetic and biochemical characteristics of all subjects

Variables	**All Subjects**(n=10)
Age (ys)	48.5±4.7
Weight (kg)	103.4±15.5
BMI (Kg/m^2)	35.3±5.7
Waist circunference (cm)	113.6±9.6
Glucose (mmol/L)	7.16±1.20
HbA1c (%)	6.4±0.7
Insulin (pmol/L)	121±95.4
HOMA_IR	5.2±3.7
Leptin (µg/L)	14.5±17.3
T (nmol/L)	9.34±2.08
FT (pmol/L)	242±105
DHT (nmol/L)	0.69±0.34
E2 (pmol/L)	93.9±34.5
FE2 (pmol/L)	1,47±0.73
SHBG (nmol/L)	20.2±9.2
LH (IU/L)	3.6±1.6
FSH (IU/L)	5.2±1.3
AMH (pmol/L)	31.4±17.1
IB (ng/L)	45.1±24.8
AR-CAG(n)	18.8±3.3(9)

All data are expressed as Mean ± Standard deviation (SD)

For steroid reference intervals in healthy adult see Fanelli *et al* (18)

Table 2. Clinical characteristics, metabolic parameters and hormonal values before and after each treatment period in all subjects

	CC/Met			**Plac/Met**			
	Pre	*Post*		*Pre*	*Post*		
	m±SD	*m±SD*	*p*	*m±SD*	*m±SD*	*p*	*p* (Δ**)**
Weight (kg)	103.9±18.2	103.5±17	0.078	103.4±15.6	105±18.4	0.797	0.142
BMI (Kg/m^2)	35.6±6.6	35.5±6.3	0.745	35.3±5.8	35.8±6.6	0.111	0.180
Waist circunf. (cm)	115.1±13.9	115.3±15.9	0.819	113.7±10.1	115.1±10.7	0.141	0.384
Glucose (mmol/L)	6.88±1.32	6.79±1.48	0.767	6.94±1.18	6.45±1.27	0.09	0.323
HbA1c (%)	6.3±0.8	6.3±0.9	0.950	6.3±0.7	6.3±0.7	0.593	0.746
Insulin (pmol/L)	101±50	80±50	0.262	119±114	99±965	0.244	0.991
HOMA_IR	4.4±2.5	3.3±1.8	0.139	4.9±4.4	4.0±2.7	0.165	0.901
Leptin (µg/L)	12.0±10.2	13±9.8	0.370	15.3±17.3	13.3±10.9	0.723	0.680
T (nmol/L)	9.31±2.08	17.0±4.16	**<.001**	9.72±2.43	10.8±1.74	0.177	**<.001**
FT (pmol/L)	242±105	448±143	**<.001**	245±105	292±95	0.111	**0.013**
DHT (nmol/L)	0.72±0.38	0.96±0.52	**<.001**	0.62±0.31	0.69±0.34	0.059	0.151
E2 (pmol/L)	80±33	135±42	**<.001**	98±18	80±25	0.467	**0.001**
FE2 (pmol/L)	1.17±0.51	1.87±0.70	**0.001**	1.51±0.29	1.21±0.48	0.829	**0.006**
SHBG (nmol/L)	21.9±9.9	24.9±9.7	**0.001**	21.9±9.6	21.7±8.7	0.774	**0.005**
LH **(IU/L)**	3.2±1.2	5.2±2.4	**0.026**	3.4±1.7	3.5±1.0	0.89	0.113
FSH **(IU/L)**	6.0±1.2	11.0±5.6	**0.001**	5.5±2.3	7.1±2.7	0.168	0.059

For the abbreviations. see the text.

P values represent the intra-treatment differences and p (Δ) values represent the comparison of the changes between treatments. For weight and leptin p values refers to their logarithmic transformation.

Statically significant differences are in bold.

Table 3. Estimate effect of clinical characteristics, metabolic parameters and hormonal values on IB. T and FSH variation during each treatment period.

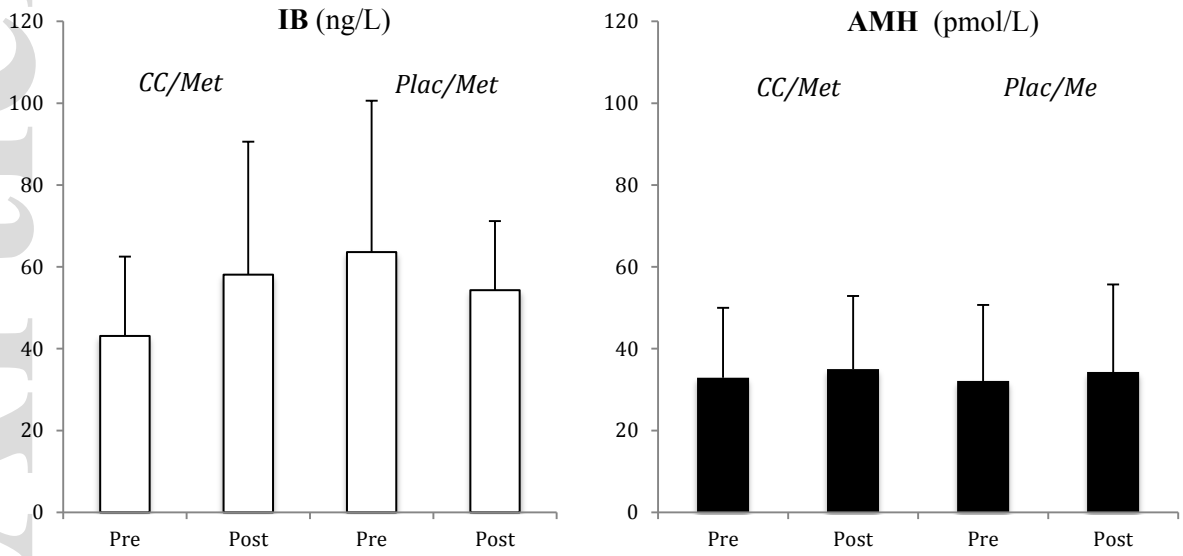
	IB			T			FSH		
	CC/Met	***Plac/Met***	***p (Δ)***	***CC/Met***	***Pla/Metc***	***p (Δ)***	***CC/Met***	***Plac/Met***	***p (Δ)***
Weight	-0.439	0.783	0.216	-0.030 *	0.000	0.130	-0.118	-0.032	0.395
BMI	-1.854	2.734	0.080	-0.072	-0.014	0.282	-0.247	-0.101	0.608
Waist circunference	-0.610	1.475	0.140	-0.038 *	-0.002	0.208	-0.151	-0.052	0.498
Glucose	-0.300	-0.224	0.918	0.014	-0.005	0.222	0.076	0.001	0.340
HbA1c	-17.986	-7.380	0.632	0.139	0.051	0.861	0.002	0.109	0.966
Insulin	-1.293	1.192	0.158	-0.036	0.004	0.340	0.046	-0.026	0.729
HOMA_IR	-4.569	4.715	0.071	0.005	0.016	0.927	0.592	-0.074	0.271
Leptin	-3.581	18.636	0.227	-0.789 *	0.039	**0.031**	-3.132 *	-0.751	0.219
T	21.769 *	-29.118 *	**0.003**	/	/	/	-1.684	-0.251	0.490
FT	0.302	-0.501	0.136	-0.010	-0.011	0.972	-0.016	0.007	0.704
DHT	130.204	-204.410	**0.038**	1.319	1.416	0.979	0.571	1.517	0.960
E2	1.352	-4.404	**0.049**	-0.079 †	0.032	0.055	-0.315 *	-0.004	0.349
FE2	54.259	-161.410	0.257	-4.194 *	2.965	0.069	-12.83	8.131	0.357
SHBG	1.393	-1.546	0.076	-0.006	0.020	0.496	-0.173	-0.067	0.551
LH	-0.526	12.176	0.250	-0.217	-0.048	0.515	-0.572	-0.065	0.701
FSH	-11.794	4.493	0.145	0.464 *	0.006	**0.048**	/	/	/
IB	/	/	/	-0.003	0.001	0.789	0.002	-0.011	0.876
AMH	3.673	-1.031	0.463	-0.155	-0.028	0.407	-1.171 *	0.026	0.101
AR-CAG	-0.303	-5.169	0.350	-0.097	-0.092	0.960	-0.146	-0.107	0.948

For the abbreviations of each clinical, genetic and hormonal parameter see the text

* $p<0.05$; † $p<0.01$; statically significant differences between treatment arms are in bold

Figure 1

IB and AMH values before and after each treatment period in all subjects



Inhibin B levels (IB) (ng/L);■AMH levels (pmolL) at baseline (Pre) and after (Post) each treatment period No significant differences within and between each treatment phase was observed)