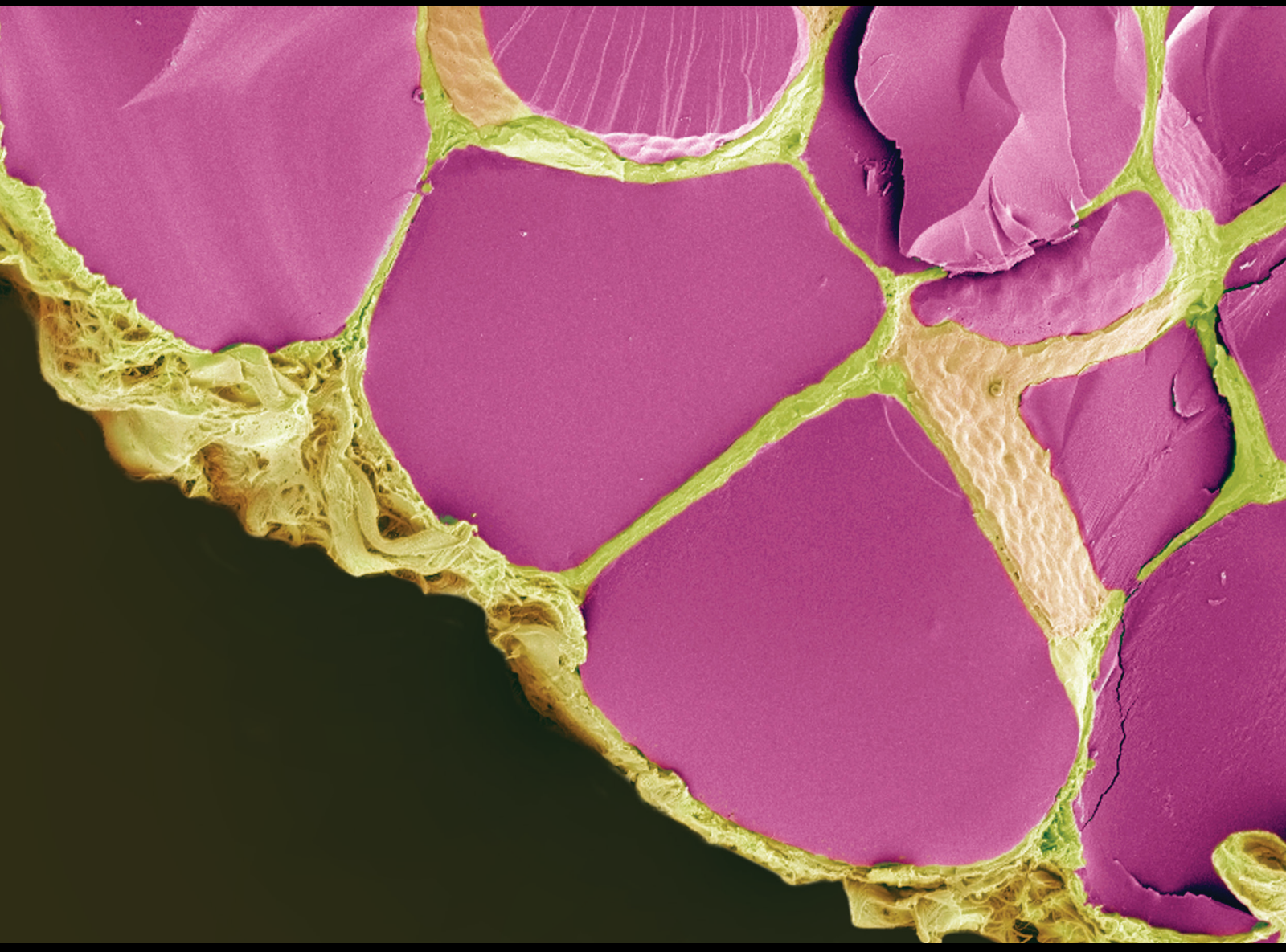
International Journal of Endocrinology

Male Hypogonadism and DHEA Deficiency: A Focus on Controversies in Replacement Therapy

Lead Guest Editor: Antonio Mancini
Guest Editors: Andrea Silvestrini and Vito A. Giagulli



Male Hypogonadism and DHEA Deficiency: A Focus on Controversies in Replacement Therapy

International Journal of Endocrinology

Male Hypogonadism and DHEA Deficiency: A Focus on Controversies in Replacement Therapy

Lead Guest Editor: Antonio Mancini
Guest Editors: Andrea Silvestrini and Vito A. Giagulli

Contents

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 5278040, 1 page
https://doi.org/10.1155/2020/5278040

Corrigendum

Corrigendum to "Anabolic Hormone Deficiencies in Heart Failure with Reduced or Preserved Ejection Fraction and Correlation with Plasma Total Antioxidant Capacity"

Antonio Mancini,[1] Angela Maria Rita Favuzzi,[2] Carmine Bruno,[1] Maria Anna Nicolazzi,[2] Edoardo Vergani,[1] Nunzia Ciferri,[1] Andrea Silvestrini,[3] Elisabetta Meucci,[3] Nicola Nicolotti,[4] Roberta D'Assante,[5] and Antonio Cittadini[5]

[1]*Operative Unit of Endocrinology, Fondazione Policlinico Universitario A Gemelli IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy*
[2]*Operative Unit of Internal Medicine and Vascular Diseases, Division of Internal Medicine and Cardiovascular Diseases, Fondazione Policlinico Universitario A Gemelli IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy*
[3]*Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, Rome, Italy*
[4]*Medical Management, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy*
[5]*Department of Translational Medical Sciences, Federico II University of Naples, Naples, Italy*

Correspondence should be addressed to Antonio Mancini; me4224@mclink.it and Andrea Silvestrini; andrea.silvestrini@unicatt.it

Received 22 August 2020; Accepted 22 August 2020; Published 27 October 2020

In the article titled "Anabolic Hormone Deficiencies in Heart Failure with Reduced or Preserved Ejection Fraction and Correlation with Plasma Total Antioxidant Capacity" [1], the name of the author Angela Maria Rita Favuzzi was given incorrectly as Angela Maria Rita Fuvuzzi. The revised authors' list is shown above.

References

[1] A. Mancini, A. M. R. Fuvuzzi, C. Bruno et al., "Anabolic hormone deficiencies in heart failure with reduced or preserved ejection fraction and correlation with plasma total antioxidant capacity," *International Journal of Endocrinology*, vol. 2020, Article ID 5798146, 7 pages, 2020.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 1942126, 8 pages
https://doi.org/10.1155/2020/1942126

Research Article

Increased DHEAS and Decreased Total Testosterone Serum Levels in a Subset of Men with Early-Onset Androgenetic Alopecia: Does a Male PCOS-Equivalent Exist?

Rossella Cannarella,[1] Rosita A. Condorelli,[1] Federica Dall'Oglio,[2] Sandro La Vignera,[1] Laura M. Mongioì,[1] Giuseppe Micali,[2] and Aldo E. Calogero[1]

[1]*Department of Clinical and Experimental Medicine, 95123 Catania, Italy*
[2]*Dermatology Clinic, University of Catania, 95123 Catania, Italy*

Correspondence should be addressed to Aldo E. Calogero; acaloger@unict.it

Received 7 September 2019; Accepted 23 January 2020; Published 12 February 2020

Guest Editor: Antonio Mancini

Background. Increased dehydroepiandrosterone sulfate (DHEAS) levels have been reported in men with early-onset (<35 years) androgenetic alopecia (AGA). It has been suggested that a male polycystic ovarian syndrome- (PCOS-) equivalent, defined as an endocrine syndrome with a metabolic background and a PCOS-like hormonal pattern, predisposing to type II diabetes mellitus (DM II), cardiovascular and prostate diseases later in life, may occur in at least a part of these men. The gonadal function, including sperm parameters and total testosterone (TT) levels, has been investigated in a low number of these men. *Objective*. The aim of the study was to assess gonadal and adrenal function in a subset of men with early-onset AGA and controls. *Methods*. 43 men with early-onset AGA and 36 controls were screened for DHEAS, TT, glycaemia, insulin, gonadotropins, 17α-hydroxyprogesterone (17αOH-P), testicular volume (TV), fat mass percentage, and sperm conventional parameters. Among men with AGA, we identified Group 1 ($n = 21$), as those with at least one of the following parameters: body mass index (BMI) >25 kg/m^2, insulin resistance (IR), and/or SHBG <25 nmol/l. *Results*. Patients with early-onset AGA had higher mean (±SD) BMI (25.5 ± 3.8 vs. 23.7 ± 3.0 kg/m^2; $P < 0.05$) and 17αOH-progesterone (2.1 ± 0.9 vs. 1.5 ± 0.6 ng/ml; $P < 0.05$) compared to controls. Group 1 had higher BMI (27.9 ± 3.8 vs. 23.7 ± 3.8 ml; $P < 0.05$), fat-mass percentage (17.5 ± 4.1 vs. 13.2 ± 5.3; $P < 0.05$), HOMA index (2.5 ± 1.8 vs. 1.5 ± 0.7; $P < 0.05$), DHEAS (323.3 ± 112.6 vs. 257.8 ± 107.1 μg/dl; $P < 0.05$), seminal fluid volume (4.2 ± 2.8 vs. 2.8 ± 1.3 ml; $P < 0.05$), lower TT (5.16 ± 1.70 vs. 6.47 ± 4.30 ng/ml; $P = 0.016$), and left TV (12.3 ± 2.8 vs. 15.0 ± 4.3 ml; $P < 0.05$) compared to controls. *Conclusion*. Men with early-onset AGA and at least one among BMI >25 kg/m^2, IR, and SHBG <25 nmol/l have increased DHEAS levels and a worse gonadal steroidogenesis. They might have a greater risk to develop gonadal dysfunction later in life. These criteria may be used to define male PCOS-equivalent.

1. Introduction

Male hypogonadism is defined as a gonadal dysfunction resulting in low total testosterone (TT) serum levels [1]. Dehydroepiandrosterone sulfate (DHEAS) is a molecule with a weak androgenic activity mainly secreted by the adrenal gland and, by a lesser extent, by the gonads, whose role in the pathogenesis of hypogonadism remains debated.

Meta-analytic data provide evidence for increased DHEAS levels and a worse glycolipid profile in male patients with early-onset (<35 years) androgenic alopecia (AGA), defined by a grade of alopecia higher than III according to the Hamilton–Norwood scale [2, 3], compared to controls [4]. Early-onset AGA may represent a feature of a male polycystic ovarian syndrome- (PCOS-) equivalent [5], a syndrome which causes gonadal dysfunction in the female gender.

The existence of a male PCOS-equivalent, whose features are summarized in Table 1, has been hypothesized based on the evidence of a genetic component in the etiology of female

Table 1: Clinical, biochemical and anamnestic features of the male equivalent of PCOS [5].

Age	Features of the male PCOS-equivalent
<35 years	(i) Clinical signs of hyperandrogenism (early-onset AGA and/or acne and/or hypertrichosis) (ii) PCOS-like hormonal pattern (increased DHEAS, AMH, 17α-OH-progesterone, FAI, decreased FSH) (iii) Metabolic abnormalities (insulin resistance, low SHBG levels, hyperglycaemia, hyperinsulinemia) and/or a trend towards higher BMI values (iv) A familiar history positive for PCOS
Elderly men	Diabetes mellitus, cardiovascular diseases, benign prostatic hyperplasia, prostatitis, prostate cancer

PCOS [6–10]. Accordingly, a higher prevalence of early-onset AGA has been observed in the first-degree male relatives of women with PCOS [11], in which hormonal, metabolic abnormalities [8, 12–14], and endothelial dysfunction [15] have been identified. Therefore, early-onset AGA has been investigated as a clinical sign of the male PCOS-equivalent [16–18]. In addition, a hormonal PCOS-like profile, consisting of decreased follicle-stimulating hormone (FSH), increased luteinizing hormone (LH), TT, androstenedione, and 17α-hydroxyprogesterone (17αOH-P) levels, hypertension, the presence of insulin resistance (IR), high body mass index (BMI), and low sex hormone binding globulin (SHBG), has been found in men with early-onset AGA [19–28].

A higher risk with aging to develop type II diabetes mellitus (T2DM) and coronary heart disease has also been observed in men with early-onset AGA [5]. Hence, AGA has been proposed as an independent predictor of mortality for T2DM and cardiovascular diseases (CVDs) [29]. These long-term issues resemble those known to be present in female PCOS [30–34]. They are also similar to those occurring in male hypogonadism [35, 36].

However, whether metabolic and cardiologic impairment is due to a concomitant hypogonadism in men with AGA is not known as studies assessing the gonadal function of these patients are lacking. In addition, likely female PCOS does not affect all women with hirsutism/acne and irregular menses, male PCOS-equivalent may occur only in a subset of men with early-onset AGA. Indeed, early-onset AGA has been estimated in approximately the 30% of men [37], whereas the prevalence of PCOS in women ranges between 4 and 7% [38–41]. As such, like in female PCOS, biochemical and/or clinical criteria are needed to identify a male PCOS-equivalent among men with early-onset AGA. Furthermore, the testicular function needs to be explored in these men.

Therefore, the aim of this study was to evaluate whether BMI >25 kg/m^2, IR and/or SHBG <25 nmol/l could be used to identify the presence of a male PCOS-equivalent among men with early-onset AGA and to assess their gonadal function.

2. Methods

This case-control study was conducted at the Division of Andrology and Endocrinology and the Dermatology Clinic, of the teaching hospital "G. Rodolico," University of Catania (Catania, Italy). The inclusion criteria were men with early-onset (before 35 years) AGA. The diagnosis of AGA was performed by clinical observation and by trichoscopy, a noninvasive *in vivo* technique that allows scalp and hair examination at high magnification (10–100x). In particular, trichoscopy was performed in the area delineated at the cross between the nose line and the ear implantation line, confirming the diagnosis of early-onset AGA by showing a hair diameter diversity >20%, a sign of progressive hair miniaturization [42].

Patients with at least one of the following were excluded: positive clinical history for cryptorchidism, testicular focal injury/injuries, testicular microlithiasis, varicocele, azoospermia, head injury, endocrine abnormalities (Cushing syndrome, acromegaly, and hypopituitarism), systemic diseases (kidney and/or liver diseases) and diabetes mellitus, consumption of alcohol and ingestion of drugs that may interfere with spermatogenesis (immunosuppressors, chemotherapy, steroids, and finasteride) during the previous 6 months, smoking and bacterial urogenital infection (evaluated by quantitative sperm and urethral swab culture), and previous chemotherapy.

At baseline, all patients were checked for LH, FSH, TT, albumin, DHEAS, 17αOH-P, SHBG, glycaemia, and insulin serum levels. The hormone measurement was performed by electrochemiluminescence (Hitachi-Roche equipment, Cobas 6000, Roche Diagnostics, Indianapolis, IN, USA). Free-T (fT) and bioavailable T (bio-T) were calculated using the Vermeulen formula [43], as recommended by a Task Force of the Endocrine Society [44]. IR was evaluated through the calculation of the HOMA index (fasting plasma glucose (mg/dl) × insulin (μUI/l)/405), and a value above 2.5 was considered indicative of IR. Patients also underwent conventional (performed according to the WHO criteria [45]) and biofunctional sperm parameters, bioelectrical impedance analysis (BIA), and scrotal ultrasound scan. Left and right testicular volumes (TV) were also measured, and total TV was obtained through the summation of both TV. Monolateral TV was considered normal when it ranged between 15 and 25 ml [46]. The BMI was calculated applying the formula: weight (kg)/(height (m) × height (m)).

Among early AGA patients, two groups were identified: Group 1, having one of the following parameters BMI >25 kg/m^2, IR, and/or SHBG <25 nmol/l or a combination thereof, and Group 2, negative for such findings.

The protocol was approved by the internal International Review Board, and an informed written consent was obtained from each patient. This work was carried out in accordance with the Declaration of Helsinki.

2.1. Statistical Analysis. The data were analyzed by Student's *t*-test and chi-squared test, as appropriate. A *P* value lower than 0.05 was accepted as statistically significant. A trend was assumed for *P* values ranging from 0.05 to 0.099.

3. Results

Eighty-one men were evaluated for eligibility, and two of them were excluded because they had azoospermia. Forty-three men with early-onset AGA (mean age: 24.3 ± 0.5 years; range: 14–30 years) were recruited and matched for age to thirty-six healthy subjects (mean age: 23.5 ± 0.5 years; range: 18–29 years) with no sign of AGA (Figure 1). The mean age of AGA onset was 20.8 ± 3.0 years (range: 16–26 years).

All patients with early AGA had significantly higher BMI and 17αOH-P serum levels compared to controls ($P < 0.05$); an upward trend for DHEAS, a downward trend for TT, and a higher sperm apoptosis percentage compared to controls ($P < 0.05$) were also found. The hormone/metabolic profile, conventional and biofunctional sperm parameters, and TV of patients and controls are summarized in Tables 2 and 3.

Among early AGA patients, we identified Group 1 ($n = 21$), having one of the following parameters BMI >25 kg/m^2 ($n = 7$), IR ($n = 4$), and/or SHBG <25 nmol/l ($n = 2$) or a combination thereof ($n = 8$), and Group 2 ($n = 22$), negative for such parameters (Figure 1).

Results from Group 1 compared to Group 2 showed significantly higher levels of insulin ($P < 0.01$) and LH ($P < 0.05$); lower TT levels ($P < 0.05$) and smaller left TV ($P < 0.05$); an upward trend for the percentage of fat mass, triglycerides (TGL), and sperm progressive motility; and a downward trend for the percentage of fat-free mass, total TV, and sperm concentration.

Results from Group 1 compared to controls showed significantly higher percentage of fat mass ($P < 0.05$), DHEAS ($P < 0.05$), and seminal fluid ($P = 0.027$); lower TT ($P = 0.016$), left TV ($P < 0.05$), and leukocyte concentration ($P < 0.05$); an upward trend for LH, 17αOH-P levels; and the percentage of spermatozoa in apoptosis. Notably, Group 1 showed a lower but not significant different value of the right TV (14.6 ± 2.6 ml).

As far the conventional sperm parameters, Group 1 showed an increased volume compared with Group 2 and controls ($P < 0.05$), a downward trend towards a lower sperm concentration compared to Group 2 ($0.05 < P < 0.1$), an upward trend towards an increased total motility compared to controls ($0.05 < P < 0.1$), and lower concentration of leukocytes in the seminal fluid compared to controls ($P < 0.05$) (Table 3). Finally, the biofunctional sperm parameters showed that the percentage of apoptotic spermatozoa was higher in patients with AGA compared to controls ($P < 0.05$) (Table 3). Results are summarized in Figure 2.

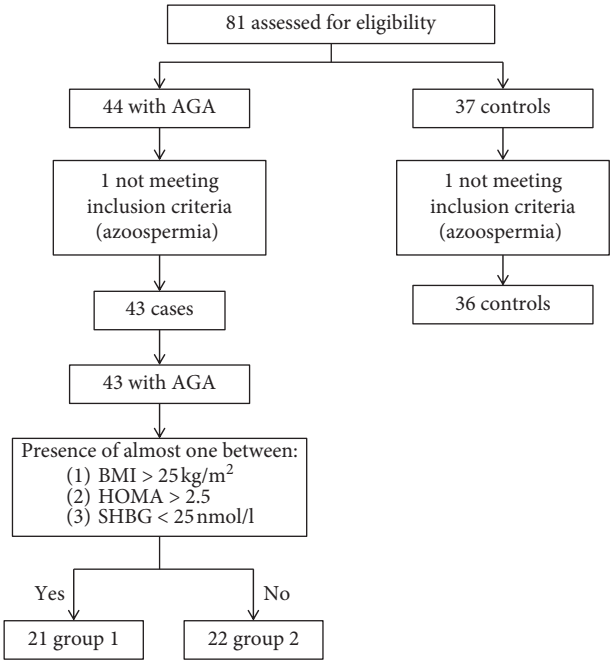


Figure 1: Study design. Eighty-one men younger than thirty years were enrolled in this study. Among them, forty-three had androgenetic alopecia (AGA) and were recruited as patients, and thirty-six made up the group of controls. Patients with AGA and at least one between BMI >25 kg/m^2, HOMA index >2.5, and SHBG <25 nmol/l were included in Group 1 ($n = 21$). The remaining men made up Group 2 ($n = 22$). Group 1 included 7 patients having BMI >25 kg/m^2, 2 patients having SHBG <25 nmol/l, 4 with IR, and 8 a combination of such alterations.

4. Discussion

Early-onset AGA has been proposed as a phenotypic sign of the male PCOS-equivalent. Previous studies have described the presence of a hormonal PCOS-like pattern in men with early-onset AGA, including low levels of SHBG [20–22, 24, 29, 47] and FSH [20–22, 29], and increased levels of 17αOH-P [21] and DHEAS [23, 24]. Data on TT and fT serum levels are contradictory: low fT levels [19, 28], increased free-androgen index (FAI) [20, 21, 29, 48], or subnormal TT [20, 21] values have been described. Patients may also show hyperglycaemia [40], IR [24–28], hyperinsulinemia [21, 47], metabolic syndrome [47], hypercholesterolemia [28], higher mean diastolic blood pressure [25, 26], and higher BMI levels [24]. An increased prevalence of T2DM and CVDs has also been reported in these men [5]. Since women with PCOS also present similar metabolic abnormalities and a higher risk for CVDs [49], these findings seem to support the hypothesis of the existence of a male PCOS-equivalent.

Male PCOS-equivalent may be considered as a "new" or previously unrecognized endocrine syndrome affecting the male gender. The delay in its recognition might be due to the sex-related perception of the phenotypic signs. Accordingly, suggested features include clinical signs of hyperandrogenism, such as early-onset AGA, acne, and/or hypertrichosis. While women readily perceive irregular menses, hirsutism, acne, and/or defluvium, early-onset AGA may be seen as a part of the normal masculine virilization [5]. Other features occurring before the age of 35 include PCOS-like hormonal pattern, metabolic abnormalities, and/or trend towards higher BMI values [5]. A family history positive for female PCOS may be also suggestive for the syndrome, which may expose to higher risk of developing DM II, CVD, and prostate diseases later in life [5].

In this context, the pathogenesis of early-onset AGA has been addressed to the peripheral conversion of weak adrenocortical androgens (e.g., DHEAS) into stronger

Table 2: Clinical features and serum hormone levels in patients with early-onset androgenetic alopecia (AGA) and in controls.

Parameter	Patients with AGA: $n = 43$	Group 1[a]: $n = 21$	Group 2[b]: $n = 22$	Control subjects: $n = 36$
Age (year)	24.3 (3.4)	24.0 (3.9)	24.5 (2.8)	23.3 (2.7)
BMI (kg/m^2)	25.5 (3.8)*	27.9 (3.8)*†	23.0 (1.6)*	23.7 (3)
Fat mass (%)	15.6 (4.8)	17.5 (4.1)^{*d}	12.4 (4.7)	13.2 (5.3)
Cholesterol (mg/dl)	167.5 (31.4)	163.6 (35.4)	171.2 (27.8)	170.1 (38.2)
HDL cholesterol (mg/dl)	49.2 (13.3)	45.4 (10.9)	52.7 (14.6)	47.7 (9.1)
LDL cholesterol (mg/dl)	104.4 (25.0)	101.7 (28.5)	106.9 (21.9)	100.4 (45.6)
Triglycerides (mg/dl)	69.9 (38.5)	82.5 (46.0)^b	58.1 (26.1)	68.7 (35.6)
Glycaemia (mg/dl)	84.4 (8.0)	85.1 (8.9)	83.7 (7.1)	83.8 (6.1)
Insulin (μU/ml)	8.8 (6.2)	11.8 (7.7)†	6.0 (2.1)*	7.3 (3.2)
HOMA index	1.9 (1.4)	2.5 (1.8)*†	1.3 (0.5)*	1.5 (0.7)
LH (mIU/ml)	4.3 (1.8)	4.9 (2.1)†	3.8 (1.4)	3.9 (2.2)
FSH (mIU/ml)	3.1 (2.0)	3.1 (1.8)	3.2 (2.2)	3.3 (2.5)
TT (ng/ml)	5.8 (1.71)^b	5.2 (1.7)*†	6.3 (1.6)	6.5 (2.0)
17β-Estradiol (pg/ml)	24.5 (9.6)	22.9 (10.4)	26.0 (8.7)	25.6 (7.7)
Androstenedione (ng/ml)	2.5 (1.1)	2.6 (1.2)	2.4 (1.0)	2.3 (0.9)
DHEAS (μg/dl)	304 (17.1)^d	321.5 (112.6)	290.3 (97.0)	257.8 (107.1)
17αOH-P (ng/ml)	2.1 (0.9)$^{\dagger *}$	2.0 (1.1)^c	2.1 (0.7)	1.5 (0.6)
Cortisol (nmol/l)	429.6 (125.3)	429.8 (132.9)	429.3 (120.4)	383.1 (119.0)
SHBG (nmol/l)	32.9 (10.3)	28.9 (9.7)^d	36.6 (9.9)	34.7 (13.7)
Bio-T (%)	44.8 (8.1)	46.9 (7.6)	43.0 (8.4)	45.3 (11.1)
Free-T	1.8 (0.4)	2.0 (0.3)	1.7 (0.4)	1.8 (0.5)

Abbreviations: 17αOH-P = 17α-hydroxyprogesterone; Bio-T = bioavailable testosterone; BMI = body mass index; Free-T = free testosterone; FSH = follicle-stimulating hormone; LH = luteinizing hormone; SHBG = Sex hormone-binding globulin; TT = total testosterone. [a]Patients with early-onset AGA with at least one of the following parameters: body mass index (BMI) >25 kg/m^2, insulin-resistance (IR), and/or sex hormone-binding globulin (SHBG) <25 nmol/l. [b]Patients with early-onset AGA which did not satisfy any of the aforementioned criteria. $^*P < 0.05$ compared to controls; $^\dagger P < 0.05$ compared to Group 2; $^c P < 0.1$ compared to controls; $^d P < 0.1$ compared to Group 2 (Student's t-test). Results are expressed as mean value (standard deviation).

Table 3: Sperm parameters and testicular volume in patients with early-onset androgenetic alopecia (AGA) and in controls.

Parameters	Patients with AGA: $n = 43$	Group 1[a]: $n = 21$	Group 2[b]: $n = 22$	Control subjects: $n = 36$
Conventional sperm parameters				
Volume (ml)	3.6 (2.2)	4.2 (2.8)*†	3.0 (1.5)	2.8 (1.3)
pH	7.9 (1.3)	8.0 (0.3)	7.7 (1.8)	8.0 (0.3)
Sperm concentration (mil/ml)	66.6 (49.8)	51.7 (42.4)^d	79.9 (53.1)	52.4 (33.2)
Total sperm count (mil/ejaculate)	193.8 (142.8)	51.7 (42.7)	79.9 (53.1)	147.7 (104.3)
Forward motility (%)	18.7 (10.6)	21.3 (10.2)	16.3 (10.6)	15.9 (10.8)
Total motility (%)	56.6 (14.5)	60.9 (9.9)^c	52.8 (17.0)	59.5 (8.3)
Normal morphology (%)	6.3 (2.9)	7.2 (3.0)	5.6 (2.7)	5.8 (3.3)
Leukocyte concentration (mil/ml)	2.1 (3.8)	1.2 (1.2)	3.0 (5.0)	2.5 (5.7)
Total leukocyte count (mil/ejaculate)	2.9 (8.2)	0.6 (0.7)*	5.1 (11.1)	2.7 (3.6)
Biofunctional sperm parameters				
Low mitochondrial membrane potential (%)	29.0 (20.8)	26.2 (17.5)	31.4 (23.6)	19.8 (17.8)
Abnormal chromatin compactness (%)	25.1 (8.1)	25.7 (5.7)	24.8 (9.5)	25.9 (8.2)
Alive spermatozoa (%)	73 (12.0)	72.2 (12.9)	73.8 (11.6)	75.5 (14.2)
Phosphatidylserine externalization (%)	1.9 (1.9)	2.4 (2.3)	1.5 (1.3)	1.8 (1.5)
Sperm apoptosis (%)	10.7 (12.6)**	7.8 (7.5)^{c,d}	13.5 (16.0)	3.4 (3.7)
DNA fragmentation (%)	2.9 (3.5)	1.7 (1.1)	4.2 (4.8)	1.2
Testicular volume				
Total testicular volume (ml)	29.5 (7.5)	24.9 (4.7)	32.6 (9.1)	30.9 (7.9)
Left testicular volume (ml)	13.7 (3.7)	12.3 (2.8)*†	15.4 (4.1)	15.0 (4.3)
Right testicular volume (ml)	15.8 (4.3)	14.6 (2.6)	17.2 (5.6)	15.8 (4.0)

[a]Patients with early-onset AGA with at least one of the following parameters: body mass index (BMI) >25 Kg/m^2, insulin-resistance (IR), and/or sex hormone-binding globulin (SHBG) <25 nmol/l. [b]Patients with early-onset AGA which did not satisfy any of the aforementioned criteria. $^*P < 0.05$ compared to controls; $^\dagger P < 0.05$ compared to Group 2; $^c P < 0.1$ compared to controls; $^d P < 0.1$ compared to Group 2 (Student's t-test). The DNA fragmentation was evaluated through TUNEL test. Results are expressed as mean value (standard deviation).

androgens. Furthermore, hyperinsulinemia or IR could promote microcirculatory insufficiency, hypoxia, and hair follicle miniaturization into the scalp [5]. However, insulin could also negatively impact on testicular steroidogenesis, decreasing testosterone production [50], and on Sertoli cell function as well [51]. Hence, hyperinsulinemia might hypothetically lead to increase of DHEAS and decrease of TT serum levels.

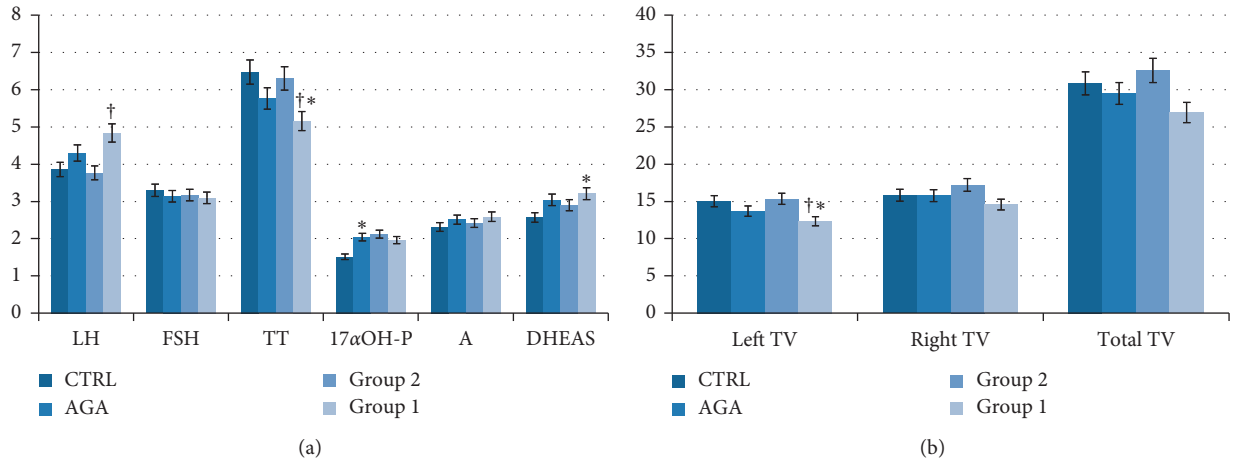


Figure 2: Hormonal parameters in Group 1, Group 2, AGA, and control group (a). Patients with early-onset androgenetic alopecia (AGA) had higher 17α-hydroxyprogesterone (17αOH-P) and an uptrend towards DHEAS values compared to controls. Group 1 had lower total testosterone (TT) values compared both to Group 2 and to controls. $^{*}P < 0.05$ compared to control; $^{\dagger}P < 0.05$ compared to Group 2 (Student's t-test). Left, right, and total testicular volume in Group 1, Group 2, AGA, and control group (b). No difference in total, left, and right testicular volume (TV) was observed in patients with early-onset AGA compared to controls. Group 1 showed lower left TV compared both to Group 2 and to controls. A downward trend for total TV was found in patients of Group 1 compared to those of Group 2. $^{*}P < 0.05$ compared to control; $^{\dagger}P < 0.05$ compared to Group 2 (Student's t-test).

Not all women with clinical signs of hyperandrogenism satisfy female PCOS diagnostic criteria. Similarly, it is likely that not all men with early-onset AGA are affected by the syndrome. Thus, additional markers are needed to identify those who may have the male PCOS-equivalent syndrome among men with early-onset AGA.

Based on previous reports that showed the presence of IR, higher BMI, and lower SHBG levels in men with early-onset AGA [19, 21, 24–28], we classified patients into two groups: those with at least one among these signs: BMI >25 Kg/m^2, IR and/or SHBG <25 nmol/l (Group 1) and those who did not have any of them (Group 2). The TV values found in Group 1 support the hypothesis that patients with early-onset AGA who might have a male PCOS-equivalent syndrome do show a distinct gonadal profile compared with their not-at-risk counterparts.

As for the spermatogenetic testicular function, a downward trend for sperm concentration in Group 1 compared to Group 2 was found, but serum FSH levels did not differ significantly among all groups. However, abnormal levels of FSH have been described both in men with early-onset AGA and to controls [19–21, 46] and in first-degree male relatives of women with PCOS [52].

Group 1 also showed higher fat mass percentage compared to controls and an upward trend compared to Group 2, in agreement with a previous study [53].

These findings support the possible role of BMI, IR, and SHBG as candidate diagnostic criteria to identify the male PCOS-equivalent syndrome among men with early-onset AGA. Indeed, the lower TT and TV levels, along with the higher LH values in patients with early-onset AGA and at least one among BMI >25 Kg/m^2, IR, and SHBG <25 nmol/l compared to controls indicate a worse gonadal profile in this subset of men. Similar to women [49], it might represent a feature of the male PCOS-equivalent. The occurrence of a worse gonadal function in these men may predispose to the development of CVDs [54, 55] and/or DM type II later in life, as it happens for women with PCOS [30–34]. In addition, a higher risk of CVDs has been described in men with AGA [56]. The early detection of hypogonadism may prevent the onset of such comorbidities.

5. Conclusions

The results of our study suggest the presence of a distinct gonadal profile in men with early-onset AGA and presenting with at least one among BMI >25 Kg/m^2, IR, and SHBG <25 nmol/l. Similarly, to the female PCOS which negatively impact on the ovarian function, such distinct gonadal profile may be associated to a male PCOS-equivalent in a subset of patients, including increased DHEAS and reduced TT serum levels. Therefore, patients with early-onset AGA for increased BMI, IR, and/or SHBG serum levels should undergo to a more accurate control of the gonadal function, although larger and well-powered studies are needed to confirm our findings.

Abbreviations

AGA: Androgenetic alopecia;
bio-T: Bio-available T;
BIA: Bioelectrical impedance analysis;
BMI: Body mass index;
CVDs: Cardiovascular diseases;
DHEAS: Dehydroepiandrosterone sulfate;
DM II: Type II diabetes mellitus;
FAI: Free androgen index;
FSH: Follicle-stimulating hormone;
fT: Free testosterone;
17αOH-P: 17α-hydroxyprogesterone;
IR: Insulin resistance;

LH: Luteinizing hormone;
PCOS: Polycystic ovary syndrome;
SHBG: Sex hormone-binding globulin;
TGL: Triglycerides;
TT: Total testosterone;
TV: Testicular volume.

Data Availability

Data used to support the findings of this study have not been made available because they belong to a wider project on the male PCOS-equivalent that is currently going on.

Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] A. M. Isidori, G. Balercia, A. E. Calogero et al., "Outcomes of androgen replacement therapy in adult male hypogonadism: recommendations from the Italian society of endocrinology," *Journal of Endocrinological Investigation*, vol. 38, no. 1, pp. 103–112, 2015.

[2] J. B. Hamilton, "Patterned loss of hair in man: types and incidence," *Annals of the New York Academy of Sciences*, vol. 53, no. 3, pp. 708–728, 1951.

[3] O. T. Norwood, "Male pattern baldness: classification and incidence," *Southern Medical Journal*, vol. 68, no. 11, pp. 1359–1365, 1975.

[4] R. Cannarella, S. La Vignera, R. A. Condorelli, and A. E. Calogero, "Glycolipid and hormonal profiles in young men with early-onset androgenetic alopecia: a meta-analysis," *Scientific Reports*, vol. 7, no. 1, p. 7801, 2017.

[5] R. Cannarella, R. A. Condorelli, L. M. Mongioì, S. La Vignera, and A. E. Calogero, "Does a male polycystic ovarian syndrome equivalent exist?," *Journal of Endocrinological Investigation*, vol. 41, no. 1, pp. 49–57, 2018.

[6] R. S. Legro, D. Driscoll, J. F. Strauss III, J. Fox, and A. Dunaif, "Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome," *Proceedings of the National Academy of Sciences*, vol. 95, no. 25, pp. 14956–14960, 1998.

[7] R. S. Legro, D. Finegood, and A. Dunaif, "A fasting glucose to insulin ratio is a useful measure of insulin sensitivity in women with polycystic ovary syndrome 1," *The Journal of Clinical Endocrinology & Metabolism*, vol. 83, no. 8, pp. 2694–2698, 1998.

[8] R. S. Legro, A. R. Kunselman, L. Demers, S. C. Wang, R. Bentley-Lewis, and A. Dunaif, "Elevated dehydroepiandrosterone sulfate levels as the reproductive phenotype in the brothers of women with polycystic ovary syndrome," *The Journal of Clinical Endocrinology & Metabolism*, vol. 87, no. 5, pp. 2134–2138, 2002.

[9] S. Franks and M. McCarthy, "Genetics of ovarian disorders: polycystic ovary syndrome," *Reviews in Endocrine and Metabolic Disorders*, vol. 5, no. 1, pp. 69–76, 2004.

[10] A. E. Calogero, V. Calabrò, M. Catanuso, R. A. Condorelli, and S. La Vignera, "Understanding polycystic ovarian syndrome pathogenesis: an updated of its genetic aspects," *Journal of Endocrinological Investigation*, vol. 34, no. 8, pp. 630–644, 2011.

[11] O. Lunde, P. Magnus, L. Sandvik, and S. Høglo, "Familial clustering in the polycystic ovarian syndrome," *Gynecologic and Obstetric Investigation*, vol. 28, no. 1, pp. 23–30, 1989.

[12] R. Benítez, T. Sir-Petermann, A. Palomino et al., "Prevalence of metabolic disorders among family members of patients with polycystic ovary syndrome," *Revista Medica de Chile*, vol. 129, no. 7, pp. 707–712, 2001.

[13] M. Yilmaz, N. Bukan, R. Ersoy et al., "Glucose intolerance, insulin resistance and cardiovascular risk factors in first degree relatives of women with polycystic ovary syndrome," *Human Reproduction (Oxford, England)*, vol. 20, no. 9, pp. 2414–2420, 2005.

[14] R. J. Norman, S. Masters, and W. Hague, "Hyperinsulinemia is common in family members of women with polycystic ovary syndrome," *Fertility and Sterility*, vol. 66, no. 6, pp. 942–947, 1996.

[15] R. Kaushal, N. Parchure, G. Bano, J.-C. Kaski, and S. S. Nussey, "Insulin resistance and endothelial dysfunction in the brothers of Indian Subcontinent Asian women with polycystic ovaries," *Clinical Endocrinology*, vol. 60, no. 3, pp. 322–328, 2004.

[16] A. H. Carey, K. L. Chan, F. Short, D. White, R. Williamson, and S. Franks, "Evidence for a single gene effect causing polycystic ovaries and male pattern baldness," *Clinical Endocrinology*, vol. 38, no. 6, pp. 653–658, 1993.

[17] A. Govind, M. S. Obhrai, and R. N. Clayton, "Polycystic ovaries are inherited as an autosomal dominant trait: analysis of 29 polycystic ovary syndrome and 10 control families," *The Journal of Clinical Endocrinology & Metabolism*, vol. 84, no. 1, pp. 38–43, 1999.

[18] R. S. Legro, "Is there a male phenotype in polycystic ovary syndrome families?," *Journal of Pediatric Endocrinology and Metabolism*, vol. 13, no. 5, pp. 1307–1309, 2000.

[19] L. Starka, M. Duskova, I. Cermakova, J. Vrbiková, and M. Hill, "Premature androgenic alopecia and insulin resistance. Male equivalent of polycystic ovary syndrome?," *Endocrine Regulations*, vol. 39, no. 4, pp. 127–131, 2005.

[20] L. Stárka, I. Čermáková, M. Dušková, M. Hill, M. Doležal, and V. Poláček, "Hormonal profile of men with premature balding," *Experimental and Clinical Endocrinology & Diabetes*, vol. 112, no. 1, pp. 24–28, 2004.

[21] M. Dusková, I. Cermáková, M. Hill, M. Vanková, P. Sámalíková, and L. Stárka, "What may be the markers of the male equivalent of polycystic ovary syndrome?," *Physiological research*, vol. 53, no. 3, pp. 287–295, 2004.

[22] R. L. Pitts, "Serum elevation of dehydroepiandrosterone sulfate associated with male pattern baldness in young men," *Journal of the American Academy of Dermatology*, vol. 16, no. 3, pp. 571–573, 1987.

[23] S. Sanke, R. Chander, A. Jain, T. Garg, and P. Yadav, "A comparison of the hormonal profile of early androgenetic alopecia in men with the phenotypic equivalent of polycystic ovarian syndrome in women," *JAMA Dermatology*, vol. 152, no. 9, pp. 986–991, 2016.

[24] V. Matilainen, P. Koskela, and S. Keinänen-Kiukaanniemi, "Early androgenetic alopecia as a marker of insulin resistance," *The Lancet*, vol. 356, no. 9236, pp. 1165-1166, 2000.

[25] J. G. González-González, L. G. Mancillas-Adame, M. Fernández-Reyes et al., "Androgenetic alopecia and insulin resistance in young men," *Clinical Endocrinology*, vol. 71, no. 4, pp. 494–499, 2009.

[26] S. Arias-Santiago, M. T. Gutiérrez-Salmerón, A. Buendía-Eisman, M. S. Girón-Prieto, and R. Naranjo-Sintes, "Sex hormone-binding globulin and risk of hyperglycemia in patients with androgenetic alopecia," *Journal of the American Academy of Dermatology*, vol. 65, no. 1, pp. 48–53, 2011.

[27] L.-H. Su and T. H.-H. Chen, "Association of androgenetic alopecia with metabolic syndrome in men: a community-based survey," *British Journal of Dermatology*, vol. 163, no. 2, pp. 371–377, 2010.

[28] C. Mumcuoglu, T. R. Ekmekci, and S. Ucak, "The investigation of insulin resistance and metabolic syndrome in male patients with early-onset androgenetic alopecia," *European Journal of Dermatology*, vol. 21, no. 1, pp. 79–82, 2011.

[29] S. Narad, S. Pande, S. Chari, and M. Gupta, "Hormonal profile in Indian men with premature androgenetic alopecia," *International Journal of Trichology*, vol. 5, no. 2, pp. 69–72, 2013.

[30] D. A. Ehrman, R. B. Barnes, R. L. Rosenfield, M. K. Cavaghan, and J. Imperial, "Prevalence of impaired glucose tolerance and diabetes in women polycystic ovary syndrome," *Diabetes Care*, vol. 22, no. 1, pp. 141–146, 1999.

[31] R. S. Legro, A. R. Kunselman, W. C. Dodson, and A. Dunaif, "Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: a prospective, controlled study in 254 affected women 1," *Journal of Clinical Endocrinology & Metabolism*, vol. 84, no. 1, pp. 165–169, 1999.

[32] R. A. Wild, R. D. Painter, P. B. Coulson, K. B. Carruth, and R. B. Ranney, "Lipoprotein lipid concentrations as cardiovascular risk in patients with polycystic ovary syndrome," *The Journal of Clinical Endocrinology and Metabolism*, vol. 61, no. 5, pp. 946–950, 1985.

[33] S. Wild, T. Pierpoint, H. McKeigue, and H. Jacobs, "Cardiovascular disease in women with polycystic ovary syndrome at long-term follow-up: a retrospective cohort study," *Clinical Endocrinology*, vol. 52, no. 5, pp. 595–600, 2000.

[34] R. S. Legro, "Polycystic ovary syndrome and cardiovascular disease: a premature association?," *Endocrine Reviews*, vol. 24, no. 3, pp. 302–312, 2003.

[35] N. S. Malipatil, G. Yadegarfar, M. Lunt et al., "Male hypogonadism: 14-year prospective outcome in 550 men with type 2 diabetes," *Endocrinology, Diabetes & Metabolism*, vol. 2, no. 3, Article ID e00064, 2019.

[36] F. Saad, M. Caliber, G. Doros, K. S. Haider, and A. Haider, "Long-term treatment with testosterone undecanoate injections in men with hypogonadism alleviates erectile dysfunction and reduces risk of major adverse cardiovascular events, prostate cancer, and mortality," *The Aging Male*, vol. 23, no. 1, pp. 81–92, 2020.

[37] R. D. Sinclair and R. P. R. Dawber, "Androgenetic alopecia in men and women," *Clinics in Dermatology*, vol. 19, no. 2, pp. 167–178, 2001.

[38] E. S. Knochenhauer, T. J. Key, M. Kahsar-Miller, W. Waggoner, L. R. Boots, and R. Azziz, "Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study," *Journal of Clinical Endocrinology & Metabolism*, vol. 83, no. 9, pp. 3078–3082, 1998.

[39] E. Diamanti-Kandarakis, C. R. Kouli, A. T. Bergiele et al., "A survey of the polycystic ovary syndrome in the Greek Island of Lesbos: hormonal and metabolic profile," *The Journal of Clinical Endocrinology & Metabolism*, vol. 84, no. 11, pp. 4006–4011, 1999.

[40] M. Asuncion, R. M. Calvo, J. L. San Millán, J. Sancho, S. Avila, and H. F. Escobar-Morreale, "A prospective study of the prevalence of the polycystic ovary syndrome in unselected Caucasian women from Spain," *Journal of Clinical Endocrinology & Metabolism*, vol. 85, no. 7, pp. 2434–2438, 2000.

[41] R. Azziz, K. S. Woods, R. Reyna, T. J. Key, E. S. Knochenhauer, and B. O. Yildiz, "The prevalence and features of the polycystic ovary syndrome in an unselected population," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 6, pp. 2745–2749, 2004.

[42] F. Lacarrubba, G. Micali, and A. Tosti, "Scalp dermoscopy or trichoscopy," in *Alopecias—Practical Evaluation and Management*, vol. 47, no. 47, pp. 21–32, Karger, Basel, Switzerland, 2015.

[43] A. Vermeulen, L. Verdonck, and J. M. Kaufman, "A critical evaluation of simple methods for the estimation of free testosterone in serum," *The Journal of Clinical Endocrinology & Metabolism*, vol. 84, no. 10, pp. 3666–3672, 1999.

[44] S. Bhasin, G. R. Cunningham, F. J. Hayes et al., "Testosterone therapy in men with androgen deficiency syndromes: an endocrine society clinical practice guideline," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 6, pp. 2536–2559, 2010.

[45] WHO, *Laboratory Manual for the Examination and Processing of Human Semen*, World Health Organization, Geneva, Switzerland, 5th edition, 2010.

[46] C. Foresta, A. Garolla, A. C. Frigo et al., "Anthropometric, penile and testis measures in post-pubertal Italian males," *Journal of Endocrinological Investigation*, vol. 36, no. 36, pp. 287–292, 2013.

[47] N. Pengsalae, C. Tanglertsampan, T. Phichawong, and S. Lee, "Association of early-onset androgenetic alopecia and metabolic syndrome in Thai men: a case-control study," *Journal of the Medical Association of Thailand*, vol. 96, no. 8, pp. 947–951, 2013.

[48] M. Dusková, M. Hill, and L. Stráka, "The polycystic ovary syndrome and its male equivalent," *CasLek Cesk*, vol. 146, no. 3, pp. 251–255, 2007.

[49] N. F. Goodman, R. H. Cobin, W. Futterweit, J. S. Glueck, R. S. Legro, and E. Carmina, "American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and PCOS Society Disease State Clinical review: guide to the best practices in the evaluation and treatment of polycystic ovary syndrome—part 1," *Endocrine Practice*, vol. 21, no. 11, pp. 1291–1300, 2015.

[50] S. W. Ahn, G.-T. Gang, Y. D. Kim et al., "Insulin directly regulates steroidogenesis via induction of the orphan nuclear receptor DAX-1 in testicular Leydig cells," *Journal of Biological Chemistry*, vol. 288, no. 22, pp. 15937–15946, 2013.

[51] R. Cannarella, I. Arato, R. A. Condorelli et al., "Effects of insulin on porcine neonatal Sertoli cell responsiveness to FSH *in vitro*," *Journal of Clinical Medicine*, vol. 8, no. 6, p. 809, 2019.

[52] L. C. Torchen, A. Kumar, B. Kalra et al., "Increased anti-müllerian hormone levels and other reproductive endocrine changes in adult male relatives of women with polycystic ovary syndrome," *Fertility and Sterility*, vol. 106, no. 1, pp. 50–55, 2016.

[53] D. A. Dumesic, A. L. Akopians, V. K. Madrigal et al., "Hyperandrogenism accompanies increased intra-abdominal fat storage in normal weight polycystic ovary syndrome women," *The Journal of Clinical Endocrinology & Metabolism*, vol. 101, no. 11, pp. 4178–4188, 2016.

[54] G. M. C. Rosano, I. Sheiban, R. Massaro et al., "Low testosterone levels are associated with coronary artery disease in

male patients with angina," *International Journal of Impotence Research*, vol. 19, no. 2, pp. 176–182, 2007.

[55] L. Antonio, F. C. W. Wu, T. W. O'Neill et al., "Associations between sex steroids and the development of metabolic syndrome: a longitudinal study in European men," *The Journal of Clinical Endocrinology & Metabolism*, vol. 100, no. 4, pp. 1396–1404, 2015.

[56] S.-Y. Park, S. S. Oh, and W.-S. Lee, "Relationship between androgenetic alopecia and cardiovascular risk factors according to BASP classification in Koreans," *The Journal of Dermatology*, vol. 43, no. 11, pp. 1293–1300, 2016.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 9649838, 9 pages
https://doi.org/10.1155/2020/9649838

Review Article

Is There Room for SERMs or SARMs as Alternative Therapies for Adult Male Hypogonadism?

Vito A. Giagulli,[1] Andrea Silvestrini,[2] Carmine Bruno,[3] Vincenzo Triggiani,[1] Alvaro Mordente,[2] and Antonio Mancini[3]

[1]*Internal Medicine, Geriatrics, Endocrinology and Rare Disease, Interdisciplinary Department of Medicine, University of Bari, School of Medicine, Policlinico, Bari, Italy*
[2]*Dipartimento di Scienze Biotecnologiche di Base, Cliniche Intensivologiche e Perioperatorie, Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario A Gemelli IRCCS, Rome, Italy*
[3]*Dipartimento di Medicina e Chirurgia Traslazionale, Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario A Gemelli IRCCS, Rome, Italy*

Correspondence should be addressed to Andrea Silvestrini; andrea.silvestrini@unicatt.it and Antonio Mancini; me4224@mclink.it

Received 12 September 2019; Revised 25 November 2019; Accepted 2 December 2019; Published 21 January 2020

Academic Editor: Michael Horowitz

Hypogonadotropic hypogonadism (HH) can be sustained by organic or functional alterations of the hypothalamic-pituitary-testicular axis. Functional HH is related to systemic alterations, such as obesity or chronic inflammatory diseases, but could contribute to a negative course of the illness. For such situation, according to results obtained in infertile women, the administration of selective estrogen receptor modulators (SERMs) has been proposed in males too, with positive results on both metabolic and sexual function. This class of medications increases gonadotropin levels via antagonism to the estrogenic receptor; similar biological effects are also exerted by aromatase inhibitors (AIs), despite different mechanism of action. After a brief review of trials regarding SERMs and AIs use in male HH, we describe the structure and function of the androgen receptor (AR) as a basis for clinical research about compounds able to bind to AR, in order to obtain specific effects (SARMs). The tissue selectivity and different metabolic fate in comparison to testosterone can potentiate anabolic versus androgenic effects; therefore, they might be a valid alternative to testosterone replacement therapy avoiding the negative effects of testosterone (i.e., on prostate, liver, and hematopoiesis). Trials are still at an early phase of investigation and, at the moment, the application seems to be more useful for chronic disease with catabolic status while the validation as replacement for hypogonadism requires further studies.

1. Introduction

Hypogonadotropic hypogonadism, also known as secondary hypogonadism, is the most common form of hypogonadism in adult and elderly man [1], related to an absolute or relative defective secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus and/or gonadotropin secretion by the pituitary gland. It recognizes two main etiologies: the organic and the functional one. The organic form is characterized by a strong genetic stigma; expansive lesions of the hypothalamic-pituitary region, traumatic events, or, more commonly, infectious or infiltrative diseases can cause it. On the contrary, the functional form appears to be acquired and underpinned by multiple metabolic and inflammatory mechanisms [2]. Based on that evidence, most of the International Societies in the field stated that Testosterone replacement therapy (TRT) should be considered only for the organic forms, independently of the patients' age at the time of onset.

As functional hypogonadism is concerned, specific treatments for the underlying conditions inducing the T decrease (i.e., obesity, metabolic syndrome, diabetes mellitus, and so on) have been suggested [3, 4]. In these conditions, hypogonadism is related to a progressive worsening of the disease, despite an adequate therapy (for instance, the failure of diet in obese men) [5], contributing to the evolution by an unfavourable vicious circle. In addition, given the concerns about the benefit of TRT (for instance, in

younger men willing to maintain their fertility) or the not fully proven safety of TRT for a long time, especially in fragile elderly subjects, other strategies have been object of investigation, for instance those that can activate the androgen receptor (AR) present in specific target tissues.

Hence, according to the results obtained in infertile women treated with either selective estrogen receptor modulators (SERMs) (i.e., clomiphene citrate) or aromatase inhibitors (AIs) (i.e., anastrozole or letrozole), which proved to enhance the serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), men with secondary functional hypogonadism were treated with the same drugs in order to increase the endogenous T levels [6]. On the contrary, both SERMs and AIs maintain the fertility, avoiding the block of spermatogenesis, as TRT does [7, 8].

The knowledge about androgen receptors also allowed to develop another class of drugs, directly acting at this receptor, but with differential effects in comparison to T supplementation (selective androgen receptor modulators, SARMs). In fact, they are considered as a new promising class of compounds to have anabolic effects for a number of clinical indications, without causing negative effects on the prostate, red blood cells, and cardiovascular system. In addition, they could be employed in several functional limitations, often associated with chronic disease, frailty, cancer cachexia, and osteoporosis.

The aim of the present review is to update information about both SERMs and SARMs in the treatment of male hypogonadism. Many clinical trials are reported on SERMs, while the field of SARMs is still largely at an early phase of investigation since pharmacological aspects of a number of new molecules have been recently made available. After a brief review of trials concerning SERMs use, we focus the attention on the androgen receptor as the basis of the expanding field of SARMs. It is known that TRT can have undesired effects as discussed in the following; therefore, the search for such compounds could be promising as an alternative therapy.

2. SERMs and AIs: Mechanism of Action and Clinical Practice Uses

2.1. Mechanism of Action. SERMs and AIs are able to elicit the secretion of LH and FSH with different mechanism: a direct action at the hypothalamus (SERMs), where they block estrogen receptors (such as clomiphene citrate, CC), or an indirect one (AIs), decreasing plasma oestrogens levels via a block of aromatase [7]. LH acts, consequently, at the testes to increase T secretion, while FSH may positively coregulate Sertoli cells function and the spermatogenesis. Due to their activity, SERMs and AIs are also considered as indirect androgen doping due to their ability to increase T, being thus prohibited both in amateur and professional athletes [9].

The raise in LH and FSH, however, requires a normal functional hypothalamic-pituitary-testis axis that can be elicited independently of the age of the subjects treated. Indeed, a retrospective study indicated that a testicular volume <14 cc and LH levels >6 UI/ml might be considered as the strong predictors of a good response to CC treatment [10], highlighting that all forms of primary hypogonadism and the secondary organic ones might be unresponsive to that therapy [11]. In young adults and elderly men, CC (50 mg/day), anastrozole (1 mg/day), and letrozole (2.5 mg/die) were able to raise plasma levels of LH and FSH, highlighting the key inhibiting role of oestrogens levels on gonadotropins secretion at the hypothalamus-pituitary level [12].

Furthermore, since the effects of both drugs were similar in young and elderly men, it can be hypothesized that an increased negative feedback by endogenous oestrogens is not instrumental in age-related decline of Leydig cell function in elderly men [13]. Similarly, letrozole (2.5 mg/day for 6 months) was able to promote the development of puberty in boys with constitutional delay of growth and puberty, further emphasizing the functional role of the impairment of the hypothalamic-pituitary-testis axis in this clinical condition [6].

2.2. Impact on Circulating Steroid Levels. Although both SERMs and AIs can raise gonadotropin levels, they induce a different pattern of circulating steroid levels, as suggested by an important difference in the serum T/17β oestradiol (E2) ratio, depending on the kind of drug employed [14]. Depending on their action, in fact, AIs can reduce circulating oestrogens, while CC does not. On the other hand, it is commonly known that E2 has a crucial role in male reproduction and sexual function [15] as well as in the brain functions and the metabolic activity. Indeed, in a hormonal milieu characterized by a normal/high T/E2 ratio by means of letrozole (2.5 mg/day) therapy, positive behavioural effects can be observed (fear and risk taking) in healthy adult men [16].

2.3. Metabolic Effects. A normal/high T/E2 ratio has proven to be able to reduce insulin resistance by lowering insulin and glucose levels, without body composition changing in nonobese adult men [17], highlighting a direct and specific role of T in coregulating the insulin secretion and activity [18]. CC also shares positive metabolic effects. In a recent double-blind placebo-controlled study, CC (25 mg/day) improved fasting glycaemia, insulin levels, and homeostasis model assessment estimate of insulin-resistance (HOMA-IR) in obese men with functional secondary hypogonadism, regardless of their glucose tolerance state (impaired glucose tolerance or type 2 diabetes mellitus) [19]. Interestingly, similar results were obtained by chronic T supplementation in a mixed population of normal men or male subjects affected by secondary hypogonadism. This study, in fact, showed that TRT enhances the body composition by increasing lean mass at the expense of fat mass, indicating the crucial role of T as a coregulator of body composition [20].

2.4. SERMs and AIs as Therapy for Hypogonadism. In addition to hormonal and metabolic outcomes, there is a large amount of evidence about the symptomatic improvement of hypogonadal men on CC therapy. As a matter of fact, even in comparison with TRT, the treatment with CC was able to improve the A questionnaire and satisfaction in hypogonadal

men [21]. Conversely, few studies with inconclusive data have been carried out on CC and AIs effect on erectile dysfunction (ED) in men so far [22]. Guay and coll, in fact, evaluated CC effect in secondary hypogonadic men and ED, showing that CC improved sexual function. At variance with these results, both a retrospective study evaluating 77 hypogonadal and infertile men treated with CC [23] and a double-blind placebo-controlled study involving 17 subjects did not show any improvement in sexual function during CC therapy [24]. Similarly, Leder and coll. did not find any significant difference in ED assessed by the International Index of Erectile Function during anastrozole (1 mg/day) therapy in a placebo-controlled study conducted in elderly men [25].

The common side effects reported with the use of both classes of drugs are scarce since this topic in particular is less explored when compared with TRT. They include hot flashes, insomnia, and weight gain for AIs and gynecomastia, dizziness, and headache for CC. However, AIs could determine a reduction in bone mineral density owing to their suppressive effect on serum oestrogens [26], whereas CC therapy caused a markedly lower incidence of secondary polycythaemia in comparison with TRT in hypogonadal men [27].

Anyway, both classes of drugs have proven to be capable to reactivate the hypothalamus-pituitary-testis axis independently of age (pubertal and adult men) in all clinical conditions without an organic damage but with only a partial and/or functional block of gonadotropin secretion. Moreover, given that they do not have relevant side effects, AIs and SERMs seem to be an attractive and quite safe alternative therapy for male secondary hypogonadism and for infertile men without organic damages at the hypothalamic-pituitary-testis axis. However, it is desirable that more studies involving large cohorts of patients and a longer period of observation should be carried out, with the aim to draw definitive evidence about those therapies, especially in the light to compare SERMs and AIs results with those obtained with T substitution in men with secondary hypogonadism.

3. SARMs: Biochemical Action and Therapeutic Effects

Active androgens, T and its 5α-hydroxy-end metabolite [dihydrotestosterone (DHT)], carry out their main effects by means of AR. Selective androgen receptor modulators (SARMs) are compounds which can bind AR similarly to active androgens, still retaining their androgen effect and displaying a tissue-selective activation of AR without showing those important negative effects of T supplementation especially in the prostate tissue, red blood cells, high-density lipoprotein (HDL), and cardiovascular system. Thus, they might be especially used in adult and elderly men suffering from the impairment of important organs such as heart, kidney, prostate, bone, and muscle. To improve the knowledge on SARMs mechanism of action, the characteristics of AR are briefly updated.

3.1. Androgen Receptor: Structure and Function. The androgen receptor (AR), also known as NR3C4 (nuclear receptor subfamily 3, group C, gene 4), is a member of the steroid receptor subfamily of the nuclear receptors (NRs) superfamily. It plays pivotal roles in the development and maintenance of homeostasis of the reproductive, musculoskeletal, cardiovascular, immune, neural, and haematopoietic tissues.

In human beings, the androgen receptor (MW 110 kDa; 919-920 amino acids) is encoded by the AR gene located on the long arm chromosome X at the locus Xq11- Xq12 [28, 29]. Like other members of the NRs superfamily, AR comprises three main functional domains: (1) an amino-terminal domain (NTD, residues 1–555) that shows little sequence homology with other steroid receptors and contains two independent activation regions (i.e., activation function 1 and activation function 5) essential for the transcriptional activation of AR; (2) a DNA-binding domain (DBD, residues 555–623) which is highly conserved among the steroid receptors and consists of two zinc finger motifs that recognise specific DNA consensus sequences, thus playing a crucial role in AR binding to androgen response elements (AREs); and (3) a carboxyl-terminal domain (CTD, residues 665–919) which includes the ligand-binding domain (LBD) and contains the ligand-binding pocket (LBP).

The DBD and LBD regions are connected by a flexible hinge region (residues 628–669) [30], called nuclear localization signal (NLS), that is, a lysine rich sequence and is important for nuclear localization of the AR receptor and is also a major target site for posttranslational modifications such as acetylation, methylation, ubiquitylation, and phosphorylation [31].

AR is expressed in several reproductive (breast, testes, prostate, ovary, and endometrium) and nonreproductive tissues (hair follicles, bone, brain, liver, and cardiovascular) [32].

Several different mutations of the AR gene have been identified so far. Some of them might lower its function leading to the androgen insensitivity syndrome [33]; they encompass pathologic conditions which occur with different clinical phenotypes: spinal and bulbar muscular atrophy and partial or complete androgen resistance (i.e., Morris syndrome). Conversely, some other mutations of AR can be activating as it does in prostate cancer.

Immunohistochemistry studies have revealed that AR has been expressed in 75% of breast tumours. Consequently, AR is suggested as a new marker and potential therapeutic target in the treatment of patients against breast cancer [34].

When an active androgen (e.g., testosterone; T) crosses the cell membrane, it either directly binds the inactive AR or is converted into the more potent dihydrotestosterone (DHT) by 5-α-reductase (Figure 1). Moreover, DHT binds the inactive form of AR strongly than T. In fact, intracellular DHT is a more potent androgenic agonist than T, and its existence in specific reproductive tissues (external genitalia, seminal vesicle, and prostate) is required for organ development and their correct functions. Additionally, at low circulating androgen levels, DHT binding to AR is favourite in respect to T [35].

The biochemical mechanisms of androgenic activity include several sequential steps that are illustrated in Figure 2. Briefly, in the absence of androgen, AR is localized in

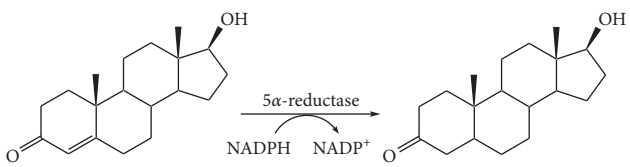


Figure 1: Testosterone (left) is converted by 5α-reductase into 5α-dihydrotestosterone (DHT) (right) in a NADPH-dependent redox reaction.

the cytoplasm as an inactive complex with heat shock proteins (e.g., HSP70 and HSP90) and chaperone proteins, which help to maintain the apo state of the AR competent to bind the ligand. When androgen (A) enters the cell, it directly binds the inactive AR. Due to the binding of androgens to AR, it undergoes a conformational change which leads to the dissociation of HSP and other proteins from the inactive AR that is converted in the active form [36]. The activated AR (i.e., active AR which binds with A or DHT) complex is translocated to the nucleus and dimerizes as a AR homodimer (AR-dimer) that acts as a transcriptional regulator activating the gene expression by binding to the specific sequence, the androgen response element (ARE), site in DNA [37]. Homodimerization of AR is mediated by the dimerization box located in the DBD region to achieve an intermolecular DBD-DBD link and N/C-terminal interactions through the FXXLF motif [38]. The AR-DNA-binding domain is responsible for the binding to DNA that takes place through a palindromic consensus sequence 5′-GGTACAnnnTGTTCT-3′, the so-called androgen response element (ARE) that is recognized by the AR-dimer.

Moreover, the AR-dimer can form signalling complexes with coregulated proteins to enhance or depress transcription of the target gene (i.e., modulates gene transcription of the AR-target genes), thus exerting androgen effect. AR-activated genes comprise KLK3 that encode the prostate-specific antigen (PSA), KLK2, FKBP5, transmembrane protease serine 2 (TMPRSS2), and several other transcripts [39].

While androgens are important for normal development of various tissues, under definite circumstances they also could prompt pathological effects on the prostate, the liver, and the myocardium. Interestingly, the AR agonists were proven to be beneficial for anabolic deficiencies. The ideal AR ligand for the treatment of anabolic deficiencies should be tissue selective. Such AR ligands were defined as selective androgen receptor modulators (SARMs). SARMs were first described and subsequently developed to facilitate selective tissue activation of the AR [40]. Several of the SARMs designed so far possess a nonsteroidal structure and have the ability to activate the AR in the muscle and bone, with a reduced activation of the AR in the prostate. Thus, SARMs may serve as therapeutic options for treatment of numerous diseases, including muscle weakness, osteoporosis, and breast cancer.

3.2. SARMs: Biochemistry. Based on this knowledge about the androgen receptor, a large field of research has been developed about compounds able to bind AR in order to obtain specific effects (SARMs). Some of these are obtained by modification of the steroid structure, but others are nonsteroidal; it is an old concept, the myotrophic/androgenic index [41] as a major tool to evaluate androgen potency. The aim of this kind of investigations is to find compounds with anabolic activity without negative effects of androgenic activity. Moreover, nonsteroidal SARMs are not metabolized by aromatase or 5-α-reductase.

The ideal profile of an effective SARM, for the treatment of hypogonadism, should include a comfortable administration (oral and once daily administration), ability to stimulate physical and psychological sexual items, anabolic effects (body composition, muscle strength, and bone growth), avoiding undesirable side effects, such as liver toxicity, fluid retention, and blood pressure increase, overstimulation of erythropoiesis, and induction of gynecomastia [42]. Moreover, the effects on the prostate gland should be carefully monitored, avoiding adverse effects associated to proliferative stimulation which are related to 5-α-reductase; therefore, the different metabolism in comparison to T is among the desired characteristics. The aim to further ameliorate anabolic effects should be on the other hand preferentially pursued for the extended indications to SARM therapy, especially osteoporosis in elderly men [43, 44], glucocorticoid-induced osteoporosis [43], HIV sarcopenia and cancer cachexia [45], anaemia, and muscular dystrophies [42, 46]. These aspects could be also extended to women.

It is known that the idea to develop SARM, modifying the T structure, is not new (steroidal SARMs) [47]. The main strategies were

(i) The 17-α-alkyl substitution, with extension of its half-life and the possibility of oral administration; the hepatotoxicity and unfavourable effects on lipid pattern limited this approach;

(ii) Removal of the 19-methyl group, with enhancement of anabolic activity (nandrolone series); also metabolism is influenced since these derived molecules are not aromatizable but are transformed by 5-α-reductase in less potent androgens; a further 7-α-alkyl substitution increases anabolic effects and reduces effects on prostate;

(iii) Esterification of the 17-β-hydroxyl group, with extension of duration of action;

(iv) A 17-α-alkyl substitution has been realized also starting by DHT; substitution of a second carbon with oxygen increases its stability and anabolic activity, without aromatization to oestrogens.

The modern era started with the synthesis on nonsteroidal SARMs [40, 42, 48–51]. The first generation of ligands was obtained by structural modifications of arylpropionamide analogs, bicalutamide, and hydroxyflutamide.

Then, different classes of SARMs have been obtained, including propionamide, quinolinone, tetrahydroquinoline, bicyclic-hydantoin, pyrazoline, imidazolpyrazole, benzimidazole, and aniline [52]. As discussed before, AR has a widespread distribution in reproductive and nonreproductive tissues and has a complex regulation related to recruitment and activation of coactivators and corepressors, followed by tissue-specific

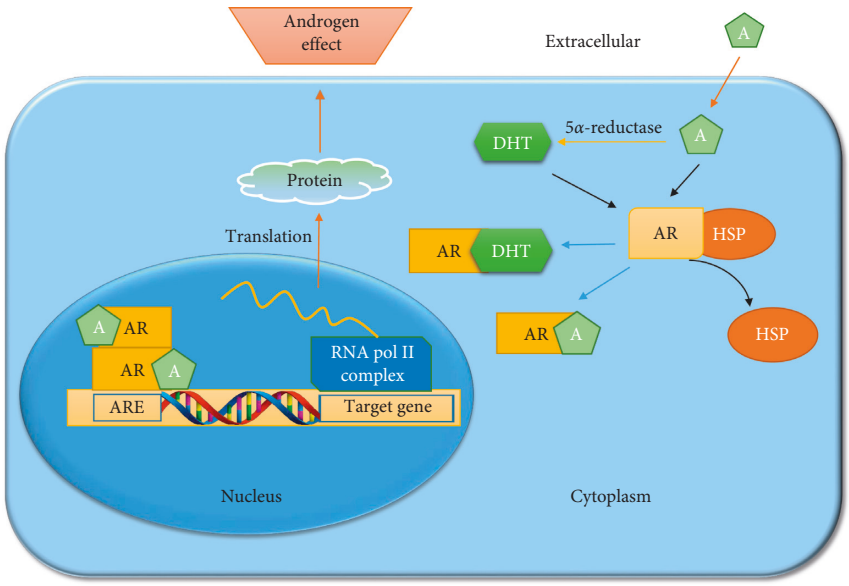


Figure 2: Illustration of the AR signalling pathway. When A enters into the cell, it is converted into the more potent DHT by 5α-reductase and binds to the ligand-binding pocket of AR forming AR-A and AR-DHT complexes, respectively. Consequently, the AR-A or AR-DHT is translocated into the nucleus with help of other proteins to form the AR-dimer (or AR-DHT dimer, not shown) that is able to bind the ARE region in DNA to regulate the expression of downstream genes thus initiating the transcription of specific protein that exert the androgen effect.

gene activation. Interestingly, three hypotheses are discussed about selectivity of SARMs [47]:

(i) A coactivator hypothesis, based on differences in transcriptional activity, related to the role played by activators and repressors;

(ii) A conformational hypothesis, based on the induction of changes in ligand-binding domains, modulation of surface topology, and subsequent protein-protein interaction (both at cytosolic level with nongenomic effects and at regulators of gene expression);

(iii) Differences in tissue distribution and metabolic fate of the different SARMs.

3.3. Biological Effects and Clinical Applications. The activity of SARMs is confirmed by both in vitro and in vivo models. A well-known model is the demonstration of AR-dependent agonist activity in castrated rats (Hershberger assay).

From a pathophysiological point of view, the interest is focused on catabolic conditions, such as muscle atrophy as observed in sepsis, ageing, cancer, AIDS [53], and bone catabolism in osteoporosis; the effects of nonsteroidal SARM have been explored in castrated male Sprague Dawley rats and compared with glucocorticoid-induced muscle atrophy [53]. SARMs, but also T, counteracted the upregulation of ubiquitin ligases. Molecular mechanism in the other model (castration) was instead a blunting effect on other signalling, such as upregulation of ligases but especially downregulation of IGF-1. Recently, we described the interaction IGF-1 gonadal function [54]. Interestingly, another promising field of investigation involves the activation of AR signalling in the skeletal muscle, which is also indirect, mediated by upregulation of follistatin and a consequent cross-communication from the Wnt-β catenin pathway to the TGFβ-SMAD pathway. These interactions are specific for the muscle, and therefore could be a strategy to a further development of anabolic drugs [47, 55].

In addition, bone metabolism is greatly influenced by gonadal milieu. It is widely accepted that patients with low levels of estradiol present bone metabolism disorders and increased risk of fractures [56, 57]. This phenomenon could depend on a reduced aromatization of testosterone into estradiol. Different studies support this view showing severe male osteoporosis in patients affected by mutations of the estrogen receptor or mutations of the aromatase [58, 59]. However, both testosterone and estradiol seem to be fundamental for bone metabolism, even with different mechanisms. Various evidences suggested a dominant role of estrogen on bone resorption, whereas both estrogen and testosterone are involved in maintaining bone formation [60].

In a gonadectomised model of skeletally-mature male rats, an increase in biochemical markers of bone reabsorption rapidly develops; anyhow, SARMs administration counteracted these effects, as confirmed by biochemical studies but also histomorphometric and mechanical analyses [61]. These studies could be the basis for a clinical application in the treatment of osteoporosis.

3.4. Clinical Trials. A number of clinical trials are ongoing as shown in Table 1.

Among the published trials, a 12-week double-blind, placebo-controlled, phase II clinical trial evaluated GTx-024 (enobosarm) effects in 120 healthy elderly men (age > 60

Table 1: Selective androgen receptor agonists (SARMs): ongoing clinical trials.

Status	Study title	Conditions	Interventions	Locations
Not yet recruiting	Multimodality Intervention for Function and Metabolism in SCI	(i) Spinal cord injuries	(i) Drug: SARM (ii) Behavioral: hybrid exercise	
Active, not recruiting	Study to Evaluate the Safety and Efficacy of 13 weeks of the Selective Androgen Receptor Modulator (SARM) GSK2881078 in Chronic Obstructive Pulmonary Disease (COPD)	(i) Cachexia	(i) Drug: GSK2881078 (ii) Placebo	USA
Active, not recruiting	Pembrolizumab and Enobosarm in Treating Patients With Androgen Receptor Positive Metastatic Triple Negative Breast Cancer	(i) Androgen receptor positive (ii) Estrogen receptor negative (iii) HER2/neu negative (iv) And 3 more	(i) Drug: enobosarm (ii) Pembrolizumab	USA
Recruiting	A Selective Androgen Receptor Modulator for Symptom Management in Prostate Cancer	(i) Prostate cancer	(i) Drug: LY2452473 (ii) Drug: placebo	USA

Data from https://clinicalltrials.gov.

years) and postmenopausal women; a significant dose-dependent increase in total lean body mass evaluated by DEXA was reported. Also, physical function and insulin resistance improved. Adverse effect did not differ in comparison to the placebo. Interestingly, the abovementioned effects persisted although there was a reduction of serum total testosterone due to the significant lowering plasma levels of SHBG [62].

Another study evaluated safety, tolerability, pharmacodynamics, and pharmacokinetics of Gsk2881078 in healthy young men. A decrease in HDL and SHBG was described with dose-related effects. Adverse effects were mild [63].

The same drug was tested in a randomized placebo-controlled parallel group repeat dose, a dose escalation study in healthy older men; this study confirms good tolerability without serious adverse event. Transient elevations of alanine amino transferase were observed; the main focus was the increase of lean mass assessed by DEXA and MRI cross-sectional thigh scans [64].

In the above-reported studies, however, a greater response was observed in women.

The purpose of most cited studies is the treatment of unfavourable metabolic status in patients with systemic diseases; use of SARMs as replacement therapy for hypogonadism is not well defined since in this case also androgenic properties should be maintained; the objective is still to identify molecules with good androgenic and myotropic properties only avoiding tissue-selective undesired actions; also the modality of administration should be comfortable and well accepted. Indeed, in a recent placebo-controlled study that lasted 21 days, conducted in 76 healthy young men (21–50 years) who were randomized to placebo or oral different doses of LGD-4033 (0.1, 0.3, or 1.0 mg per day) showed a dose-dependent increase of lean body mass and presented a significant reduction of serum HDL, indicating a potential negative effect on the lipoprotein profile [65]. With the aim to reduce the above negative effect on HDL levels that can allow to also use SARMs in elderly hypogonadal men, a recent transdermal SARM (LY305) has been proposed in the preclinical study [66]. LY305, in fact, was found to be a safe and well-tolerated transdermal nonsteroidal androgen modulator which offers a potential therapeutic option for elderly patients suffering from signs and symptoms of hypogonadism.

4. Conclusion

Although there are different mechanisms of action, both SERMs and SARMs can find an appropriate role in treating hypogonadism condition in adult and elderly men. While SERMs can reactivate the hypothalamic-pituitary-testis axis whenever it is functionally blocked owing to chronic diseases (metabolic and/or not metabolic conditions), SARMs, overcoming the hypothalamic-pituitary-testis axis function, act by directly stimulating AR. On the contrary, both families of drugs appear not to cause important side effects as TRT could do during chronic therapy. However, concerning SERMs, there are several observational and randomized studies which have confirmed their use in clinical setting, while about SARMs there are some preclinical studies which point out interesting results in adult hypogonadal men suffering from severe form of catabolic processes such as muscle atrophy, cachexia, and severe osteoporosis. Based on this evidence, although preclinical data suggest a positive outcome for SARMs, further clinical trials with larger sample sizes and randomized design are needed before using them in the clinical practice particularly in elderly hypogonadal men.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] A. Tajar, G. Forti, T. W. O'Neill et al., "Characteristics of secondary, primary, and compensated hypogonadism in aging men: evidence from the European male ageing study," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 4, pp. 1810–1818, 2010.

[2] M. Grossmann and A. M. Matsumoto, "A perspective on middle-aged and older men with functional hypogonadism:

focus on holistic management," *The Journal of Clinical Endocrinology & Metabolism*, vol. 102, no. 3, pp. 1067–1075, 2017.

[3] S. Bhasin, J. P. Brito, G. R. Cunningham et al., "Testosterone therapy in men with hypogonadism: an endocrine society ∗ clinical practice guideline," *The Journal of Clinical Endocrinology & Metabolism*, vol. 103, no. 5, pp. 1715–1744, 2018.

[4] B. B. Yeap, M. Grossmann, R. I. McLachlan et al., "Endocrine society of Australia position statement on male hypogonadism (part 2): treatment and therapeutic considerations," *Medical Journal of Australia*, vol. 205, no. 5, pp. 228–231, 2016.

[5] C. M. Apovian, L. J. Aronne, D. H. Bessesen et al., "Pharmacological management of obesity: an endocrine society clinical practice guideline," *The Journal of Clinical Endocrinology & Metabolism*, vol. 100, no. 2, pp. 342–362, 2015.

[6] T. Varimo, H. Huopio, L. Kariola et al., "Letrozole versus testosterone for promotion of endogenous puberty in boys with constitutional delay of growth and puberty: a randomised controlled phase 3 trial," *The Lancet Child & Adolescent Health*, vol. 3, no. 2, pp. 109–120, 2019.

[7] E. M. Lo, K. M. Rodriguez, A. W. Pastuszak, and M. Khera, "Alternatives to testosterone therapy: a review," *Sexual Medicine Reviews*, vol. 6, no. 1, pp. 106–113, 2018.

[8] M. E. Chua, K. G. Escusa, S. Luna, L. C. Tapia, B. Dofitas, and M. Morales, "Revisiting oestrogen antagonists (clomiphene or tamoxifen) as medical empiric therapy for idiopathic male infertility: a meta-analysis," *Andrology*, vol. 1, no. 5, pp. 749–757, 2013.

[9] D. J. Handelsman, "Indirect androgen doping by oestrogen blockade in sports," *British Journal of Pharmacology*, vol. 154, no. 3, pp. 598–605, 2008.

[10] C. R. Mazzola, D. J. Katz, N. Loghmanieh, C. J. Nelson, and J. P. Mulhall, "Predicting biochemical response to clomiphene citrate in men with hypogonadism," *The Journal of Sexual Medicine*, vol. 11, no. 9, pp. 2302–2307, 2014.

[11] Y. Liel, "Clomiphene citrate IN the treatment of idiopathic or functional hypogonadotropic hypogonadism IN men: a case series and review of the literature," *Endocrine Practice*, vol. 23, no. 3, pp. 279–287, 2017.

[12] J. S. Tenover and W. J. Bremner, "The effects of normal aging on the response of the pituitary-gonadal axis to chronic clomiphene administration in men," *Journal of Andrology*, vol. 12, no. 4, pp. 258–263, 2019.

[13] G. G. T'Sjoen, V. A. Giagulli, H. Delva, P. Crabbe, D. De Bacquer, and J.-M. Kaufman, "Comparative assessment in young and elderly men of the gonadotropin response to aromatase inhibition," *The Journal of Clinical Endocrinology & Metabolism*, vol. 90, no. 10, pp. 5717–5722, 2005.

[14] S. Helo, J. Ellen, C. Mechlin et al., "A randomized prospective double-blind comparison trial of clomiphene citrate and anastrozole in raising testosterone in hypogonadal infertile men," *The Journal of Sexual Medicine*, vol. 12, no. 8, pp. 1761–1769, 2015.

[15] M. Schulster, A. Bernie, and R. Ramasamy, "The role of estradiol in male reproductive function," *Asian Journal of Andrology*, vol. 18, no. 3, p. 435, 2016.

[16] A. E. Goudriaan, B. Lapauw, J. Ruige et al., "The influence of high-normal testosterone levels on risk-taking in healthy males in a 1-week letrozole administration study," *Psychoneuroendocrinology*, vol. 35, no. 9, pp. 1416–1421, 2010.

[17] F. W. Gibb, N. Z. M. Homer, A. M. M. Faqehi et al., "Aromatase inhibition reduces insulin sensitivity in healthy men," *The Journal of Clinical Endocrinology & Metabolism*, vol. 101, no. 5, pp. 2040–2046, 2016.

[18] B. Lapauw, M. Ouwens, L. M. 't Hart et al., "Sex steroids affect triglyceride handling, glucose-dependent insulinotropic polypeptide, and insulin sensitivity: a 1-week randomized clinical trial in healthy young men," *Diabetes Care*, vol. 33, no. 8, pp. 1831–1833, 2010.

[19] C. Pelusi, V. A. Giagulli, M. Baccini et al., "Clomiphene citrate effect in obese men with low serum testosterone treated with metformin due to dysmetabolic disorders: a randomized, double-blind, placebo-controlled study," *PLoS One*, vol. 12, no. 9, Article ID e0183369, 2017.

[20] G. Corona, V. A. Giagulli, E. Maseroli et al., "Therapy of endocrine disease: testosterone supplementation and body composition: results from a meta-analysis study," *European Journal of Endocrinology*, vol. 174, no. 3, pp. R99–R116, 2016.

[21] R. Ramasamy, J. M. Scovell, J. R. Kovac, and L. I. Lipshultz, "Testosterone supplementation versus clomiphene citrate for hypogonadism: an age matched comparison of satisfaction and efficacy," *Journal of Urology*, vol. 192, no. 3, pp. 875–879, 2014.

[22] A. T. Guay, J. Jacobson, J. B. Perez, M. B. Hodge, and E. Velasquez, "Clomiphene increases free testosterone levels in men with both secondary hypogonadism and erectile dysfunction: who does and does not benefit?," *International Journal of Impotence Research*, vol. 15, no. 3, pp. 156–165, 2003.

[23] J. C. Chandrapal, S. Nielson, D. P. Patel et al., "Characterising the safety of clomiphene citrate in male patients through prostate-specific antigen, haematocrit, and testosterone levels," *BJU International*, vol. 118, no. 6, pp. 994–1000, 2016.

[24] A. T. Guay, S. Bansal, and G. J. Heatley, "Effect of raising endogenous testosterone levels in impotent men with secondary hypogonadism: double blind placebo-controlled trial with clomiphene citrate," *The Journal of Clinical Endocrinology & Metabolism*, vol. 80, no. 12, pp. 3546–3552, 1995.

[25] B. Z. Leder, J. L. Rohrer, S. D. Rubin, J. Gallo, and C. Longcope, "Effects of aromatase inhibition in elderly men with low or borderline-low serum testosterone levels," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 3, pp. 1174–1180, 2004.

[26] S.-A. M. Burnett-Bowie, E. A. McKay, H. Lee, and B. Z. Leder, "Effects of aromatase inhibition on bone mineral density and bone turnover in older men with low testosterone levels," *The Journal of Clinical Endocrinology & Metabolism*, vol. 94, no. 12, pp. 4785–4792, 2009.

[27] K. M. Wheeler, R. P. Smith, R. A. Kumar, S. Setia, R. A. Costabile, and P. K. Kavoussi, "A comparison of secondary polycythemia in hypogonadal men treated with clomiphene citrate versus testosterone replacement: a multi-institutional study," *Journal of Urology*, vol. 197, no. 4, pp. 1127–1131, 2017.

[28] D. B. Lubahn, D. R. Joseph, M. Sar et al., "The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate," *Molecular Endocrinology*, vol. 2, no. 12, pp. 1265–1275, 1988.

[29] E. P. Gelmann, "Molecular biology of the androgen receptor," *Journal of Clinical Oncology*, vol. 20, no. 13, pp. 3001–3015, 2002.

[30] Q. Wang, J. Lu, and E. L. Yong, "Ligand- and coactivator-mediated transactivation function (AF2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region," *Journal of Biological Chemistry*, vol. 276, no. 10, pp. 7493–7499, 2001.

[31] L. Clinckemalie, D. Vanderschueren, S. Boonen, and F. Claessens, "The hinge region in androgen receptor control," *Molecular and Cellular Endocrinology*, vol. 358, no. 1, pp. 1–8, 2012.

[32] N. Kimura, A. Mizokami, T. Oonuma, H. Sasano, and H. Nagura, "Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues," *Journal of Histochemistry & Cytochemistry*, vol. 41, no. 5, pp. 671–678, 1993.

[33] R. L. Batista, E. M. F. Costa, A. de Santi Rodrigues et al., "Androgen insensitivity syndrome: a review," *Archives of Endocrinology and Metabolism*, vol. 62, no. 2, pp. 227–235, 2018.

[34] C. M. Venema, R. D. Bense, T. G. Steenbruggen et al., "Consideration of breast cancer subtype in targeting the androgen receptor," *Pharmacology & Therapeutics*, vol. 200, pp. 135–147, 2019.

[35] Z. X. Zhou, M. V. Lane, J. A. Kemppainen, F. S. French, and E. M. Wilson, "Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability," *Molecular Endocrinology*, vol. 9, no. 2, pp. 208–218, 1995.

[36] L. Q. Cano, D. N. Lavery, and C. L. Bevan, "Mini-review: foldosome regulation of androgen receptor action in prostate cancer," *Molecular and Cellular Endocrinology*, vol. 369, no. 1-2, pp. 52–62, 2013.

[37] J. Prescott and G. A. Coetzee, "Molecular chaperones throughout the life cycle of the androgen receptor," *Cancer Letters*, vol. 231, no. 1, pp. 12–19, 2006.

[38] D. Xu, Y. Zhan, Y. Qi et al., "Androgen receptor splice variants dimerize to transactivate target genes," *Cancer Research*, vol. 75, no. 17, pp. 3663–3671, 2015.

[39] C. D. Chen, D. S. Welsbie, C. Tran et al., "Molecular determinants of resistance to antiandrogen therapy," *Nature Medicine*, vol. 10, no. 1, pp. 33–39, 2004.

[40] J. T. Dalton, A. Mukherjee, Z. Zhu, L. Kirkovsky, and D. D. Miller, "Discovery of nonsteroidal androgens," *Biochemical and Biophysical Research Communications*, vol. 244, no. 1, pp. 1–4, 1998.

[41] A. T. Kicman, "Pharmacology of anabolic steroids," *British Journal of Pharmacology*, vol. 154, no. 3, pp. 502–521, 2008.

[42] A. Negro-Vilar, "Selective androgen receptor modulators (SARMs): a novel approach to androgen therapy for the new millennium," *The Journal of Clinical Endocrinology & Metabolism*, vol. 84, no. 10, pp. 3459–3462, 1999.

[43] E. S. Orwoll and R. F. Klein, "Osteoporosis in men," *Endocrine Reviews*, vol. 16, no. 1, pp. 87–116, 1995.

[44] P. J. Snyder, H. Peachey, P. Hannoush et al., "Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age," *The Journal of Clinical Endocrinology & Metabolism*, vol. 84, no. 8, pp. 2647–2653, 1999.

[45] S. Bhasin, T. W. Storer, N. Asbel-Sethi et al., "Effects of testosterone replacement with a nongenital, transdermal system, androderm, in human immunodeficiency virus-infected men with low testosterone levels [1]," *The Journal of Clinical Endocrinology & Metabolism*, vol. 83, no. 9, pp. 3155–3162, 1998.

[46] C. J. Bagatell and W. J. Bremner, "Androgens in men—uses and abuses," *New England Journal of Medicine*, vol. 334, no. 11, pp. 707–715, 1996.

[47] S. Bhasin and R. Jasuja, "Selective androgen receptor modulators as function promoting therapies," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 12, no. 3, pp. 232–240, 2009.

[48] J. P. Edwards, S. J. West, C. L. F. Pooley, K. B. Marschke, L. J. Farmer, and T. K. Jones, "New nonsteroidal androgen receptor modulators based on 4-(trifluoromethyl)-2(1H)-pyrrolidino[3,2-g] quinolinone," *Bioorganic & Medicinal Chemistry Letters*, vol. 8, no. 7, pp. 745–750, 1998.

[49] L. G. Hamann, N. S. Mani, R. L. Davis, X.-N. Wang, K. B. Marschke, and T. K. Jones, "Discovery of a potent, orally active, nonsteroidal androgen receptor agonist: 4-Ethyl-1,2,3,4-tetrahydro-6- (trifluoromethyl)-8-pyridono[5,6- g]-quinoline (LG121071)," *Journal of Medicinal Chemistry*, vol. 42, no. 2, pp. 210–212, 1999.

[50] J. N. Miner, W. Chang, M. S. Chapman et al., "An orally active selective androgen receptor modulator is efficacious on bone, muscle, and sex function with reduced impact on prostate," *Endocrinology*, vol. 148, no. 1, pp. 363–373, 2007.

[51] Y. He, D. Yin, M. Perera et al., "Novel nonsteroidal ligands with high binding affinity and potent functional activity for the androgen receptor," *European Journal of Medicinal Chemistry*, vol. 37, no. 8, pp. 619–634, 2002.

[52] X. Zhang, J. C. Lanter, and Z. Sui, "Recent advances in the development of selective androgen receptor modulators," *Expert Opinion on Therapeutic Patents*, vol. 19, no. 9, pp. 1239–1258, 2009.

[53] A. Jones, D.-J. Hwang, R. Narayanan, D. D. Miller, and J. T. Dalton, "Effects of a novel selective androgen receptor modulator on dexamethasone-induced and hypogonadism-induced muscle atrophy," *Endocrinology*, vol. 151, no. 8, pp. 3706–3719, 2010.

[54] A. Mancini, C. Bruno, A. Palladino, E. Vergani, and E. Giacchi, "GH–IGF1 axis in spermatogenesis and male fertility," in *Molecular Signaling in Spermatogenesis and Male Infertility*, R. Singh, Ed., , CRC Press, Boca Raton, FL, USA, 1st edition, 2019.

[55] R. Singh, S. Bhasin, M. Braga et al., "Regulation of myogenic differentiation by androgens: cross talk between androgen receptor/β-catenin and follistatin/transforming growth factor-β signaling pathways," *Endocrinology*, vol. 150, no. 3, pp. 1259–1268, 2009.

[56] P. Szulc, F. Munoz, B. Claustrat et al., "Bioavailable estradiol may be an important determinant of osteoporosis in men: the minos study 1," *The Journal of Clinical Endocrinology & Metabolism*, vol. 86, no. 1, pp. 192–199, 2001.

[57] E. Barrett-Connor, J. E. Mueller, D. G. von Mühlen, G. A. Laughlin, D. L. Schneider, and D. J. Sartoris, "Low levels of estradiol are associated with vertebral fractures in older men, but not women: the rancho bernardo study 1," *The Journal of Clinical Endocrinology & Metabolism*, vol. 85, no. 1, pp. 219–223, 2000.

[58] A. Belgorosky, G. Guercio, C. Pepe, N. Saraco, and M. A. Rivarola, "Genetic and clinical spectrum of aromatase deficiency in infancy, childhood and adolescence," *Hormone Research in Paediatrics*, vol. 72, no. 6, pp. 321–330, 2009.

[59] A. Morishima, M. M. Grumbach, E. R. Simpson, C. Fisher, and K. Qin, "Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens," *The Journal of Clinical Endocrinology & Metabolism*, vol. 80, no. 12, pp. 3689–3698, 1995.

[60] S. Khosla, "Pathogenesis of age-related bone loss in humans," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 68, no. 10, pp. 1226–1235, 2013.

[61] J. Rosen and A. Negro-Vilar, "Novel, non-steroidal, selective androgen receptor modulators (SARMs) with anabolic

activity in bone and muscle and improved safety profile," *Journal of Musculoskeletal and Neuronal Interactions*, vol. 2, no. 3, pp. 222–224, 2002.

[62] J. T. Dalton, K. G. Barnette, C. E. Bohl et al., "The selective androgen receptor modulator GTx-024 (enobosarm) improves lean body mass and physical function in healthy elderly men and postmenopausal women: results of a double-blind, placebo-controlled phase II trial," *Journal of Cachexia, Sarcopenia and Muscle*, vol. 2, no. 3, pp. 153–161, 2011.

[63] R. V. Clark, A. C. Walker, S. Andrews, P. Turnbull, J. A. Wald, and M. H. Magee, "Safety, pharmacokinetics and pharmacological effects of the selective androgen receptor modulator, GSK2881078, in healthy men and postmenopausal women," *British Journal of Clinical Pharmacology*, vol. 83, no. 10, pp. 2179–2194, 2017.

[64] D. Neil, R. V. Clark, M. Magee et al., "GSK2881078, a SARM, produces dose-dependent increases in lean mass in healthy older men and women," *The Journal of Clinical Endocrinology & Metabolism*, vol. 103, no. 9, pp. 3215–3224, 2018.

[65] S. Basaria, L. Collins, E. L. Dillon et al., "The safety, pharmacokinetics, and effects of LGD-4033, a novel nonsteroidal oral, selective androgen receptor modulator, in healthy young men," *The Journals of Gerontology: Series A*, vol. 68, no. 1, pp. 87–95, 2013.

[66] V. Krishnan, N. J. Patel, J. G. Mackrell et al., "Development of a selective androgen receptor modulator for transdermal use in hypogonadal patients," *Andrology*, vol. 6, no. 3, pp. 455–464, 2018.

Hindawi
International Journal of Endocrinology
Volume 2020, Article ID 5798146, 7 pages
https://doi.org/10.1155/2020/5798146

Research Article

Anabolic Hormone Deficiencies in Heart Failure with Reduced or Preserved Ejection Fraction and Correlation with Plasma Total Antioxidant Capacity

Antonio Mancini,[1] Angela Maria Rita Fuvuzzi,[2] Carmine Bruno,[1] Maria Anna Nicolazzi,[2] Edoardo Vergani,[1] Nunzia Ciferri,[1] Andrea Silvestrini,[3] Elisabetta Meucci,[3] Nicola Nicolotti,[4] Roberta D'Assante,[5] and Antonio Cittadini[5]

[1]*Operative Unit of Endocrinology, Fondazione Policlinico Universitario A Gemelli IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy*
[2]*Operative Unit of Internal Medicine and Vascular Diseases, Division of Internal Medicine and Cardiovascular Diseases, Fondazione Policlinico Universitario A Gemelli IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy*
[3]*Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, Rome, Italy*
[4]*Medical Management, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy*
[5]*Department of Translational Medical Sciences, Federico II University of Naples, Naples, Italy*

Correspondence should be addressed to Antonio Mancini; me4224@mclink.it and Andrea Silvestrini; andrea.silvestrini@unicatt.it

Received 28 June 2019; Revised 16 October 2019; Accepted 3 December 2019; Published 3 January 2020

Academic Editor: Małgorzata Kotula-Balak

Background. While anabolic hormone deficit is a common finding in heart failure with reduced ejection fraction (HFrEF), few data are available in heart failure with preserved ejection fraction (HFpEF). *Methods*. Blood samples were collected for metabolic (total cholesterol, HDL cholesterol, LDL cholesterol, creatinine, and glucose) and hormonal (IGF-1, DHEA-S, TSH, fT3, fT4, and T) determination, comparing 30 patients with HFpEF and 20 patients with HFrEF. Total antioxidant capacity was evaluated by using the spectrophotometric method using the latency time in the appearance of the radical species of a chromogen (LAG, sec) as a parameter proportional to antioxidant content of the sample. Echocardiographic parameters were also assessed in the two groups. *Results*. A high prevalence of testosterone (32% in HFrEF and 72% in HFpEF, $p < 0.05$) and DHEA-S deficiencies was observed in HFpEF patients. Echocardiographic parameters did not correlate with hormone values. A significant direct correlation between T ($r^2 = 0.25$, $p < 0.05$) and DHEA-S ($r^2 = 0.19$, $p < 0.05$) with LAG was observed only in HFpEF. *Conclusion*. Anabolic hormone deficiency is clearly shown in HFpEF, as already known in HFrEF. Although longitudinal studies are required to confirm the prognostic value of this observation, our data suggest different mechanisms in modulating antioxidants in the two conditions, with possible therapeutic implications.

1. Introduction

Chronic heart failure (CHF) is defined as a clinical syndrome based on an unbalance between cardiac output and metabolic requirements of organism [1]. Any structural or functional disorders such as coronary heart disease, hypertension, diabetes, cardiomyopathies, heart valve diseases, arrhythmias, congenital heart defects, anaemia, cocaine abuse, AIDS, thyroid disorders, radiation, and chemotherapy that reduce the ability of the ventricle to fill with or eject an adequate volume of blood may be the cause of this condition. It is a staggering plague for our times since its prevalence is around 1-2% of the adult population in developed countries, with a peak ≥10% among people >70 years of age [2–4].

The main classification of CHF relies on left ventricular ejection fraction (LVEF), evaluated by echocardiography, or, less frequently, myocardial scintigraphy and magnetic resonance of the heart; its measurement identifies three classes

of CHF: from the well known and classic heart failure with reduced ejection fraction (HFrEF), which includes patients with left ventricular ejection fraction (LVEF) <40%, to heart failure with preserved ejection fraction (HFpEF), which comprises patients with LVEF <50%, to the grey area of LVEF in the range of 40–49%, which describes the new entity of heart failure with midrange ejection fraction [1]. HFrEF and HFpEF are the most known subtypes, with a relevant and increasing literature concerning the last one [5, 6]. They are different syndromes, with different pathogenesis, pathophysiologies, and therapy. In HFrEF, the hinge point is a direct damage to the heart that leads to reduced left ventricle contraction [7], whereas in HFpEF, diastolic dysfunction is the main mechanism involved, with other features contributing to this scenario such as left atrial dysfunction, right ventricular dysfunction, pulmonary hypertension, and increased vascular stiffness [8–12].

Both the conditions present with a high prevalence of multihormonal deficiencies [13, 14]. The impairment of major anabolic systems (somatotropic, adrenal, and gonadal) does not appear to represent a mere epiphenomenon but is involved in the CHF pathophysiology; especially low serum testosterone (T), dehydroepiandrosterone-sulfate (DHEA-S), and insulin-like growth factor (IGF)-1 levels have been correlated to the symptoms severity and the adverse outcomes in men suffering from CHF [15–19]. On the contrary, T, DHEA-S, and IGF-1 are known to regulate oxidative stress in different manners [20], exerting pro-oxidative effects or exhibiting an antioxidant power. CHF is a syndrome in which inflammation and OS play a fundamental role, and, in turn, this may point to a pivotal role of these hormonal alterations both in the pathogenesis of CHF and in its treatment.

Total antioxidant capacity (TAC) expresses the whole effects of nonproteic nonenzymatic antioxidants, as widely discussed in previous studies [21, 22]. Previously, we have shown the modulatory action of anabolic hormones on this parameter and its variations in CHF [20, 23].

Thus, the aim of the present study was to explore the correlation between anabolic hormones, echocardiographic parameters, and TAC in HFpEF and HFrEF and correlate them with metabolic parameters (with the aim to better understand the possible molecular consequences of hormonal derangement in these conditions).

2. Materials and Methods

50 subjects involved in this study were admitted to the University Hospital "Policlinico Gemelli" Dept. of Internal Medicine and were enrolled after being given an explanation of purposes and nature of the study, conducted in accordance with the Declaration of Helsinki, as revised in 2013. The study protocol was approved by the Institutional review board of "Medical Pathology" of our University Hospital.

Twenty patients with HFrEF, aged 42–88 years (mean 69.5), and thirty patients with HFpEF, aged 59–90 years (mean 77.7), were recruited. The diagnosis of HFpEF was established according to the current guidelines of the European Society of Cardiology [1]. Patients with end-stage renal disease, liver cirrhosis, and neoplastic or autoimmune diseases were excluded. All patients were nonsmokers or had stopped smoking for at least a year. Clinical, anthropometric, and echocardiographic evaluations were achieved, including the main risk factors for cardiovascular disease. Prevalence of comorbidities (T2DM, hypertension, atrial fibrillation, peripheral atherosclerosis, non-end-stage chronic kidney disease, and COPD) was evaluated. The two groups were not significantly different for age, BMI, and NYHA classes (all belonged to class II-III).

Between 08.30 and 09.00 a.m., after an overnight fast, a polyethylene catheter was inserted into the antecubital vein of one forearm and the blood was collected using a 6 mL vacutainer blood collection tube containing lithium heparin and immediately centrifuged (4°C at 3000 ×g for 15 min) with aliquots stored at −80°C until assayed.

We evaluated metabolic (glycaemia, insulinemia, and total-HDL-LDL cholesterol) and hormonal pattern (fT3, fT4, TSH, IGF-1, T, DHEA-S, and NT-proBNP).

Fasting glucose and insulin levels were quantified with commercial kits using ADVIA automatic analyser (Siemens, Italy).

Plasmatic concentrations of NT-proBNP, TSH, fT3, fT4, DHEA-S, T, and IGF-1 were measured by using immunochemiluminometric assays on a Roche Modular E170 analyser (Roche Diagnostics, Indianapolis, IN, USA). The intra- and interassay CV for all hormones were, respectively, <5.0% and <7.0%.

Normal ranges in our laboratory were NT-proBNP (>126 pg/ml), TSH (0.4–3.2 μUI/ml), fT3 (2.4–4.2 pg/ml), fT4 (8.5–16.5 pg/ml), DHEA-S (800–3500 ng/ml), and T (2.5–8.4 ng/ml). Values equal or below the lower limit of normal ranges were used to define as deficiency. For IGF-1, due to the age-related variations, we applied, to define IGF-1 deficiency, criteria of the TOSCA registry referring to the 33th percentile of a population of men with chronic heart failure (i.e., 122 ng/ml for age range under 55 years, 109 ng/ml for age range 55–64 years, 102 ng/ml for age range 65–74, and 99 ng/ml for age range older than 75 years) [13, 17].

A complete echocardiographic evaluation was performed (Echocardiography Philips, Affiniti 70c), measuring the following parameters: left ventricular ejection fraction (EF), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LV-ESD), left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LV-ESV), septal thickness (IVS), posterior wall thickness (LV-PW), peak E-wave velocity (E), peak A-wave velocity (A), E/A ratio, pulsed-wave TDI E′ velocity (E′), E/E′ ratio, deceleration time (DT), left atrial volume (LAV), indexed left atrial volume (LAVI), systolic pulmonary artery pressure (SPAP), tricuspid annular plane systolic excursion (TAPSE), and tricuspid peak velocity (TPV).

Total antioxidant capacity (TAC) was evaluated with the method of Rice-Evans and Miller [21], modified as previously reported [24]. The method is based on the interaction between the system H_2O_2-metmyoglobin with the chromogen ABTS, whose radical form is spectroscopically detectable. The latency time (LAG in sec.) before the appearance of radical species is proportional to the antioxidant concentration in the sample.

HOMA-IR was used as an index of insulin resistance and was obtained from the fasting blood insulin (immunoreactive insulin (IRI)) concentration and the fasting blood sugar (FBS) level early in the morning, based on the following equation: HOMA-IR = (IRI × FBS)/405.

To estimate the sample sizes, the estimated decrease prevalence of T deficiency between the two groups was set at 20%, based on the only reported work that, at the best of our knowledge, evaluated these data in HFrEF vs HFpEF patients [14], with a type I error rate of 0.05 and a type II error rate of 0.20 (i.e., power of 0.80). Due to the expected effect size, a total of 44 patients were considered adequate.

The Mann–Whitney U test was employed to evaluate differences between the two groups of subjects. A p value of 0.05 was considered statistically significant. Linear regression analysis was employed to correlate TAC with hormonal parameters. The X square test was used to compare percent differences between the two groups, when considering the prevalence of hormone deficiencies and comorbidities.

3. Results

Table 1 shows the echocardiographic parameters: other than ejection fraction, which was different, by definition, other differences were found in LVEDV, LVESV, and LAV, all significantly higher in HFrEF ($p < 0.05$). On the contrary, the A wave was significantly higher in HFpEF ($p < 0.05$).

Comorbidities, as expected, were more prevalent in HFpEF patients (41% T2DM, 72% hypertension, 36% atrial fibrillation, 68% peripheral atherosclerosis, 63% non-end-stage chronic kidney disease, and 36% COPD) than in HFrEF patients (30% T2DM, 39% hypertension, 44% atrial fibrillation, 5% peripheral atherosclerosis, 33% non-end-stage chronic kidney disease, and 16% COPD). X square analysis showed a significant difference only in hypertension and peripheral atherosclerotic disease ($p < 0.05$).

Table 2 shows the metabolic and hormonal parameters in the two groups. Significant differences were observed in NT-proBNP, total cholesterol, and T, with higher levels in HFrEF. LAG values were not significantly different between the two groups despite higher prevalence of comorbidities in HFpEF.

Figure 1 shows the graphical representation of percent prevalence of T deficiency, and DHEA-S levels were under the normal range in all but two patients. The prevalence of T deficiency was significantly different between the two groups using the X square test ($p < 0.05$).

No significant correlation was present when correlating T or DHEA-S with echocardiographic parameters. On the contrary, both T and DHEA-S significantly correlated with LAG values, but only in patients with HFpEF (Figure 2).

Table 1: Echocardiographic parameters in the two subgroups of heart failure, with preserved or reduced ejection fraction (HFpEF and HFrEF, respectively).

	HFpEF	HFrEF	p
LV-EDV (ml)	48.6 ± 1	146 ± 13	<0.05
LV-ESV (ml)	31.1 ± 1.8	91 ± 10	<0.05
LV-PW (mm)	10.9 ± 0.6	10.6 ± 0.6	n.s.
E (mm/s)	575.3 ± 74.6	347 ± 152	n.s.
A (mm/s)	789.6 ± 63.3	334 ± 110.74	<0.05
EF (%)	55.7 ± 0.8	36.91 ± 2	<0.05
E/A	0.8 ± 0.1	1.10 ± 0.38	n.s.
E/e′	11.9 ± 1.2	12.42 ± 2.86	n.s.
LAV (ml)	86.4 ± 8.9	117.86 ± 12	<0.05
TAPSE (mm)	21.3 ± 0.9	19.5 ± 1.21	n.s.
SPAP (mmHg)	35.9 ± 2.4	42 ± 4.84	n.s.

LV-EDV = left ventricular end-diastolic volume; LV-ESV = left ventricular end-systolic volume; LV-PW = left ventricular posterior wall thickness; E = E wave; A = A wave; e′ = e′ wave; EF = ejection fraction; LAV = left atrial volume; TAPSE = tricuspid annular plane systolic excursion; SPAP = systolic pulmonary arterial pressure.

Table 2: Mean ± SEM values of clinical, metabolic, and hormonal values in the two subgroups of heart failure, with preserved or reduced ejection fraction (HFpEF and HFrEF, respectively).

	HFpEF	HFrEF	p
NHYA class	II ($n = 12$) III ($n = 18$)	II ($n = 10$) III ($n = 10$)	
NT-proBNP (pg/ml)	2862.2 ± 488.5	7500.16 ± 2459.5	<0.05
Total cholesterol (mg/dl)	125.5 ± 6.9	153.6 ± 10.9	<0.05
HDL-C (mg/dl)	34.4 ± 2	44.47 ± 5.2	n.s.
LDL-C (mg/dl)	75.7 ± 7.3	91.36 ± 7.3	n.s.
Creatinine (mg/dl)	1.3 ± 0.1	1.27 ± 0.1	n.s.
Insulin (μUI/ml)	11.8 ± 1.4	25.32 ± 9.9	n.s.
Glucose (mg/dl)	88.5 ± 4.7	94.95 ± 8.4	n.s.
HOMA-IR	2.8 ± 0.5	3.68 ± 1.3	n.s.
BMI (kg/m^2)	28.7 ± 1.1	28.03 ± 1.2	n.s.
IGF-1 (ng/ml)	89.4 ± 7.1	112.78 ± 11.8	n.s.
DHEA-S (ng/ml)	438.9 ± 61.1	564.1 ± 140.3	n.s.
TSH (μU/ml)	2.5 ± 0.5	2.82 ± 1.0	n.s.
fT3 (pg/ml)	2.4 ± 0.1	2.5 ± 0.1	n.s.
fT4 (pg/ml)	11.3 ± 0.5	11.28 ± 0.5	n.s.
T (ng/ml)	2.4 ± 0.2	3.55 ± 0.5	<0.05
LAG (sec)	83 ± 6.7	75.77 ± 7.5	n.s.

4. Discussion

Our data confirm a high prevalence of anabolic hormones deficit in the two HF subgroups, thus expanding the concept that anabolic deficiencies are a common finding not only in HFrEF but also in HFpEF, a poorly explored condition in this concern. We found a significant difference in the prevalence of low T in HFpEF; this datum is not fully in agreement with the only report of hormone evaluation in HFpEF [13]; however, the difference in age of patients could contribute to these results. Nevertheless, ageing seems not to be the only factor influencing hormone picture in these patients since T levels correlated with LAG in this specific group, suggesting a possible link between T, oxidative stress, and cardiac function.

Focusing on T levels, there are several evidences that, in HFrEF, low levels can represent a bad prognostic sign [18, 25]. At the state of knowledge, the same conclusion in HFpEF cannot be sustained due to the lack of large longitudinal studies.

We have neither found correlations between T or DHEA-S levels and echocardiographic parameters nor

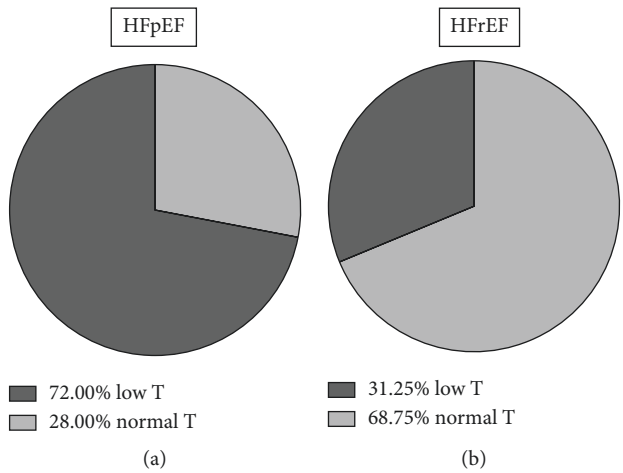


Figure 1: Percentage of patients with low testosterone levels in the two forms of heart failure (preserved or reduced ejection fraction, HFpEF and HFrEF, respectively).

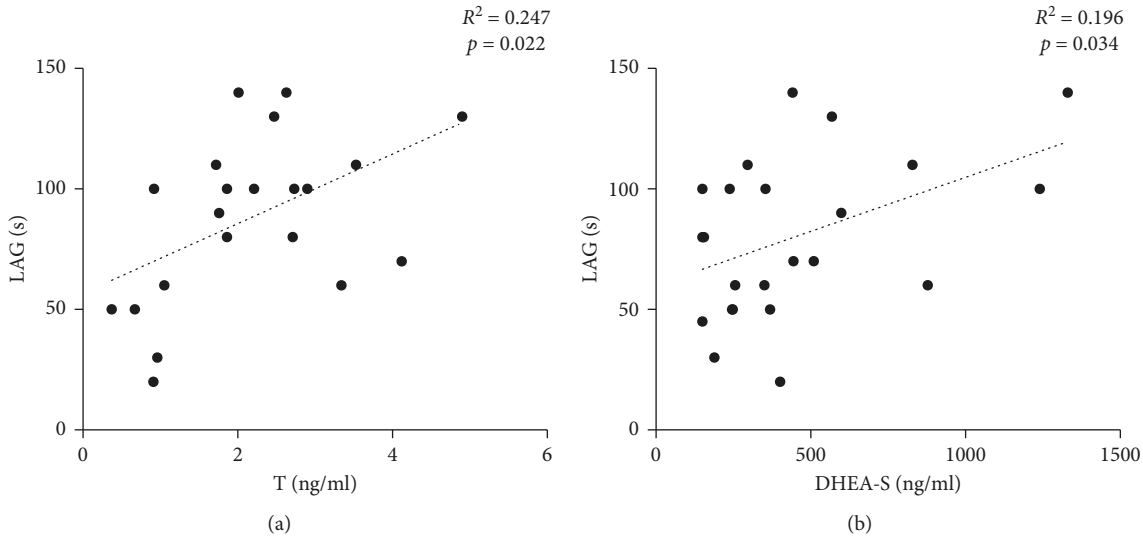


Figure 2: Graphical representation of correlation between LAG (latency time in the appearance of radical species, see text for the method), as a parameter of total antioxidant capacity and levels of testosterone T (a) or DHEA-S (b) in patients with heart failure with preserved ejection fraction (HFpEF).

differences comparing patients with low or normal T and low or normal DHEA-S.

On the contrary, interesting data emerge when correlating hormonal levels with TAC. Previously, we have shown that antioxidant systems can counteract OS in patients with HFrEF when one single hormonal deficit is detected, while such compensation is less effective when more deficits ensue [20].

This is the first report on TAC in HFpEF. We have found a significant correlation between T and DHEA-S with LAG in such model, while this is not evident in HFrEF.

Two consequences can be argued from these data: first, an important role of anabolic hormones in modulating antioxidant systems in HFpEF; second, different pathophysiological mechanisms underlying the two models of HF.

For both hormones, conflicting data are reported in literature showing pro-oxidant or antioxidant effects, depending on concentration or different models studied. For instance, DHEA-S administration can induce oxidative stress in hearts of male wistar rats, defining various histologic cardiac lesions in rats, such as misshapen cell nuclei, leukocytic infiltrates, disorganized myocardial fibers, and echocardiographic alterations (increased LV-PW and LV-ESD) [26, 27], while it exerts a protective role in the liver of diabetic rats [28] and rats with obstructive jaundice [29]; in ovariectomized rats, it improves nitric oxide (NO) production, vascular function, and blood pressure levels [30]. The results of *in vivo* and *in vitro* studies have shown that DHEA-S limits lipid peroxidation [31, 32]. Moreover, oxidative stress parameters in plasma and in peripheral blood mononuclear cells in diabetic subjects are significantly decreased by DHEA-S treatment [33].

The same double-faced effects are attributed to T. In fact, ROS production in vascular smooth muscle cells in culture is

stimulated by T, especially in hypertensive animal models [34]; T also stimulates xanthine oxidase and therefore superoxide generation [35]; finally, acute administration of T in supraphysiological doses increased NO urinary metabolites in healthy subjects [36]. However, it well established a protective role in ischaemic cardiopathy [37–39]; it is also known that T has a vasodilatory property via nongenomic mechanisms [40].

The literature concerning the T evaluation in CHF has been extensively reviewed, also for the therapeutic implication. T deficiency has a key role in some pathophysiological aspects of CHF, such as reduced muscle mass, abnormal energy handling, dyspnoea, and fatigue [41]. The so-called "muscle hypothesis" is based on functional and structural alterations of myocytes, which are strongly influenced by anabolic hormones [42, 43]. Metabolic influence of T has been also reported [25, 44]. However, some data are still contrasting: total and free T levels have been shown to decrease in elderly patients and related to CHF severity, but they were not independent predictors for mortality [45]. Long-term epidemiological trials were in favour of a protective effect of T treatment in the reduction of major adverse cardiovascular events and mortality [44], even if other meta-analyses raised doubts on this topic [46–49].

Our study clearly shows the correlation of the two hormones with antioxidant systems and is therefore in favour of a positive role on OS, which is one of the main players in the pathophysiology of HF.

According to literature, our patients with HFpEF, with a higher prevalence of obesity and other metabolic comorbidities, exhibited a trend toward increased LAG values. It can be speculated that a further increase in oxidative stress condition could induce a compensatory increase in antioxidant systems, possibly influencing T levels with a reciprocal vicious circle due to the modulatory role of T itself on antioxidants. Such correlation is not evident in the HFrEF group in which a worse cardiac performance or systemic catabolic status is present in CHF. Therefore, two different pathophysiological models seem to be involved in the two kinds of CHF. As recently proposed, in HFrEF, the process starts with primary ischaemic or oxidative damage of cardiomyocytes, whereas in HFpEF, a cascade of events is increased by the systemic proinflammatory state related to multiple comorbidities. The resultant endothelial damage leads to microvascular coronary inflammation and, ultimately, to myocardial dysfunction [50].

Nevertheless, there are some potential limitations of the present study. Firstly, the number of subjects is relatively small, and our findings need to be validated in a larger cohort. It is not possible to express a cause-effect relation between anabolic hormones and antioxidant status. Moreover, only one parameter of antioxidant status has been evaluated although, in our previous study, the positive effect of T replacement therapy on TAC in hypogonadal patients was described [51].

In conclusion, deficit of anabolic hormones is clearly revealed in HFpEF, as already known in HFrEF. Although longitudinal studies are needed to confirm a prognostic value of this observation, our data suggest a different mechanism in modulating antioxidant systems in these two conditions. Moreover, a possible therapeutic role of antioxidants needs to be investigated, particularly when T therapy is contraindicated.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This manuscript was presented as abstract at the American Society of Andrology 44th Annual Conference, Chicago (IL, USA), April 6–9th 2019.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] P. Ponikowski, A. A. Voors, S. D. Anker et al., "2016 ESC guidelines for the diagnosis and treatment of acute and chronic heart failure," *European Heart Journal*, vol. 37, no. 27, pp. 2129–2200, 2016.

[2] A. Mosterd and A. W. Hoes, "Clinical epidemiology of heart failure," *Heart*, vol. 93, no. 9, pp. 1137–1146, 2007.

[3] G. Bleumink, A. Knetsch, M. Sturkenboom et al., "Quantifying the heart failure epidemic: prevalence, incidence rate, lifetime risk and prognosis of heart failure—the Rotterdam study," *European Heart Journal*, vol. 25, no. 18, pp. 1614–1619, 2004.

[4] M. M. Redfield, S. J. Jacobsen, J. C. Burnett Jr., D. W. Mahoney, K. R. Bailey, and R. J. Rodeheffer, "Burden of systolic and diastolic ventricular dysfunction in the community," *JAMA*, vol. 289, no. 2, p. 194, 2003.

[5] B. A. Borlaug and W. J. Paulus, "Heart failure with preserved ejection fraction: pathophysiology, diagnosis, and treatment," *European Heart Journal*, vol. 32, no. 6, pp. 670–679, 2011.

[6] C. Andersson and R. S. Vasan, "Epidemiology of heart failure with preserved ejection fraction," *Heart Failure Clinics*, vol. 10, no. 3, pp. 377–388, 2014.

[7] M. W. Bloom, B. Greenberg, T. Jaarsma et al., "Heart failure with reduced ejection fraction," *Nature Reviews Disease Primers*, vol. 3, no. 1, 2017.

[8] C. S. P. Lam, V. L. Roger, R. J. Rodeheffer, B. A. Borlaug, F. T. Enders, and M. M. Redfield, "Pulmonary hypertension in heart failure with preserved ejection fraction. A community-based study," *Journal of the American College of Cardiology*, vol. 53, no. 13, pp. 1119–1126, 2009.

[9] B. A. Borlaug and D. A. Kass, "Ventricular-vascular interaction in heart failure," *Cardiology Clinics*, vol. 29, no. 3, pp. 447–459, 2011.

[10] P. H. Brubaker and D. W. Kitzman, "Prevalence and management of chronotropic incompetence in heart failure," *Current Cardiology Reports*, vol. 9, no. 3, pp. 229–235, 2007.

[11] T. T. Phan, G. N. Shivu, K. Abozguia et al., "Impaired heart rate recovery and chronotropic incompetence in patients with heart failure with preserved ejection fraction," *Circulation: Heart Failure*, vol. 3, no. 1, pp. 29–34, 2010.

[12] D. W. Kitzman, B. Nicklas, W. E. Kraus et al., "Skeletal muscle abnormalities and exercise intolerance in older patients with heart failure and preserved ejection fraction," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 306, no. 9, pp. H1364–H1370, 2014.

[13] M. Arcopinto, A. Salzano, E. Bossone et al., "Multiple hormone deficiencies in chronic heart failure," *International Journal of Cardiology*, vol. 184, no. 1, pp. 421–423, 2015.

[14] A. Salzano, A. M. Marra, F. Ferrara et al., "Multiple hormone deficiency syndrome in heart failure with preserved ejection fraction," *International Journal of Cardiology*, vol. 225, pp. 1–3, 2016.

[15] P. E. Kontoleon, M. I. Anastasiou-Nana, P. D. Papapetrou et al., "Hormonal profile in patients with congestive heart failure," *International Journal of Cardiology*, vol. 87, no. 2-3, pp. 179–183, 2003.

[16] J. E. Nettleship, R. D. Jones, K. S. Channer, and T. H. Jones, "Testosterone and coronary artery disease," *Frontiers of Hormone Research*, vol. 37, pp. 91–107, 2008.

[17] M. Arcopinto, J. Isgaard, A. M. Marra et al., "IGF-1 predicts survival in chronic heart failure. Insights from the T.O.S.CA. (Trattamento Ormonale Nello Scompenso CArdiaco) registry," *International Journal of Cardiology*, vol. 176, no. 3, pp. 1006–1008, 2014.

[18] E. A. Jankowska, B. Biel, J. Majda et al., "Anabolic deficiency in men with chronic heart failure: prevalence and detrimental impact on survival," *Circulation*, vol. 114, no. 17, pp. 1829–1837, 2006.

[19] Y. Moriyama, H. Yasue, M. Yoshimura et al., "The plasma levels of dehydroepiandrosterone sulfate are decreased in patients with chronic heart failure in proportion to the severity," *Journal of Clinical Endocrinology & Metabolism*, vol. 85, no. 5, pp. 1834–1840, 2000.

[20] A. Mancini, E. Vergani, C. Bruno et al., "Oxidative stress as a possible mechanism underlying multi-hormonal deficiency in chronic heart failure," *European Review for Medical and Pharmacological Sciences*, vol. 22, no. 12, pp. 3936–3961, 2018.

[21] C. Rice-Evans and N. J. Miller, "[241 total antioxidant status in plasma and body fluids," *Methods in Enzymology*, vol. 234, pp. 279–293, 1994.

[22] A. Mancini, S. Raimondo, M. Persano et al., "Estrogens as antioxidant modulators in human fertility," *International Journal of Endocrinology*, vol. 2013, Article ID 607939, 6 pages, 2013.

[23] A. Mancini, R. Festa, V. Donna et al., "Hormones and antioxidant systems: role of pituitary and pituitary-dependent axes," *Journal of Endocrinological Investigation*, vol. 33, no. 6, pp. 422–433, 2010.

[24] A. Mancini, E. Leone, R. Festa et al., "Evaluation of antioxidant systems (coenzyme Q10 and total antioxidant capacity) in morbid obesity before and after biliopancreatic diversion," *Metabolism*, vol. 57, no. 10, pp. 1384–1389, 2008.

[25] V. A. Giagulli, E. Guastamacchia, G. D. Pergola, M. Iacoviello, and V. Triggiani, "Testosterone deficiency in male: a risk factor for heart failure," *Endocrine, Metabolic & Immune Disorders-Drug Targets*, vol. 13, no. 1, pp. 92–99, 2013.

[26] M. H. V. M. Jacob, D. d. R. Janner, A. Belló-Klein, S. F. Llesuy, and M. F. M. Ribeiro, "Dehydroepiandrosterone modulates antioxidant enzymes and Akt signaling in healthy Wistar rat hearts," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 112, no. 1–3, pp. 138–144, 2008.

[27] E. Emer, O. Yildiz, M. Seyrek et al., "High-dose testosterone and dehydroepiandrosterone induce cardiotoxicity in rats: assessment of echocardiographic, morphologic, and oxidative stress parameters," *Human & Experimental Toxicology*, vol. 35, no. 5, pp. 562–572, 2016.

[28] M. Aragno, E. Tamagno, V. Gatto et al., "Dehydroepiandrosterone protects tissues of streptozotocin-treated rats against oxidative stress," *Free Radical Biology and Medicine*, vol. 26, no. 11-12, pp. 1467–1474, 1999.

[29] F. Çelebi, I. Yilmaz, H. Aksoy, M. Gümüş, S. Taysi, and D. Oren, "Dehydroepiandrosterone prevents oxidative injury in obstructive jaundice in rats," *Journal of International Medical Research*, vol. 32, no. 4, pp. 400–405, 2004.

[30] J. P. G. Camporez, E. H. Akamine, A. P. Davel, C. R. Franci, L. V. Rossoni, and C. R. de Oliveira Carvalho, "Dehydroepiandrosterone protects against oxidative stress-induced endothelial dysfunction in ovariectomized rats," *The Journal of Physiology*, vol. 589, no. 10, pp. 2585–2596, 2011.

[31] G. Boccuzzi, M. Aragno, M. Seccia et al., "Protective effect of dehydroepiandrosterone against copper induced lipid peroxidation in the rat," *Free Radical Biology and Medicine*, vol. 22, no. 7, pp. 1289–1294, 1997.

[32] A. Khalil, J.-G. Lehoux, R. J. Wagner et al., "Dehydroepiandrosterone protects low density lipoproteins against peroxidation by free radicals produced by gamma-radiolysis of ethanol-water mixtures," *Atherosclerosis*, vol. 136, no. 1, pp. 99–107, 1998.

[33] E. Brignardello, C. Runzo, M. Aragno et al., "Dehydroepiandrosterone administration counteracts oxidative imbalance and advanced glycation end product formation in type 2 diabetic patients," *Diabetes Care*, vol. 30, no. 11, pp. 2922–2927, 2007.

[34] A. Z. Chignalia, E. Z. Schuldt, L. L. Camargo et al., "Testosterone induces vascular smooth muscle cell migration by NADPH oxidase and c-Src-dependent pathways," *Hypertension*, vol. 59, no. 6, pp. 1263–1271, 2012.

[35] Y. Puttabyatappa, J. N. Stallone, A. Ergul et al., "Peroxynitrite mediates testosterone-induced vasodilation of microvascular resistance vessels," *Journal of Pharmacology and Experimental Therapeutics*, vol. 345, no. 1, pp. 7–14, 2013.

[36] C. Skogastierna, M. Hotzen, A. Rane, and L. Ekström, "A supraphysiological dose of testosterone induces nitric oxide production and oxidative stress," *European Journal of Preventive Cardiology*, vol. 21, no. 8, pp. 1049–1054, 2014.

[37] V. A. Cameron, T. J. Mocatta, A. P. Pilbrow et al., "Angiotensin type-1 receptor A1166C gene polymorphism correlates with oxidative stress levels in human heart failure," *Hypertension*, vol. 47, no. 6, pp. 1155–1161, 2006.

[38] C. J. Howe, M. M. LaHair, J. A. McCubrey, and R. A. Franklin, "Redox regulation of the calcium/calmodulin-dependent protein kinases," *Journal of Biological Chemistry*, vol. 279, no. 43, pp. 44573–44581, 2004.

[39] J. R. Erickson, M.-l. A. Joiner, X. Guan et al., "A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation," *Cell*, vol. 133, no. 3, pp. 462–474, 2008.

[40] R. D. Jones, K. M. English, P. J. Pugh, A. H. Morice, T. H. Jones, and K. S. Channer, "Pulmonary vasodilatory action of testosterone: evidence of a calcium antagonistic action," *Journal of Cardiovascular Pharmacology*, vol. 39, no. 6, pp. 814–823, 2002.

[41] M. Volterrani, G. Rosano, and F. Iellamo, "Testosterone and heart failure," *Endocrine*, vol. 42, no. 2, pp. 272–277, 2012.

[42] A. J. S. Coats, A. L. Clark, M. Piepoli, M. Volterrani, and P. A. Poole-Wilson, "Symptoms and quality of life in heart failure: the muscle hypothesis," *Heart*, vol. 72, no. 2 Suppl, pp. S36–S39, 1994.

[43] M. F. Piepoli, A. Kaczmarek, D. P. Francis et al., "Reduced peripheral skeletal muscle mass and abnormal reflex physiology in chronic heart failure," *Circulation*, vol. 114, no. 2, 2006.

[44] T. Jones and D. Kelly, "Randomized controlled trials—mechanistic studies of testosterone and the cardiovascular system," *Asian Journal of Andrology*, vol. 20, no. 2, p. 120, 2018.

[45] H.-Y. Wu, X.-F. Wang, J.-H. Wang, and J.-Y. Li, "Testosterone level and mortality in elderly men with systolic chronic heart failure," *Asian Journal of Andrology*, vol. 13, no. 5, pp. 759–763, 2011.

[46] R. A. Kloner, C. Carson, A. Dobs, S. Kopecky, and E. R. Mohler, "Testosterone and cardiovascular disease," *Journal of the American College of Cardiology*, vol. 67, no. 5, pp. 545–557, 2016.

[47] O. M. Calof, A. B. Singh, M. L. Lee et al., "Adverse events associated with testosterone replacement in middle-aged and older men: a meta-analysis of randomized, placebo-controlled trials," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 60, no. 11, pp. 1451–1457, 2005.

[48] R. M. Haddad, C. C. Kennedy, S. M. Caples et al., "Testosterone and cardiovascular risk in men: a systematic review and meta-analysis of randomized placebo-controlled trials," *Mayo Clinic Proceedings*, vol. 82, no. 1, pp. 29–39, 2007.

[49] M. M. Fernández-Balsells, M. H. Murad, M. Lane et al., "Clinical review 1: adverse effects of testosterone therapy in adult men: a systematic review and meta-analysis," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 6, pp. 2560–2575, 2010.

[50] W. J. Paulus and C. Tschöpe, "A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation," *Journal of the American College of Cardiology*, vol. 62, no. 4, pp. 263–271, 2013.

[51] A. Mancini, E. Leone, R. Festa et al., "Effects of testosterone on antioxidant systems in male secondary hypogonadism," *Journal of Andrology*, vol. 29, no. 6, pp. 622–629, 2008.

Hindawi
International Journal of Endocrinology
Volume 2019, Article ID 6743489, 6 pages
https://doi.org/10.1155/2019/6743489

Research Article

Gonadotropin-Induced Spermatogenesis in CHH Patients with Cryptorchidism

Zhaoxiang Liu,[1,2] Jiangfeng Mao,[1] Hongli Xu,[1] Xi Wang,[1] Bingkun Huang,[1] Junjie Zheng,[1] Min Nie,[1] Hongbing Zhang,[3] and Xueyan Wu[1]

[1]*Key Laboratory of Endocrinology, Department of Endocrinology, Peking Union Medical College Hospital, National Health Commission of People's Republic of China, Beijing, China*
[2]*Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China*
[3]*Department of Physiology, State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences and School of Basic Medicine, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China*

Correspondence should be addressed to Jiangfeng Mao; maojiangfeng88@vip.sina.com and Xueyan Wu; wuxueyan@pumch.ac.cn

Received 29 July 2019; Accepted 22 October 2019; Published 18 December 2019

Academic Editor: Vito Angelo Giagulli

Congenital hypogonadotropic hypogonadism (CHH) patients with cryptorchidism history usually have poor spermatogenesis outcome, while researches focusing on this population are rare. This study retrospectively evaluated gonadotropin-induced spermatogenesis outcome in CHH patients with cryptorchidism ($n = 40$). One hundred and eighty-three CHH patients without cryptorchidism were served as control. All patients received combined gonadotropins therapy (HCG and HMG) and were followed up for at least 6 months. The median follow-up period was 24 (15, 33) months (totally 960 person-months). Sperm (>0/ml) initially appeared in semen at a median of estimated 24 months (95% confidence interval (CI) 17.8–30.2). Twenty (20/40, 50%) patients succeeded in producing sperms, and the average time to produce first sperm was 19 ± 8 months. Five pregnancies were achieved in 9 (5/9, 56%) couples who desired for children. Compared with CHH patients without cryptorchidism ($n = 183$), cryptorchid patients had longer median time for sperm appearance in semen (24 months vs. 15 months, $P < 0.001$), lower rate of spermatogenesis (50% vs. 67%, $P = 0.032$), and lower mean sperm concentration (1.9 (0.5, 8.6) million/ml vs. 11.1(1.0, 25.0) million/ml, $P = 0.006$) at the last visit. In conclusion, CHH patients with cryptorchidism require a longer period for gonadotropin-induced spermatogenesis. The successful rate and sperm concentration were lower than patients without cryptorchidism.

1. Introduction

Congenital hypogonadotropic hypogonadism (CHH), caused by GnRH deficiency or dysfunction, is characterized by delayed or absent puberty, development, and infertility. When fertility is desired, pulsatile GnRH infusion or combined human chorionic gonadotropin (HCG) and human menopausal gonadotropin (HMG) therapy can be used for spermatogenesis.

Cryptorchidism (particularly bilateral) has a significant negative impact on fertility potential [1, 2]. Compared with 1–3% cryptorchidism rate in full-term healthy neonates [3], the prevalence of cryptorchidism in CHH patients is about 10–20% [4, 5]. For normal male fetuses, testosterone and insulin-like factor 3 (INSL3) secreted by Leydig cells are primary regulators for testicular descent [6]. However, the normal procedure of testicular descent is disturbed due to a low level of testosterone and impaired Leydig cell function in CHH patients [7]. Abnormal position exposes testes to hyperthermia which impairs gonocyte transformation and subsequent mature cessation [8]. Therefore, it is recommended that cryptorchidism should be treated within one year after birth, hoping to maximally preserve the spermatogenic potentiality [9].

Cryptorchidism has been referred as a detrimental factor for producing sperms in CHH patients [4, 10–12]. In this

study, we report the fertility and paternity outcomes in 40 CHH patients with cryptorchidism treated with gonadotropins (HCG/HMG).

2. Materials and Methods

2.1. Subjects. This retrospective cohort study included 40 CHH patients with cryptorchidism histories referred to our hospital between 2005 and 2014. One hundred and eighty-three CHH patients without cryptorchidism treated with HCG/HMG were served as control (noncryptorchidism group). The diagnosis of CHH was made according to the criteria reported previously [13]. Cryptorchidism was defined as one or both testes remained undescended 6 months after birth.

The study protocol was reviewed and approved by the Ethics Committee of the Peking Union Medical College Hospital, and all aspects of the study comply with the Declaration of Helsinki. Ethics Committee of the Peking Union Medical College Hospital approved that no informed consent was required because data were analyzed retrospectively and anonymously.

2.2. Treatment and Data Collection. Patients discontinued androgen therapy (if used) for at least 3 months before starting gonadotropins therapy. Combined HCG (2000 U, Livzon Pharmaceutical Co, Guangdong, China) and HMG (75 U, Livzon Pharmaceutical Co.) was intramuscularly injected twice weekly at the beginning. The dosages of gonadotropins were adjusted according to serum testosterone level and sperm output. HCG dosage would increase to 2500–5000 U to maintain a serum testosterone level at 10–20 nmol/L. If sperm did not appear in seminal fluid after treatment of 6 months, HMG dosage would increase to 150 U. All patients were treated with gonadotropins for at least 6 months. Regular follow-ups were conducted at an interval of 3–6 months.

All patients were tested for their sense of smell. We prepared alcohol, vinegar, and water. If the patient could not distinguish them, he would be diagnosed as Kallmann syndrome preliminarily. Each patient had MR for pituitary and olfactory bulb and tract. Secondary sexual characteristics, testicular volumes, serum gonadotropins, serum testosterone, and sperm count were measured on each visit. For undescended testes, the testis volume was defined as 0. For descended testis (natural or after orchidopexy), the testicular volume was measured using a Prader orchidometer. The mean value of bilateral testicular volumes was used in data analysis. Semen samples were collected by masturbation and were analyzed according to the standard World Health Organization method [14]. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), and total testosterone levels were measured using commercial kits by the chemiluminescent method (ACS 180 Automatic Chemiluminescence System; Bayer, Germany). The intra- and interassay coefficients of variation of total testosterone were 5.6% and 6.6%, respectively. All the patients were intramuscularly injected with triptorelin (100 μg) at the first visit, and basal and peak LH levels were measured [15]. Sperm motility was classified as fast progressive sperm (*A*), slow progressive sperm (*B*), nonprogressive sperm, (*C*) and immotile sperm (*D*). The proportion in each motility categories was assessed.

2.3. Outcomes. The primary outcome was defined as the first sperm detection under microscopy (the semen were centrifugated if necessary). The secondary outcome was paternity. Self-reported pregnancy of the partners was noted.

2.4. Statistical Analysis. SPSS version 17.0 was used for data analysis. Normal distributive data were expressed as the mean ± SD, and nonnormal distributive data were expressed as median (quartiles). The paired *t*-test was used to compare the plasma testosterone and testicular volumes before and after the treatment. Kaplan–Meier analyses were used to estimate the median time to achieve different sperm thresholds. Cox regression models were built to analyze the predictors of successful spermatogenesis. χ^2 test was used to compare the differences between cryptorchidism and noncryptorchidism groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. The Baseline Characteristics for Groups with or without Cryptorchidism. A total of 223 CHH patients were retrospectively evaluated in this study. They were in good conditions with normal blood and urine routine test results and normal liver and renal function. Thyroid hormones, adrenal glucocorticoids, and IGF-1 levels were all in the normal range. All patients had normal pituitary MR images.

Of 40 cryptorchidism CHH patients, 25 patients (25/40, 62.5%) were diagnosed as Kallmann syndrome because they were anosmic and they had olfactory bulb aplasia in MR images. Another 15 patients were diagnosed as nCHH. The proportion of Kallmann syndrome in the noncryptorchidism group was 47.0% (86/183). The basal testicular volume in cryptorchid patients was smaller than that in noncryptorchid patients (1.6 ± 1.1 ml vs. 2.2 ± 1.6 ml, $P = 0.013$). After gonadotropin treatment, the testicular size in cryptorchid and noncryptorchid patients increased to 6.2 ± 4.1 ml vs. 8.6 ± 4.6 ml, respectively, ($P = 0.003$). The rate and duration of pretreatment androgen exposure were similar between cryptorchid and noncryptorchid patients. The age of initiating treatment and peak LH level after triptorelin stimulation was similar between two groups as well (see Table 1).

Several comorbidities were observed in all these CHH participants, such as obesity ($n = 23$), dwarfism ($n = 9$), mental retardation ($n = 5$), and unilateral agenesis of the kidney ($n = 3$) and cleft lip and palate ($n = 3$). The incidences of these comorbidities were similar between two groups (see Table 1).

3.2. Gonadotropins Boost Testosterone Level and Testicular Volume of the Patients with Cryptorchidism. For all the

Table 1: Comparison of baseline characteristics between the cryptorchidism and noncryptorchidism group.

	Cryptorchidism ($n = 40$)	Noncryptorchidism ($n = 183$)	P value
Proportion of Kallmann Syndrome	62.5% (25/40)	47.0% (86/183)	0.083
Age initiating treatment (y)	18.9 ± 4.6	20.4 ± 4.8	0.063
BMI (kg/m^2)	21.2 ± 4.1	22.6 ± 3.7	0.027*
Peak LH (IU/L)#^	0.8 (0.4, 3.3)	1.7 (0.7, 5.5)	0.812
Rate of CHH family history	7.5% (3/40)	8.2% (15/183)	0.591
Basal testicular volume (ml)	1.6 ± 1.1	2.2 ± 1.6	0.013*
Testicular volume after treatment (ml)	6.2 ± 4.1	8.6 ± 4.6	0.003*
Proportion of preandrogen therapy	72.5% (29/40)	55.7% (102/183)	0.054
Duration of androgen therapy (months)#	17 (3, 45)	3 (3, 6)	0.061
Proportion of pre-HCG therapy	37.5% (15/40)	24.0% (44/183)	0.112
Duration of pre-HCG therapy (months)#	6 (0, 23)	3 (3, 5)	0.070
Comorbidities			
Obesity	10.0% (4/40)	10.4% (19/183)	0.878
Dwarfism	5.0% (2/40)	3.8% (7/183)	0.772
Mental retardation	2.5% (1/40)	2.2% (4/183)	0.583
Unilateral agenesis of the kidney	2.5% (1/40)	1.1% (2/183)	0.672
Cleft lip and palate	0.0% (0/40)	1.6% (3/183)	0.091

*Statistical significance. $P < 0.05$ was defined as significant difference. #Data were expressed as median (25%, 75%). ^Peak LH: highest LH level after intramuscular injection of triptorelin 100 μg.

cryptorchid patients, the mean basal testicular volume was 1.6 ± 1.1 mL. Eighteen patients presented unilateral and 22 bilateral cryptorchidism. Twenty-three patients underwent orchidopexy at a mean age of 8.3 ± 6.0 years (ranged from 1 to 23 years). Baseline serum levels of LH, FSH, and testosterone were 0.2 ± 0.6 IU/L, 0.8 ± 0.9 IU/L, and 0.8 ± 0.5 nmol/l, respectively. Peak LH level after triptorelin stimulation was 4.5 ± 11.9 IU/L. Patients began gonadotropins treatment at the age of 21.8 ± 5.0 years. The median follow-up period of the patients was 24 (15, 33) months (totally 960 person-months). After gonadotropins treatment, their serum testosterone increased from 0.8 ± 0.5 nmol/L to 14.1 ± 8.2 nmol/L ($P < 0.001$) and the testicular volume enlarged from 1.6 ± 1.1 ml to 6.2 ± 4.1 mL ($P < 0.001$).

3.3. Spermatogenesis and Paternity Outcomes in CHH Patients with Cryptorchidism. The median time for first sperm appearance in semen of cryptorchid patients was 24 months (95% CI, 17.8–30.2, using the Kaplan–Meier analysis) after gonadotropin therapy. The estimated median time for sperm concentration >5, 10, and 15 million/ml could not be obtained due to insufficient number of patients who produced sperms above these levels (Figure 1). Orchidopexy history ($P = 0.054$), unilateral or bilateral cryptorchidism ($P = 0.225$), peak LH level after triptorelin stimulation ($P = 0.210$), as well as basal testicular volume ($P = 0.119$) were not significant predictors for spermatogenesis outcome according to Cox regression analysis (Table 2). For patients with orchidopexy ($n = 23$), age for surgery ($P = 0.448$) was also not a significant contributor for successful spermatogenesis.

Of the 20 (20/40, 50%) patients who succeeded in spermatogenesis, 10 had unilateral and 10 bilateral cryptorchidism. The mean time to achieve the first sperm is 19 ± 8 months. Only 2 patients (2/20, 10%) attained lower reference limit of sperm concentrations (≥15 million/ml) according to WHO standards [16]. Five patients (5/20, 25%) had normal

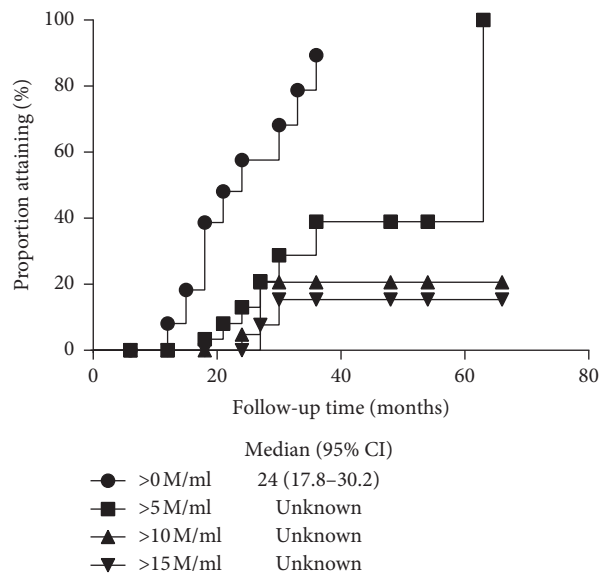


Figure 1: Median time for achieving sperm counts at different thresholds. Kaplan–Meier analysis of the median times for achieving sperm concentration more than 0, 5, 10, and 15 million/ml. $n = 40$.

sperm progressive motility ($A + B$) (≥32%). Of the 9 patients who desired for fathering children, 5 patients (56%) (2 with unilateral and 3 bilateral cryptorchidism) impregnated their partners during the treatment. Three boys and 2 girls were born with normal external genital appearance.

3.4. Comparison of Spermatogenesis between CHH Patients with and without Cryptorchidism. The mean follow-up period was similar between the two groups, cryptorchidism CHH 24 (15, 33) months and noncryptorchidism CHH 23 ± 13 months ($P = 0.438$). The success rate of spermatogenesis in the cryptorchidism group was lower than that of

Table 2: No significant predictors for successful spermatogenesis (Cox regression model).

	β	P value	95% CI Lower bound	95% CI Upper bound
Orchidopexy history (0, 1)	−1.622	0.054	0.038	1.027
Unilateral or bilateral cryptorchidism (1, 2)	−0.770	0.225	0.134	1.606
Peak LH level after triptorelin stimulation	0.025	0.210	0.986	1.066
Basal testis volume	0.528	0.119	0.873	3.294

the noncryptorchidism group (50% vs. 67%, $P = 0.032$) in responding to gonadotropins. Consistently, the cryptorchidism group had a longer median time for first sperm detection in semen (24 months vs. 15 months, $P < 0.001$) and a lower mean sperm concentration (1.9 (0.5, 8.6) million/ml vs. 11.1(1.0, 25.0) million/ml, $P = 0.006$) during the treatment. However, sperm progressive motility $(A + B)$ $((30.4 \pm 24.0)$ % vs. (37.1 ± 20.6) %, $P = 0.372)$ and total mobility $(A + B + C)$ $((34.8 \pm 24.9)$ % vs. (44.6 ± 20.5) %, $P = 0.210)$ were similar between the two groups (Table 3).

4. Discussion

It has been suggested that cryptorchidism history is a detrimental risk factor for spermatogenesis, and many clinicians empirically believed that the chance of successful spermatogenesis was extremely low in cryptorchid CHH patients [17, 18]. Our present work showed that 50% (20/40) of CHH patients with cryptorchidism history succeed in spermatogenesis, and 5 patients impregnate their wives during gonadotropin therapy.

Cryptorchid CHH patients (even bilateral) possess the potentiality of spermatogenesis and impregnating their wives [19, 20]. In this study, the successful rate of producing sperms is lower in cryptorchidism patients than noncryptorchidism patients. Several factors may contribute to this phenomenon. First, for CHH patients, absence of mini puberty may block the transformation from gonocyte into adult (dark) spermatogonia in the seminiferous tubules, leading to a significant reduction in fertility potentiality [21, 22]. Second, spermatogenesis needs an optimal scrotal temperature of 33°C. Higher temperature for undescended testis directly impairs the procession of sperm maturation [3]. Last, excessive oxidative stress and inflammatory reaction were found in undescended testis, which have negative effects on future fertility [23].

In our study, orchidopexy history, age for surgery, unilateral or bilateral cryptorchidism, as well as basal testicular volume did not correlate with spermatogenesis outcome. In order to maximally preserve the spermatogenesis capacity, it is recommended to conduct orchidopexy between 6 and 12 months of age. If the condition is diagnosed later in life, surgery should be performed at the earliest time [9, 24]. A randomized controlled study conducted by Kollin et al. [25] showed that orchidopexy performed at the age of 9 months produced a better testicular function than that performed at the age of 3 years. Earlier surgery leads to a larger number of Sertoli and germ cells and a larger testicular volume. Canavese's study [26] provided further evidence to support earlier surgery. Compared with orchidopexy performed in the second year of life, surgeries performed during the first year of life had clearly better results in total sperm counts and sperm motility. However, only 20% of cryptorchid boys in England underwent surgeries before the age of 18 months because surgeons are reluctant to conduct operations on newborns [27]. In our study, most of the participants conducted orchidopexy at the age over 2 years, possibly due to economic constraints or insufficient knowledge of the disease. It is presumed that the severely impaired testicular function could not be compensated by orchidopexy which was done too late. This may explain the finding that the age for orchiopexy had no influence on spermatogenesis in our study.

It has been reported that men undergoing bilateral orchidopexy in their childhood have poorer fertile capacity compared with those with unilateral procedure [1, 2]. The paternity rate is approximately two-thirds and less than one-third, respectively, in patients with unilateral and bilateral cryptorchidism [28]. However, our study showed no difference between these 2 groups, possibly due to small sample size and different etiology of our participants. Patients in our study are CHH with cryptorchidism, while patients in the other studies are with isolated cryptorchidism (with normal gonadotropin secretion). Furthermore, it was reported that men presenting with unilaterally undescended testis may have bilateral testicular abnormality [29].

Till now, more than two dozens of genes have been identified to cause CHH. Mutations of some genes, such as *FGFR1* and *KAL1*, may impair testicular development and spermatogenesis [30–32]. The incidence of cryptorchidism was up to 67% in patients with *KAL1* mutation [32], while 20% in patients was caused by FGFR1 mutations. One study, including 90 CHH patients treated with pulsatile GnRH infusion, showed that nearly 14% of patients had impaired testicular response to endogenous gonadotropins [33]. Therefore, the impaired testicular function in our participants may be caused by CHH-related gene mutations. As different gene mutations may markedly influence testicular function and spermatogenesis outcome, genetic screening of these patients ought to be conducted in a prospective study.

Multiple factors, such as gonadotropin deficits (i.e., absent mini puberty), temperature, oxidative stress, and age for orchidopexy, may remarkably influence the function of undescended testis. Additionally, testicular function may be impaired by surgical intervention, suggesting the surgery procedure itself is a potential confounding factor [24]. The compounding situation made it difficult to quantitatively analyze the effect of each factor on the patient's fertility outcome.

Table 3: Comparison of spermatogenesis between the cryptorchidism group and noncryptorchidism group.

	Cryptorchidism ($n = 40$)	Noncryptorchidism ($n = 183$)	P value
Spermatogenesis rate	50%	67%	0.032*
Median time for first sperm detection in semen (months)	24	15	<0.001*
Mean sperm concentration#	1.9 (0.5, 8.6)	11.1 (1.0, 25.0)	0.006*
Sperm progressive motility ($A + B$) (%)	30.4 ± 24.0	37.1 ± 20.6	0.372
Sperm total mobility ($A + B + C$) (%)	34.8 ± 24.9	44.6 ± 20.5	0.210

*Statistical significance. $P < 0.05$ was defined as significant difference. #Data were expressed as median (25%, 75%).

Some limitations should be addressed. First, since our conclusion is stemmed from CHH patients, it should be prudent to extrapolate the results to cryptorchidism population without CHH. Second, orchidopexy was done relatively late in our patients, which would bring potential selection bias. Third, as a retrospective study, HCG/HMG was the only regime prescribed to our participants during 2005–2014. Recent data revealed that HCG/rhFSH is superior to HCG/HMG for spermatogenesis [34, 35]. It is hopeful that HCG/rhFSH regime may further improve the outcome of spermatogenesis in CHH patients with cryptorchidism.

5. Conclusions

In summary, our study demonstrates that the successful rate of spermatogenesis in CHH patients with cryptorchidism is 50% and the pregnant rate is about 50% in couples desiring for children. This study provides more accurate prognostic information for CHH patients with cryptorchidism after gonadotropin treatment. Prospective studies with large cohort are needed to determine potential predictors on spermatogenesis in this population.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported in part by a grant from the National Key Research and Development Program of China (2016YFC0905102 and 2016YFA0101003) and CAMS Innovation Fund for Medical Sciences (CIFMS) (2016-I2M-1-002 and 2017-I2M-3-007).

References

[1] B. Trsinar and U. R. Muravec, "Fertility potential after unilateral and bilateral orchidopexy for cryptorchidism," *World Journal of Urology*, vol. 27, no. 4, pp. 513–519, 2009.

[2] D. Cortes, J. M. Thorup, and J. Visfeldt, "Cryptorchidism: aspects of fertility and neoplasms," *Hormone Research in Paediatrics*, vol. 55, no. 1, pp. 21–27, 2001.

[3] G. Cobellis, C. Noviello, F. Nino et al., "Spermatogenesis and cryptorchidism," *Frontiers in Endocrinology (Lausanne)*, vol. 5, p. 63, 2014.

[4] P. Y. Liu, H. W. G. Baker, V. Jayadev, M. Zacharin, A. J. Conway, and D. J. Handelsman, "Induction of spermatogenesis and fertility during gonadotropin treatment of gonadotropin-deficient infertile men: predictors of fertility outcome," *The Journal of Clinical Endocrinology & Metabolism*, vol. 94, no. 3, pp. 801–808, 2009.

[5] B. Bhagavath, R. H. Podolsky, M. Ozata et al., "Clinical and molecular characterization of a large sample of patients with hypogonadotropic hypogonadism," *Fertility and Sterility*, vol. 85, no. 3, pp. 706–713, 2006.

[6] J. M. Hutson, B. R. Southwell, R. Li et al., "The regulation of testicular descent and the effects of cryptorchidism," *Endocrine Reviews*, vol. 34, no. 5, pp. 725–752, 2013.

[7] S. Trabado, S. Lamothe, L. Maione et al., "Congenital hypogonadotropic hypogonadism and Kallmann syndrome as models for studying hormonal regulation of human testicular endocrine functions," *Annales d'Endocrinologie*, vol. 75, no. 2, pp. 79–87, 2014.

[8] J. M. Hutson, "Undescended testis: the underlying mechanisms and the effects on germ cells that cause infertility and cancer," *Journal of Pediatric Surgery*, vol. 48, no. 5, pp. 903–908, 2013.

[9] E. M. Ritzen, A. Bergh, R. Bjerknes et al., "Nordic consensus on treatment of undescended testes," *Acta Paediatrica*, vol. 96, pp. 638–643, 2007.

[10] R. Farhat, F. Al-zidjali, and A. S. Alzahrani, "Outcome of gonadotropin therapy for male infertility due to hypogonadotrophic hypogonadism," *Pituitary*, vol. 13, no. 2, pp. 105–110, 2010.

[11] Y. Miyagawa, A. Tsujimura, K. Matsumiya et al., "Outcome of gonadotropin therapy for male hypogonadotropic hypogonadism at university affiliated male infertility centers: a 30-year retrospective study," *Journal of Urology*, vol. 173, pp. 2072–2075, 2005.

[12] D. W. Warne, G. Decosterd, H. Okada, Y. Yano, N. Koide, and C. M. Howles, "A combined analysis of data to identify predictive factors for spermatogenesis in men with hypogonadotropic hypogonadism treated with recombinant human follicle-stimulating hormone and human chorionic gonadotropin," *Fertility and Sterility*, vol. 92, no. 2, pp. 594–604, 2009.

[13] T. Raivio, J. Falardeau, A. Dwyer et al., "Reversal of idiopathic hypogonadotropic hypogonadism," *New England Journal of Medicine*, vol. 357, no. 9, pp. 863–873, 2007.

[14] World Health Organization, *Laboratory Manual for the Examination and Processing of Human Semen*, World Health Organization, Geneva, Switzerland, 5th edition, 2010.

[15] X. Y. Wu, M. Nie, S. Y. Lu, and J. F. Mao, "[Clinical values of triptorelin stimulating test in assessing hypothalamus-pituitary-gonad axis function in male patients with hypothalamus-pituitary-gonad axis disorders]," *Zhonghua Yi Xue Za Zhi*, vol. 91, no. 10, pp. 679–682, 2011.

[16] T. G. Cooper, E. Noonan, S. von Eckardstein et al., "World Health Organization reference values for human semen characteristics," *Human Reproduction Update*, vol. 16, no. 3, pp. 231–245, 2010.

[17] D. M. Finkel, J. L. Phillips, and P. J. Snyder, "Stimulation of spermatogenesis by gonadotropins in men with hypogonadotropic hypogonadism," *New England Journal of Medicine*, vol. 313, no. 11, pp. 651–655, 1985.

[18] J. M. W. Kirk, M. O. Savage, D. B. Grant, P.-M. G. Bouloux, and G. M. Besser, "Gonadal function and response to human chorionic and menopausal gonadotrophin therapy in male patients with idiopathic hypogonadotrophic hypogonadism," *Clinical Endocrinology*, vol. 41, no. 1, pp. 57–63, 1994.

[19] D. Buchter, H. Behre, S. Kliesch, and E. Nieschlag, "Pulsatile GnRH or human chorionic gonadotropin/human menopausal gonadotropin as effective treatment for men with hypogonadotropic hypogonadism: a review of 42 cases," *European Journal of Endocrinology*, vol. 139, no. 3, pp. 298–303, 1998.

[20] T. H. Jones and J. F. Darne, "Self-administered subcutaneous human menopausal gonadotrophin for the stimulation of testicular growth and the initiation of spermatogenesis in hypogonadotrophic hypogonadism," *Clinical Endocrinology*, vol. 38, no. 2, pp. 203–208, 1993.

[21] D. Živković and I. Fratrić, "Disturbances of sperm maturation and minipuberty: is there a connection?" *Biomed Research International*, vol. 2014, Article ID 912746, 4 pages, 2014.

[22] F. Hadziselimovic and B. Hoecht, "Testicular histology related to fertility outcome and postpubertal hormone status in cryptorchidism," *Klinische Pädiatrie*, vol. 220, no. 5, pp. 302–307, 2008.

[23] M. Imamoğlu, S. S. Bülbül, N. Kaklikkaya, and H. Sarihan, "Oxidative, inflammatory and immunologic status in children with undescended testes," *Pediatrics International*, vol. 54, no. 6, pp. 816–819, 2012.

[24] E. Chan, C. Wayne, and A. Nasr, "Ideal timing of orchiopexy: a systematic review," *Pediatric Surgery International*, vol. 30, no. 1, pp. 87–97, 2014.

[25] C. Kollin, J. B. Stukenborg, M. Nurmio et al., "Boys with undescended testes: endocrine, volumetric and morphometric studies on testicular function before and after orchidopexy at nine months or three years of age," *The Journal of Clinical Endocrinology & Metabolism*, vol. 97, no. 12, pp. 4588–4595, 2012.

[26] F. Canavese, A. Mussa, M. Manenti et al., "Sperm count of young men surgically treated for cryptorchidism in the first and second year of life: fertility is better in children treated at a younger age," *European Journal of Pediatric Surgery*, vol. 19, no. 6, pp. 388–391, 2009.

[27] J. E. McCabe and S. E. Kenny, "Orchidopexy for undescended testis in England: is it evidence based?" *Journal of Pediatric Surgery*, vol. 43, no. 2, pp. 353–357, 2008.

[28] P. Goel, J. Rawat, A. Wakhlu, and S. Kureel, "Undescended testicle: an update on fertility in cryptorchid men," *Indian Journal of Medical Research*, vol. 141, no. 2, pp. 163–171, 2015.

[29] L. I. Lipshultz, R. Caminos-Torres, C. S. Greenspan, and P. J. Snyder, "Testicular function after orchiopexy for unilaterally undescended testis," *New England Journal of Medicine*, vol. 295, no. 1, pp. 15–18, 1976.

[30] N. O. Hadziselimovic, C. de Geyter, P. Demougin, E. J. Oakeley, and F. Hadziselimovic, "Decreased expression of FGFR1, SOS1, RAF1 genes in cryptorchidism," *Urologia Internationalis*, vol. 84, no. 3, pp. 353–361, 2010.

[31] L. Cotton, G. M. Gibbs, L. G. Sanchez-Partida, J. R. Morrison, D. M. de Kretser, and M. K. O'Bryan, "FGRF-1 signaling is involved in spermiogenesis and sperm capacitation," *Journal of Cell Science*, vol. 119, no. 1, pp. 75–84, 2006.

[32] S. Salenave, P. Chanson, H. Bry et al., "Kallmann's syndrome: a comparison of the reproductive phenotypes in men carrying KAL1 and FGFR1/KAL2 mutations," *The Journal of Clinical Endocrinology & Metabolism*, vol. 93, no. 3, pp. 758–763, 2008.

[33] G. P. Sykiotis, X.-H. Hoang, M. Avbelj et al., "Congenital idiopathic hypogonadotropic hypogonadism: evidence of defects in the hypothalamus, pituitary, and testes," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 6, pp. 3019–3027, 2010.

[34] Y. Kobori, S. Ota, H. Okada, and T. Tanaka, "Investigation of treatment for azoospermia due to male hypogonadotropic hypogonadism in Japan," *International Journal of Urology*, vol. 26, no. 1, pp. 134-135, 2019.

[35] Y. Kobori, K. Suzuki, T. Iwahata et al., "Hormonal therapy (hCG and rhFSH) for infertile men with adult-onset idiopathic hypogonadotropic hypogonadism," *Systems Biology in Reproductive Medicine*, vol. 61, no. 2, pp. 110–112, 2015.

Hindawi
International Journal of Endocrinology
Volume 2019, Article ID 7546385, 10 pages
https://doi.org/10.1155/2019/7546385

Research Article

Metabolic Effects of Testosterone Hormone Therapy in Normal and Orchiectomized Male Rats: From Indirect Calorimetry to Lipolytic Enzymes

Mahmoud Mustafa Ali Abulmeaty,[1,2] **Ali Madi Almajwal**,[1] **Mohamed Farouk ElSadek**,[1,3] **Mohamed Y Berika**,[4,5] **and Suhail Razak**[1]

[1]*Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia*
[2]*Medical Physiology Department, School of Medicine, Zagazig University, Zagazig, Egypt*
[3]*Nutrition and Food Science Department, Faculty of Home Economics, Helwan University, Helwan, Egypt*
[4]*Rehabilitation Sciences Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia*
[5]*Faculty of Medicine, Mansoura University, Mansoura, Egypt*

Correspondence should be addressed to Mahmoud Mustafa Ali Abulmeaty; dr.abulmeaty@gmail.com

Received 8 September 2019; Revised 2 October 2019; Accepted 10 October 2019; Published 28 November 2019

Guest Editor: Andrea Silvestrini

Background and Aim. Changes in total energy expenditure (TEE) and substrate metabolism may help explain the metabolic actions of testosterone (T). This study measured respiratory quotient (RQ), TEE, ghrelin, insulin, and key lipolysis enzyme concentrations in relation to body weight (wt) and food intake (FI) in both normal and bilaterally orchiectomized rats with/without T treatment. *Methods*. In total, thirty-two male Wistar rats (300–400 g) were divided into four groups ($n = 8$/group), including (a) sham-operated and vehicle-injected group (Sham), (b) T-treated sham group (T-Sham) for which sham-operated rats were injected with IM testosterone undecanoate (100 mg/kg, for one week), (c) orchiectomy and vehicle-injected group (Orch), and (d) T-replaced orchiectomy group (T-Orch). After one week, FI and wt were automatically recorded, indirect calorimetry parameters were measured, and blood samples were collected to measure T, ghrelin, insulin, growth hormone (GH), glucose, hormone-sensitive lipase (HSL), adipocyte triglyceride lipase (ATGL), free fatty acids (FFA), and lipid profiles. *Results*. Orchiectomy decreased ghrelin, GH, and insulin levels, increased TEE and RQ, and lowered FI and wt. The T-Orch group exhibited increased levels of ghrelin (3-fold), insulin, GH, blood levels of lipolysis products, TEE, and FI in addition to reduced glucose levels ($P < 0.05$). This group demonstrated no significant changes in wt. In the T-Sham group, T increased ghrelin and insulin levels ($P < 0.05$) with strong positive correlations ($r = 0.663$ and 0.644, respectively, $P < 0.05$), increased ATGL levels, RQ toward carbohydrate utilization ranges, and TEE, and reduced HSL levels ($P < 0.05$) with insignificant changes in FI or wt. *Conclusions*. T administration in orchiectomized rats significantly increased orexigenic mediators such as ghrelin and insulin without inducing any significant changes in wt. The mechanism for this finding might be the increased TEE and the stimulation of lipolysis through the ATGL enzyme. The associated rise of GH might help in interference with accumulation of lipid in adipose tissue. Apart from the effect on GH, T-Sham showed similar effects of T supplementation.

1. Introduction

Males with hypogonadism have distinctive metabolic derangements that mandate testosterone hormone replacement therapy (TRT), which indicates that testosterone (T) is a metabolic hormone with essential roles in substrate metabolism and energy homeostasis [1]. Being an anabolic hormone, it has also been investigated for use in healthy normal older men for improvement and maintenance of physical function and muscle mass [2]. The mechanisms of the stimulatory effect of T on feeding are still under investigation. Iwasa et al. [3] reported that the orexigenic effect of T is not due to raised neuropeptide Y (NPY) levels or any effects on leptin. In contrast, Jafaripour et al. [4]

showed that T increases food intake (FI) by increasing NPY and ghrelin levels. It has also been reported that T decreases preproghrelin mRNA expression in the hypothalamus and increases expression in the gut [5]. This orexigenic effect of T is not always accompanied by weight (wt) gain in either animal research models or clinical practice. Additionally, T deficiency, at least in part, may lead to clinical conditions such as obesity, type 2 diabetes, and metabolic syndrome [1]. Taken together, it can be concluded that T increases FI without increasing body wt. This apparent paradox requires further clarification.

The study of energy expenditure (EE) is an essential part of metabolic research, especially with the availability of new equipment that can accurately measure the total energy expenditure (TEE) or its components, such as the resting energy expenditure (REE), thermic effect of feeding (TEF), and thermic effect of activity (TEA). Indirect calorimetry is a gold standard for measuring TEE in humans and experimental animals [6]. Typically, new devices are based on open-circuit indirect calorimetry in which the gas is withdrawn from the atmosphere by a pump, passed through silica gel to be dried and measured, and then regulated by a flow controller before entering the box containing the animal. The box also has an outflow port with the outflow gases being dried again to remove any water vapor produced by the animal. After drying, the outflow gases are analyzed for O_2 volumes (VO_2 in liters) and CO_2 volumes (VCO_2 in liters) [7]. Both O_2 consumption and CO_2 production are used to calculate the respiratory quotient (RQ). The RQ, coupled with the amount of nitrogen excretion, enables the calculation of the relative consumption of macronutrients, especially carbohydrates and fat [8]. The TEE can be calculated using the Weir equation in which heat $\text{production}_{(\text{kcal/day})} = 1.44 \times [(3.94 \times VO_2 \text{ consumed}) + (1.1 \times VCO_2 \text{ produced})]$ [9].

Lipolysis is a pivotal process in metabolism and energy homeostasis. The regulation of lipolysis is mediated via three key enzymes, hormone-sensitive lipase (HSL), the recently discovered adipose triglyceride lipase (ATGL), and monoglyceride lipase (MGL), with the main lipase activity in adipose tissue and skeletal muscle being mediated by HSL and ATGL [10]. The mechanisms by which T affects lipolysis and consequently modulates body composition and wt remain unclear. Some investigators have reported that T reduces ATGL and HSL [11], while others have reported that T increases ATGL and HSL expression [12], and yet others found no significant effect of castration or T supplementation on these enzymes [13]. Moreover, Zang et al. [14] concluded that T reduces the expression of HSL while phosphodiesterase-3B expression is increased. Changes in EE, orexigenic mediators, and fat hydrolyzers may provide a reliable explanation for the metabolic actions of T in normal and hypogonadism conditions. Based on the findings described above, the current study aimed to measure the indirect calorimetry parameters (VO_2, VCO_2, RQ, and TEE), ghrelin and insulin levels, ATGL and HSL enzymes, and substrate concentrations (glucose, lipid panels, and free fatty acids) in the blood of both normal and orchiectomized rats with or without T treatment in relation to changes in FI and body wt.

2. Methods

2.1. Animals and Procedures. A total of 32 male Wistar rats (300–400 g, 10–12 weeks old) were obtained in January 2018 from the animal research center in the College of Pharmacy, King Saud University, to be used in this study. The rats were hygienically reared in the animal vivarium at 25°C room temperature and 20 to 45% humidity where the lights were on from 6 am to 6 pm daily [15]. The rats were fed a dietary formulation of protein (18.1%), fat (7.1%), carbohydrate (59.3%), and fiber (15.5%) with food and water being provided ad libitum. Rats were housed in groups of four, and after one week of habituation, they were divided into four groups ($n = 8$/group), including (a) the sham-operated group (Sham), which underwent a sham surgery and received intramuscular (IM) injections of normal saline (0.3 ml) and served as normal controls; (b) the T-treated Sham group (T-Sham) in which sham-operated rats received IM injections of a preparation of testosterone undecanoate (100 mg/kg) [16] daily for one week [17]; (c) the orchiectomy group (Orch) in which orchiectomized rats received IM injections of an equal volume of the vehicle (saline); and (d) the T-replaced orchiectomy group (T-Orch) in which orchiectomized rats received TRT with daily IM injections of T at the same dose and for the same duration as the T-Orch group. The body wt (g) of all the rats was recorded before treatment, after treatment, and also during and after indirect calorimetry analysis. The study protocol was approved by the ethics committee in the College of Applied Medical Sciences, King Saud University.

2.2. Surgery. General anesthesia was provided via intraperitoneal injection of a ketamine/xylazine mixture (75/2.5 mg/kg, respectively) [18]. Anesthetized animals were secured in a sterile operating theatre in a supine position. The scrotal skin was then disinfected with povidone-iodine and an eye ointment was applied to the eyes. For the Orch and T-Orch groups, a linear incision in the ventral skin of the scrotum was made, dissection of subcutaneous tissues was performed, and the tunicae were opened. The spermatic cord and main blood vessels were isolated and ligated with two adjacent ligations. The lower end of the spermatic cord was then cut between the two ligations to enable bilateral removal of the testicle and epididymis. The testicular skin was continuously sutured and a local antibiotic ointment was applied over the sutures [19]. The Sham and T-Sham groups of rats underwent midline linear incisions in the ventral skin of the scrotum and dissection of the subcutaneous tissues to reveal the testes, followed by continuous suture of the skin. To ensure the metabolic and endocrinal derangements induced by this procedure, the rats were left undisturbed for two weeks after the orchiectomy [20], prior to the study protocols being applied. In the end, rats were re-anesthetized and blood was collected via cardiac puncture and the visceral fat depots (epididymal, retroperitoneal, and mesenteric) were dissected and weighed.

2.3. Indirect Calorimetry. After treatment week, rats of all the groups were individually housed in Calo-cages of a TSE PhenoMaster system (TSE, Germany) at 25°C. The animals were left undisturbed for 24 hours before making the calorimetry measurements to allow for acclimatization to the individual housing conditions and to minimize the so-called novelty effect [21]. After gas calibration and calibration of the food sensors, the experimental protocol was executed for three days. The volumes of respiratory gases oxygen (VO_2) and carbon dioxide (VCO_2) were measured based on open-circuit indirect calorimetry. In addition, the RQ and EE per hour and per kg of body wt (Kcal/h/kg) were measured. FI was automatically recorded using a calibrated sensor at a sensitivity of 0.01 g. The measurements were taken and recorded every 15 min and those of the first six hours were omitted to ensure the stability of the recording process. Data used for the analyses included VO_2, VCO_2, RQ, TEE, FI, and body wt [22].

2.4. Blood Samples. After indirect calorimetry, all rats were fasted overnight and then euthanized and approximately 7 mL of blood was collected via cardiac puncture. The blood was collected in heparinized mini collection tubes and centrifuged, and the plasma was collected and stored at −80°C until the time of analysis.

2.5. Hormone, Enzyme, and Substrate Analysis. Measurement of total testosterone levels was done using enzyme-linked immunosorbent assay (ELISA) kits for T (catalog number MBS262661; Mybiosource, USA) as described by Njoroge et al. [23]. Based on the information from the manufacturer, the sensitivity of the T ELISA kits was 0.05 ng/ml. A total ghrelin ELISA kit (catalog number MBS731169; Mybiosource, USA) with sensitivity of 100 pg/ml was used to measure ghrelin levels according to Ali et al. [24]. Furthermore, an insulin ELISA kit for rats with a sensitivity of 0.5 mIU/L (MBS2602037; Mybiosource, USA) was used to measure the fasting insulin levels. Growth hormone (GH) levels in plasma were measured by ELISA kits (catalog number MBS2700019; Mybiosource, USA) with a sensitivity of <49.3 pg/ml. The plasma glucose concentrations and lipid panels including triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were assessed using automatic biochemical analyzers. [25]. Low-density lipoprotein cholesterol (LDL-C) levels were calculated according to the simple but accurate modified Friedewald formula [26] with the equation LDL-C = 3/4 × (TC − HDL-C), while very low-density lipoprotein (VLDL) levels were calculated by as 20% of the TG levels [27]. The insulin resistance indicated by the homeostasis model assessment-insulin resistance (HOMA-IR) was calculated according to Matthews et al. {fasting insulin (mIU/l) × fasting glucose (mmol/l)/22.5} [28]. ATGL and HSL were assessed using specific ELISA kits (catalog numbers MBS2503928 and MBS762158, respectively; Mybiosource, USA) with sensitivities of 0.469 ng/mL and <2 pg/ml, respectively. The free fatty acid (FFA) levels were measured using an FFA quantification colorimetric assay kit (catalog number MB S841629; Mybiosource, USA). The fatty acids composed of >8 carbons were quantified by either colorimetric spectrophotometry (lambda = 570 nm) or fluorometric (excitation/emission = 535/587 nm) methods with detection limits of 56.338 ng/dl of FFA. Although being an adipose tissue bound enzymes, the measurement of these enzymes in plasma is also valid [29].

2.6. Statistical Analysis. SPSS, version 24 for Windows software (SPSS Inc., Chicago, IL, USA), was used for all statistical analyses. The data were presented as the mean ± standard deviation (SD). Analysis of variance (ANOVA) with a post hoc test was used to analyze the differences in multiple comparisons. Pearson correlation coefficient was used to test the relationships among the study variables. P values <0.05 were considered to be statistically significant.

3. Results

3.1. Metabolic Characteristics of Castration as a Rat Model of Andropause. T levels in castrated rats were severely reduced in the Orch group compared to those in the normal control group, as well as decreased ghrelin, decreased insulin, decreased HOMA-IR, and GH levels. Hyperglycemia and reductions in lipid panels including low cholesterol, LDL, VLDL, and TG were also evident (Table 1). The Orch group also demonstrated higher EE, VO_2, VCO_2, and RQ in addition to lower FI, lower body wt (Table 2), and lower visceral fat depots (Figure 1). These metabolic stigmata were corrected by TRT, except for TEE, VO_2, VCO_2, and RQ, which were further increased (Table 2).

3.2. Effect of T on Ghrelin and Insulin Levels, Lipolytic Enzymes, and Substrates' Concentrations. T treatment significantly ($P < 0.001$) increased T levels in normal rats (2 fold) and restored T levels in orchiectomized rats to low points within the normal range. Ghrelin and insulin levels were significantly higher in the T-Sham group compared to those in the sham rats ($P < 0.001$) and in the T-Orch group compared to those in the orchiectomized rats ($P < 0.001$ for ghrelin and $P < 0.05$ for insulin). In addition, there was a significant reduction of ghrelin, insulin, and HOMA-IR values in the Orch group compared to those in the Sham group ($P < 0.001$). Regarding glucose, significant hyperglycemia ($P < 0.05$) was detected in the Orch group, which was normalized by TRT in the T-Orch group. All components of the lipid panel were significantly higher in the Sham-treated group compared to those in normal rats ($P < 0.001$), while in castrated rats there were significant reductions in the levels of LDL, VLDL, and TG and a significant increase in HDL. Components of the lipid profile, except HDL, increased after TRT to levels even higher than those observed in normal rats. Orchiectomy produced insignificant changes regarding HSL and FFA in adipose tissue, while ATGL showed a significant positive correlation with T levels ($r = 0.811$, $P < 0.5$). T treatment in both orchiectomized and sham-operated rats led to the significant increases in ATGL levels

Table 1: Mean ± SD of the biochemical parameters among studied groups ($n = 8$ per each group).

Parameters	Sham	T-Sham	Orch	T-Orch
Testosterone (ng/ml)	7.01 ± 0.91	14.95 ± 1.69 $P < 0.001^a$	0.94 ± 0.17 $P < 0.001^a$	4.41 ± 1.01 $P < 0.05^a$ $P < 0.001^b$
Ghrelin (pg/ml)	574.58 ± 49.11	748.26 ± 69.14 $P < 0.001^a$	233.34 ± 38.12 $P < 0.001^a$	705.26 ± 63.78 $P < 0.05^a$ $P < 0.001^b$
Growth hormone (pg/ml)	3671.67 ± 382.59	3824.17 ± 271.96 $P < 0.554^a$	2356.67 ± 671.54 $P < 0.001^a$	3318.33 ± 714.60 $P < 0.334^a$ $P < 0.001^b$
Insulin (mIU/l)	8.52 ± 0.16	9.95 ± 1.06 $P < 0.05^a$	4.49 ± 0.74 $P < 0.001^a$	7.86 ± 0.38 $P < 0.112^a$ $P < 0.001^b$
Glucose (mmol/l)	6.03 ± 0.28	5.63 ± 0.41 $P < 0.118^a$	6.74 ± 0.57 $P < 0.001^a$	5.88 ± 0.37 $P < 0.549^a$ $P < 0.05^b$
HOMA-IR	2.29 ± 0.12	2.49 ± 0.31 $P < 0.111^a$	1.34 ± 0.24 $P < 0.001^a$	2.05 ± 0.12 $P < 0.074^a$ $P < 0.001^b$
Cholesterol (mmol/l)	3.91 ± 0.24	8.03 ± 0.62 $P < 0.001^a$	3.64 ± 0.79 $P < 0.597^a$	6.92 ± 1.37 $P < 0.001^a$ $P < 0.001^b$
LDL (mmol/l)	2.29 ± 0.14	4.52 ± 0.62 $P < 0.001^a$	1.40 ± 0.62 $P < 0.05^a$	3.83 ± 1.12 $P < 0.05^a$ $P < 0.001^b$
HDL (mmol/l)	0.86 ± 0.08	2.01 ± 0.22 $P < 0.001^a$	1.77 ± 0.16 $P < 0.001^a$	1.80 ± 0.28 $P < 0.001^a$ $P < 0.756^b$
VLDL (mmol/l)	0.29 ± 0.06	0.57 ± 0.13 $P < 0.001^a$	0.07 ± 0.01 $P < 0.001$	0.48 ± 0.08 $P < 0.05^a$ $P < 0.001^b$
TG (mmol/l)	1.44 ± 0.30	2.86 ± 0.64 $P < 0.001^a$	0.33 ± 0.07 $P < 0.001^a$	2.38 ± 0.38 $P < 0.05^a$ $P < 0.001^b$
HSL (pg/ml)	123.84 ± 4.67	107.38 ± 7.67 $P < 0.001^a$	118.52 ± 3.35 $P < 0.088^a$	64.56 ± 3.68 $P < 0.001^a$ $P < 0.001^b$
ATGL (ng/ml)	4.98 ± 0.36	23.21 ± 1.67 $P < 0.001^a$	7.40 ± 0.53 $P < 0.05^a$	25.18 ± 1.07 $P < 0.001^a$ $P < 0.001^b$
FFA (ng/dl)	563.52 ± 6.64	494.34 ± 3.58 $P < 0.001^a$	569.22 ± 10.56 $P < 0.197^a$	501.16 ± 7.06 $P < 0.001^a$ $P < 0.001^b$

Sham = normal control; T-Sham = normal rats treated with IM testosterone undecanoate preparation (100 mg/kg); Orch = orchiectomized rats; T-Orch = orchiectomized rats treated with IM testosterone undecanoate preparation (100 mg/kg); LDL = low-density lipoprotein; HDL = high-density lipoprotein; VLDL = very-low-density lipoprotein; TG = triglycerides; HSL = hormone-sensitive lipase; ATGL = adipocyte triglyceride lipase; FFA = free fatty acids. [a]Significant versus Sham group. [b]Significant versus Orch group.

(3.4-fold and 4.7-fold, respectively) in addition to reduced HSL and FFA levels.

3.3. Indirect Calorimetry and Changes in FI and Body Weight. T-treated normal rats recorded significantly higher VO_2 and much higher VCO_2 and hence the RQ increased in comparison with that in normal control rats. Rats in the T-Orch group showed similar significant increases in both VO_2 and VCO_2 but led to an insignificant change in RQ in comparison with the Orch group (Table 2). T injections increased TEE in normal and orchiectomized rats, and TEE was significantly increased in the T-Sham and T-Orch groups ($P < 0.001$). Interestingly, castrated rats and those in the nontreated group also had increased TEE in comparison with the Sham group ($P < 0.001$). FI and body wt significantly decreased by orchiectomy, and T treatment failed to increase either of them (T-Sham versus Sham; $P < 0.05$). However, the T-Orch group showed significant improvement in FI with insignificant wt changes compared with that in the Orch group (Figure 2).

Table 2: Mean ± SD of indirect calorimetry parameters of all studied groups ($n = 8$/group).

Parameters	Sham	T-Sham	Orch	T-Orch
VO_2 (ml/h/kg)	1182 ± 61	1509 ± 113 $P < 0.001^a$	1397 ± 61 $P < 0.001^a$	1699 ± 94 $P < 0.001^a$ $P < 0.001^b$
VO_2 (ml/h/rat)	456 ± 23	598 ± 44 $P < 0.001^a$	441 ± 19 $P < 0.001^a$	566 ± 31 $P < 0.001^a$ $P < 0.001^b$
VCO_2 (ml/h/kg)	999 ± 50	1364 ± 116 $P < 0.001^a$	1264 ± 60 $P < 0.001^a$	1535 ± 55 $P < 0.001^a$ $P < 0.001^b$
VCO_2 (ml/h/rat)	385 ± 19	541 ± 46 $P < 0.001^a$	399 ± 19 $P < 0.001^a$	511 ± 18 $P < 0.001^a$ $P < 0.001^b$
RQ	0.84 ± 0.01	0.90 ± 0.01 $P < 0.05^a$	0.91 ± 0.06 $P < 0.05^a$	0.91 ± 0.01 $P < 0.05^a$ $P < 0.001^b$
TEE (kcal/h/kg)	5.93 ± 0.18	7.62 ± 0.76 $P < 0.001^a$	6.90 ± 0.10 $P < 0.001^a$	8.11 ± 0.01 $P < 0.001^a$ $P < 0.001^b$
TEE (kcal/h/rat)	2.10 ± 0.35	2.28 ± 0.23 $P < 0.140^a$	2.07 ± 0.03 $P < 0.845^a$	2.44 ± 0.01 $P < 0.05^a$ $P < 0.05^b$
Food intake (g/rat)	38.79 ± 9.59	39.25 ± 3.65 $P < 0.933^a$	12.98 ± 3.35 $P < 0.001^a$	41.15 ± 14.91 $P < 0.662^a$ $P < 0.001^b$
Body weight (g)	385.50 ± 26.80	396.45 ± 43.10 $P < 0.661^a$	315.83 ± 47.43 $P < 0.05^a$	332.83 ± 49.49 $P < 0.05^a$ $P < 0.498^b$

Sham = normal control; T-Sham = normal rats treated with IM testosterone undecanoate preparation (100 mg/kg); Orch = orchiectomized rats; T-Orch = orchiectomized rats treated with IM testosterone undecanoate preparation (100 mg/kg); VO_2 = volume of consumed oxygen; VCO_2 = volume of the produced carbon dioxide; RQ = respiratory quotient (VCO_2/VO_2); TEE = total energy expenditure. [a]Significant versus Sham group. [b]Significant versus Orch group.

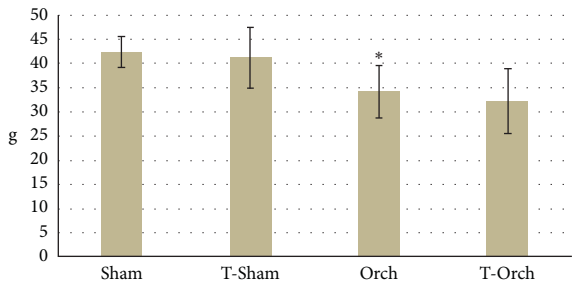


Figure 1: Weights of the visceral fat depots among all studied groups. *Significant versus control (Sham) group.

3.4. Association of T Levels with Other Study Parameters. As shown in Table 3, T levels had a strong positive correlation with ghrelin levels in the T-Sham group ($r = 0.663$, $P < 0.5$) and a strong negative correlation with ghrelin levels in orchiectomized rats ($r = 0.754$, $P < 0.5$). In normal control rats, T levels had a positive correlation with both insulin and glucose levels. A positive correlation also occurred in the T-Sham group in regard to insulin and in the T-Orch group in regard to glucose. Furthermore, T levels also showed a strong positive correlation with TEE in the T-Orch group ($r = 0.858$, $P < 0.5$), while FI showed an only moderate correlation with T levels in the Orch group ($r = 0.584$, $P < 0.5$). T treatment showed a positive correlation with FFA levels in only sham-operated rats ($r = 0.715$, $P < 0.05$). Growth hormone failed to show any significant correlations with T in all groups.

4. Discussion

Andropause and male hypogonadism are biochemical syndromes characterized by a deficiency of T with variable degrees of reduced sensitivity to androgen, which adversely affects many organs and deteriorates the quality of life. Energy homeostasis and body composition are affected by andropause and during TRT to variable degrees [30]. The current study measured EE and lipolytic enzymes under various androgenic conditions and correlated the parameters of indirect calorimetry with some adipokines and substrates.

Increased TEE in castrated rats was consistent with the short-term results reported by Wei et al. [31], which included increased TEE in cats nine days after castration, a postcastration period similar to that in the current study. However, Christoffersen et al. [32] reported insignificant changes in EE between castrated rats and normal controls after two weeks of orchiectomy. The results of Christoffersen

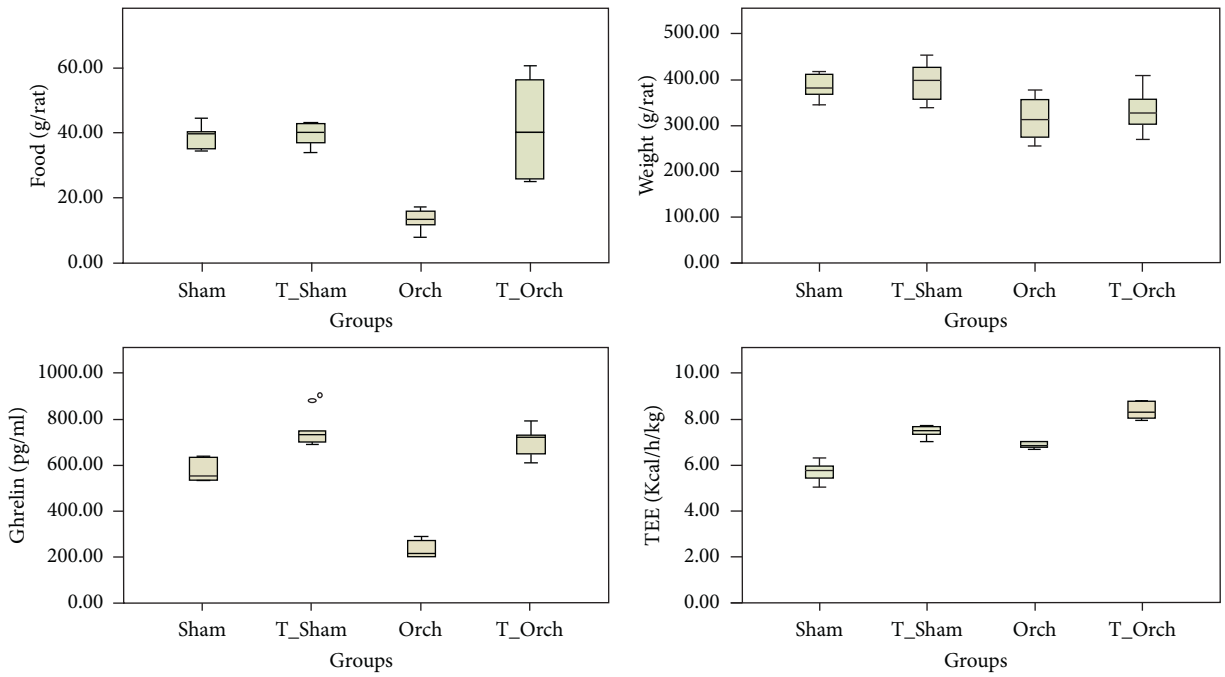


Figure 2: Boxplots of the food intake, weight (wt), ghrelin, and total energy expenditure (TEE) among all studied groups.

Table 3: Correlations of testosterone level with some measured parameters in all studied groups.

Parameters	Sham	T-Sham	Orch	T-Orch
Ghrelin	0.268	0.663*	−0.754*	−0.028
Growth hormone	0.135	0.248	0.710	0.471
Insulin	0.766*	0.644*	0.356	0.467
Glucose	0.613*	0.333	−0.275	0.732*
Cholesterol	−0.186	0.362	0.114	−0.189
Triglycerides	0.700*	0.266	0.211	0.077
HSL	−0.356	0.040	−0.462	0.166
ATGL	0.550	−0.164	0.811*	−0.353
FFA	−0.360	0.715*	0.152	0.422
VO_2	−0.193	0.150	−0.361	−0.548
VCO_2	−0.193	0.151	0.657*	−0.759
RQ	−0.022	0.319	0.761	0.438
TEE	0.280	0.149	0.329	0.858*
Food intake	−0.066	0.026	0.584*	0.023
Body weight	0.355	−0.046	0.431	0.356

Sham = normal control; T-Sham = normal rats treated with IM testosterone undecanoate preparation (100 mg/kg); Orch = orchiectomized rats; T-Orch = orchiectomized rats treated with IM testosterone undecanoate preparation (100 mg/kg); HSL = hormone-sensitive lipase; ATGL = adipocyte triglyceride lipase; FFA = free fatty acid; VO_2 = volume of O_2; VCO_2 = volume of CO_2; RQ = respiratory quotient; TEE = total energy expenditure. *The correlation is significant at the 0.05 level (2-tailed).

and his colleagues regarding glucose and lipids were more consistent with our data. Moreover, Xia et al. [33] reported that castration-induced T deficiency resulted in an increase in glucose levels and a reduction in insulin sensitivity. It is evident that, in Orch group, the insulin level was decreased leading to higher glucose level while HOMA-IR was significantly lower than the control group, i.e., more sensitive to insulin. However, the reduced insulin level is more potent on the glucose level. The reduced GH level in castrated rats might be the cause of low HOMA-IR (Table 1). This is consistent with the finding of Sharma et al. [34, 35] who proved that GH stimulate STAT5- and MEK/ERK-dependent signaling pathways leading to change in the expression of the fat-specific protein 27 (FSP27) and peroxisome proliferator-activated receptor gamma activity (PPARγ) on its promoter. As a result, GH could induce insulin resistance and lipolysis in cultured human adipocytes. Ghrelin levels decreased in one study using castrated rats [36] but increased in another study that used castrated cats [31]. The increased EE and reduced FI in orchiectomized rats produced a negative energy balance resulting in significant wt loss in the Orch group. The mechanisms of increased TEE in orchiectomized rats might include increased ATGL levels (Table 1). Haemmerle et al. [37]

reported that ATGL-knockout mice have decreased the ability to acclimatize to cold exposure. Furthermore, a reduction in ghrelin itself is able to increase EE and decrease FI [38]. De Smet et al. [39] reported that young ghrelin-null mice recorded higher whole-body EE. Conversely, systemic administration of ghrelin precursor is able to reduce RQ in mice, indicating a reduced usage of lipids as a fuel substrate [40]. Another possible mechanism of the increased TEE is the evident low level of GH in the rats of Orch group (Table 1) as a result of low ghrelin. GH is responsible for tissue maintenance in adult organisms. Furthermore, it has been reported that decreased GH signaling is associated with altered profiles of adipokines, enhanced insulin sensitivity, and increased VO_2 and TEE, which may delay the aging process [41].

Short-term intramuscular injection of T undecanoate (100 mg/kg) succeeded in raising T levels to the normal range reported by Rai et al. [42], but not to the same levels of those of the Sham group. The increase in blood lipids, such as total cholesterol, LDL, VLDL, and TG, after TRT was consistent with the results of Host et al. [43] who produced an acute TRT for chemically castrated men. In that study, the patients received gonadotropin-releasing hormone antagonist for thirty days in a randomized double-blind trial and they found that T independently increased VLDL-TG production. On the other hand, Wen and Kang [44] reported that T-treated groups at two different doses demonstrated lower concentrations of total cholesterol and LDL compared to those of castrated controls. Moreover, in that study, they also reported insignificant changes in insulin and glucose levels. Regarding the lipolytic enzymes, it may be concluded that despite the antilipolytic effects of increased insulin/ghrelin and HSL-dependent lipolytic effect of GH [45], T treatment independently induced lipolysis through increasing the levels of ATGL. However, HSL and FFA were decreased, which may have been due to increased levels of insulin [46] and ghrelin [47]. The mechanism of action of T on fat-cell lipolysis includes T effects on catecholamine signaling in adipocytes in which T increases β-adrenergic receptor-mediated transduction signals of lipolysis [48]. Furthermore, T increases ATGL mRNA expression and activity via the activation of PPARγ^2 receptors [12]. Overexpression of ATGL enzyme is able to reduce lipid droplet size and TG storage in adipose tissue [49]. The associated rise of GH might get rid of the accumulation of lipid in adipocytes. Zhang et al. [50] proved that in vivo and in vitro GH treatment of adipocyte cell culture enhances uncoupling protein-1 (UCP1) and adiponectin mRNA expression leading to negative effect on accumulation of lipid in adipose tissue cells.

In the current study, TRT increased TEE, VO_2, and VCO_2 and showed a significant positive correlation with TEE (Table 3) while having an insignificant effect on changes in body wt or mass of fat depots (Figure 2). This finding was consistent with Bauman et al. [51], who reported that TRT, even in chronic use, produces increased TEE and insignificant weight changes in hypogonadal patients with spinal cord injury. Conversely, Santosa et al. [52] reported that short-term repletion of T does not cause any changes in RQ or resting EE. Brown adipose tissue (BAT) is a logical basis for T-induced thermogenesis; however, the common mediator of UCP1, which is the primary thermogenic effector in BAT, is not directly associated with T-induced thermogenesis [53]. Santillo et al. [53] found a significantly low level of UCP-3 at one month after castration and that T administration prevented that reduction. Furthermore, T-induced thermogenesis mechanisms include the modulation of catecholamine-induced lipolysis [48] and increased levels of ATGL enzyme.

Regarding the rise of ghrelin and its positive correlation with T levels in the T-Sham group, it has been reported that T levels positively correlate with ghrelin levels in rats [34], men, and postmenopausal women [54]. Furthermore, total ghrelin levels decrease with the age of men and with declined serum testosterone levels [55]. However, in rats with a negative energy balance, a significant negative correlation exists between serum T and ghrelin [56]. Although the administration of T increased ghrelin and insulin in sham-operated rats, FI and body wt did not significantly change, indicating that T failed to increase the appetite and body wt of the rats. Skarra et al. [57] reported that, in a letrozole-induced polycystic ovary rat model, wt gain occurred secondarily to increased insulin levels and not directly to a hyperandrogenemia state. Moreover, Iwasa et al. [58] found that T-induced facilitative actions on body wt, FI, and adiposity are controlled by the coadministration of estradiol. In other words, T has inhibitory effects on wt gain and FI in the absence of estradiol supplementation. Recently, it was reported that neonatal testosterone helps in the programming of sexual differences in the hypothalamic-pituitary-gonadal-orexinergic axis, which affects serum levels of orexins (orexin A and orexin B) [59].

The current indirect calorimetry study of T-Sham rats revealed significant increases in TEE and RQ from the level of mixed substrates used to that of greater carbohydrate utilization with an insignificant reduction in blood glucose levels ($P = 0.118$). Lynn et al. [60] measured daily EE (DEE) of captive male dark-eyed juncos using a double-labeled water technique and studied the effect of subcutaneous T injection in normal males compared to controls. The results showed that elevated T levels increase some components of DEE, such as the thermic effect of locomotor activity, and lowered the contribution of others, such as resting EE. Total DEE was not higher in the T-treated males compared to that in the controls. In another investigation, Braun et al. [61] created three different androgenic levels in exercising men and found that despite the marked variation in T levels, carbohydrate oxidation, the rate of glucose disappearance, and muscular glycogen use each were highly similar across the three androgenic levels. The setting of study by Braun and colleagues was much different from the current study since we investigated resting rats. Additionally, Keller et al. [62], who investigated the relationship of androgen levels to substrate use, RQ, and EE, found that T levels do not correlate with resting EE or substrate utilization.

The effects of T on adipose tissue lipolytic enzymes may provide an explanation for the effect of T on energy homeostasis and body wt. ATGL is the initial enzyme that catalyzes TG hydrolysis into diacylglycerol (DG) inside fat droplets of adipocytes, which liberates one fatty acid. The

subsequent increase in the liberation of glycerol is associated with an upregulation of the cellular enzymes essential for the oxidation of fatty acids and the release of energy [63]. The second key enzyme is HSL, which catalyzes the further hydrolysis of DG and minor quantities of TG. HSL is localized to the cytoplasm of cells, while ATGL is present on the lipid droplets and in the cytoplasm of adipocytes [64]. The current study showed increased blood levels of ATGL and reduced levels of HSL and FFA in both sham and orchiectomized rats following T administration. This effect might reduce the mass of adipose tissue and increase EE [63]. Morak et al. [65] found that a deficiency of ATGL in BAT causes a marked reduction in the expression of other esterolytic and lipolytic enzymes that are responsible for energy production. In contrast, they found that HSL deficiency causes insignificant effects. Also, a study using ATGL-knockout (AAKO) mice confirmed the potential relationship of EE with AGTL. Schoiswoh et al. [66] found that EE and VO_2 were markedly decreased in fasted AAKO mice. Furthermore, Miyoshi et al. [49] reported that the overexpression of ATGL enzymes significantly reduces lipid droplets size and decreases TG storage, while the overexpression of HSL fails to change the size of lipid droplets or to alter TG storage in adipocytes.

Despite our findings, the current study had some limitations. The main limitation was the lack of accurate body composition analyses such as MRI to correlate our findings regarding TEE with changes in various boy compartments. Another limitation was the measurement of TEE rather than REE. Furthermore, the absence of the measurement of the luteinizing hormone and investigations of the molecular mechanisms at the intracellular level is a considerable limitation.

5. Conclusions

In this rat model of hypogonadism, low ghrelin/GH axis might play a role in the elevation of TEE, increased RQ, increased ATGL, hyperglycemia, low insulin, and reductions of food intake and body weight. T administration in orchiectomized rats significantly increased orexigenic mediators such as ghrelin and insulin without inducing any significant changes in wt. The mechanism for this finding might be the increase in TEE and stimulation of lipolysis through the ATGL enzyme. The associated rise of GH might help in interference with accumulation of lipid in adipose tissue. Apart from the effect on GH and HOMA-IR, T-Sham showed similar effects of T supplementation.

Data Availability

The indirect calorimetry and biochemical data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The study protocol was approved by the ethics committee at the College of Applied Medical Sciences, King Saud University.

Conflicts of Interest

All authors declare that they have no conflicts of interest, either financial or nonfinancial issues.

Authors' Contributions

MMAA contributed to the practical work, indirect calorimetry, and writing of the manuscript. AA participated in the study design and indirect calorimetry. ME contributed to the statistical analysis. SR performed the lab work. MBa-nalyzed the lab results and wrote the manuscript.

Acknowledgments

The authors gratefully acknowledge the Deanship of Scientific Research at King Saud University, Riyadh, Kingdom of Saudi Arabia, for funding this research (Research Group no. 1439-78).

References

[1] D. M. Kelly and T. H. Jones, "Testosterone: a vascular hormone in health and disease," *Journal of Endocrinology*, vol. 217, no. 3, pp. R47–R71, 2013.

[2] M. G. Giannoulis, F. C. Martin, K. S. Nair, A. M. Umpleby, and P. Sonksen, "Hormone replacement therapy and physical function in healthy older men. Time to talk hormones?," *Endocrine Reviews*, vol. 33, no. 3, pp. 314–377, 2012.

[3] T. Iwasa, T. Matsuzaki, M. Yiliyasi, K. Yano, and M. Irahara, "The effects of chronic testosterone administration on body weight, food intake, and fat weight were age-dependent," *Steroids*, vol. 127, pp. 18–23, 2017.

[4] D. Jafaripour, H. Khazali, H. Rokni, and H. Alipanah, "Effect of interaction between testosterone and morphine on serum ghrelin concentration in sheep fed on different dietary energy levels," *International Journal of Endocrinology & Metabolism*, vol. 10, no. 3, pp. 558–562, 2012.

[5] J. I. Bertucci, A. M. Blanco, L. F. Canosa, and S. Unniappan, "Estradiol and testosterone modulate the tissue-specific expression of ghrelin, ghs-r, goat and nucb2 in goldfish," *General and Comparative Endocrinology*, vol. 228, pp. 17–23, 2016.

[6] P. Alberts, B. G. Johansson, and R. A. McArthur, "Characterization of energy expenditure in rodents by indirect calorimetry," *Current Protocols in Neuroscience*, vol. 36, no. 1, p. 9, 2006.

[7] J. R. Speakman, "Measuring energy metabolism in the mouse—theoretical, practical, and analytical considerations," *Frontiers in Physiology*, vol. 4, p. 34, 2013.

[8] J. R. S. Arch, D. Hislop, S. J. Y. Wang, and J. R. Speakman, "Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals," *International Journal of Obesity*, vol. 30, no. 9, pp. 1322–1331, 2006.

[9] X. Xian, A. Quach, D. Bridgeman, F. Tsow, E. Forzani, and N. Tao, "Personalized indirect calorimeter for energy expenditure (EE) measurement," *Global Journal of Obesity Diabetes and Metabolic Syndrome*, vol. 2, no. 1, pp. 004–008, 2015.

[10] R. C. Noland, "Exercise and regulation of lipid metabolism," *Progress in Molecular Biology and Translational Science*, vol. 135, pp. 39–74, 2015.

[11] K. J. McInnes, L. B. Smith, N. I. Hunger, P. T. K. Saunders, R. Andrew, and B. R. Walker, "Deletion of the androgen receptor in adipose tissue in male mice elevates retinol binding protein 4 and reveals independent effects on visceral fat mass and on glucose homeostasis," *Diabetes*, vol. 61, no. 5, pp. 1072–1081, 2012.

[12] J. Karbowska and Z. Kochan, "Fat-reducing effects of dehydroepiandrosterone involve upregulation of ATGL and HSL expression, and stimulation of lipolysis in adipose tissue," *Steroids*, vol. 77, no. 13, pp. 1359–1365, 2012.

[13] L. Nikolaenko, Y. Jia, C. Wang et al., "Testosterone replacement ameliorates nonalcoholic fatty liver disease in castrated male rats," *Endocrinology*, vol. 155, no. 2, pp. 417–428, 2014.

[14] H. Zang, M. Rydén, K. Wåhlen, K. Dahlman-Wright, P. Arner, and A. L. Hirschberg, "Effects of testosterone and estrogen treatment on lipolysis signaling pathways in subcutaneous adipose tissue of postmenopausal women," *Fertility and Sterility*, vol. 88, no. 1, pp. 100–106, 2007.

[15] A. E. Arrant, N. L. Schramm-Sapyta, and C. M. Kuhn, "Use of the light/dark test for anxiety in adult and adolescent male rats," *Behavioural Brain Research*, vol. 256, pp. 119–127, 2013.

[16] K. Cui, R. Li, Y. Zhang et al., "Androgen may improve erectile function in castrated rats by regulating the ERK1/2 pathway," *Zhonghua Nan Ke Xue*, vol. 21, no. 11, pp. 967–972, 2015.

[17] G. R. Ward and A. A. Abdel-Rahman, "Effect of testosterone replacement or duration of castration on baroreflex bradycardia in conscious rats," *BMC Pharmacology*, vol. 5, no. 1, p. 9, 2005.

[18] A. M. Molina, M. R. Moyano, J. M. Serrano-Rodriguez, N. Ayala, A. J. Lora, and J. M. Serrano-Caballero, "Analyses of anaesthesia with ketamine combined with different sedatives in rats," *Veterinarni Medicina*, vol. 60, no. 7, pp. 368–375, 2015.

[19] A. I. Idris, "Ovariectomy/orchidectomy in rodents," *Methods in Molecular Biology*, vol. 816, pp. 545–551, 2012.

[20] S. Moghadami, M. Jahanshahi, H. Sepehri, and H. Amini, "Gonadectomy reduces the density of androgen receptor-immunoreactive neurons in male rat' hippocampus: testosterone replacement compensates it," *Behavior and Brain Functions*, vol. 12, no. 1, pp. 5–10, 2016.

[21] M. H. Tschöp, J. R. Speakman, J. R. S. Arch et al., "A guide to analysis of mouse energy metabolism," *Nature Methods*, vol. 9, no. 1, pp. 57–63, 2011.

[22] P. C. Even and N. A. Nadkarni, "Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 303, no. 5, pp. R459–R476, 2012.

[23] R. W. Njoroge, B. N. Macharia, D. J. Sawe, and G. K. Maiyoh, "Effects of crude kerosene on testosterone levels, aggression and toxicity in rat," *Toxicology Reports*, vol. 2, pp. 175–183, 2015.

[24] S. S. Ali, E. A. Hamed, N. N. Ayuob, A. Shaker Ali, and M. I. Suliman, "Effects of different routes of nicotine administration on gastric morphology and hormonal secretion in rats," *Experimental Physiology*, vol. 100, no. 8, pp. 881–895, 2015.

[25] L. Ding, Z. Qu, J. Chi et al., "Effects of preventative application of metformin on bile acid metabolism in high fat-fed/streptozotocin-diabetic rats," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 5, pp. 5450–5456, 2015.

[26] C. M. M. de Cordova and M. M. de Cordova, "A new accurate, simple formula for LDL-cholesterol estimation based on directly measured blood lipids from a large cohort," *Annals of Clinical Biochemistry*, vol. 50, no. 1, pp. 13–19, 2013.

[27] Y. Chen, X. Zhang, B. Pan et al., "A modified formula for calculating low-density lipoprotein cholesterol values," *Lipids in Health and Disease*, vol. 9, no. 1, p. 52, 2010.

[28] D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher, and R. C. Turner, "Homeostasis model assessment: insulin resistance and ?-cell function from fasting plasma glucose and insulin concentrations in man," *Diabetologia*, vol. 28, no. 7, pp. 412–419, 1985.

[29] S. Xu, L. Li, G. Yang, M. Yang, S. Li, and H. Liu, "Elevated adipose triglyceride lipase in newly diagnosed type 2 diabetes mellitus with hypertension," *The American Journal of the Medical Sciences*, vol. 342, no. 6, pp. 452–455, 2011.

[30] D. A. Gruenewald and A. M. Matsumoto, "Testosterone supplementation therapy for older men: potential benefits and risks," *Journal of the American Geriatrics Society*, vol. 51, no. 1, pp. 101–115, 2003.

[31] A. Wei, A. J. Fascetti, K. Kim et al., "Early effects of neutering on energy expenditure in adult male cats," *PLoS One*, vol. 9, no. 2, p. e89557, 2014.

[32] B. Christoffersen, K. Raun, O. Svendsen, C. Fledelius, and V. Golozoubova, "Evalution of the castrated male Sprague-Dawley rat as a model of the metabolic syndrome and type 2 diabetes," *International Journal of Obesity*, vol. 30, no. 8, pp. 1288–1297, 2006.

[33] F. Xia, X. Xu, H. Zhai et al., "Castration-induced testosterone deficiency increases fasting glucose associated with hepatic and extra-hepatic insulin resistance in adult male rats," *Reproductive Biology and Endocrinology*, vol. 11, no. 1, p. 106, 2013.

[34] R. Sharma, Q. Luong, V. M. Sharma et al., "Growth hormone controls lipolysis by regulation of FSP27 expression," *Journal of Endocrinology*, vol. 239, no. 3, pp. 289–301, 2018.

[35] V. M. Sharma, E. T. Vestergaard, N. Jessen et al., "Growth hormone acts along the PPARγ-FSP27 axis to stimulate lipolysis in human adipocytes," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 316, no. 1, pp. E34–E42, 2019.

[36] S. El-Sawy and A. Hashish, "Role of testosterone replacement therapy on plasma ghrelin level before and after orchiectomy in male albino rats," *Bulletin of Egyptian Society for Physiological Sciences*, vol. 28, no. 1, pp. 213–224, 2008.

[37] G. Haemmerle, A. Lass, R. Zimmermann et al., "Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase," *Science*, vol. 312, no. 5774, pp. 734–737, 2006.

[38] J. S. Zakhari, E. P. Zorrilla, B. Zhou, A. V. Mayorov, and K. D. Janda, "Oligoclonal antibody targeting ghrelin increases energy expenditure and reduces food intake in fasted mice," *Molecular Pharmaceutics*, vol. 9, no. 2, pp. 281–289, 2012.

[39] B. De Smet, I. Depoortere, D. Moechars et al., "Energy homeostasis and gastric emptying in ghrelin knockout mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 316, no. 1, pp. 431–439, 2006.

[40] W. Zhang, A. Majumder, X. Wu, and M. W. Mulholland, "Regulation of food intake and body weight by recombinant proghrelin," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 297, no. 6, pp. E1269–E1275, 2009.

[41] A. Bartke, R. Westbrook, L. Sun, and M. Ratajczak, "Links between growth hormone and aging," *Endokrynologia Polska*, vol. 64, no. 1, pp. 46–52, 2013.

[42] J. Rai, S. N. Pandey, and R. K. Srivastava, "Testosterone hormone level in albino rats following restraint stress of long

duration," *Journal of the Anatomical Society of India*, vol. 53, no. 1, pp. 17–19, 2004.

[43] C. Host, L. C. Gormsen, B. Christensen et al., "Independent effects of testosterone on lipid oxidation and VLDL-TG production: a randomized, double-blind, placebo-controlled, crossover study," *Diabetes*, vol. 62, no. 5, pp. 1409–1416, 2013.

[44] T. Wen and D. Kang, "Effects of testosterone replacement therapy on glucose and lipid metabolism in middle-aged and elderly high-fat-fed male rats," *Biomedical Research*, vol. 28, no. 7, pp. 3048–3052, 2017.

[45] A. Khieokhajonkhet, G. Kaneko, Y. Hirano, L. Wang, and H. Ushio, "Different effects of growth hormone and fasting on the induction patterns of two hormone-sensitive lipase genes in red seabream Pagrus major," *General and Comparative Endocrinology*, vol. 236, pp. 121–130, 2016.

[46] S. Meijssen, M. C. Cabezas, C. G. Ballieux, R. J. Derksen, S. Bilecen, and D. W. Erkelens, "Insulin mediated inhibition of hormone sensitive lipase activity in vivo in relation to endogenous catecholamines in healthy subjects," *Journal of Clinical Endocrinology & Metabolism*, vol. 86, no. 9, pp. 4193–4197, 2001.

[47] A. Rodríguez, "Novel molecular aspects of ghrelin and leptin in the control of adipobiology and the cardiovascular system," *Obesity Facts*, vol. 7, no. 2, pp. 82–95, 2014.

[48] P. Amer, "Effects of testosterone on fat cell lipolysis. Species differences and possible role in polycystic ovarian syndrome," *Biochimie*, vol. 87, no. 1, pp. 39–43, 2005.

[49] H. Miyoshi, J. W. Perfield II, M. S. Obin, and A. S. Greenberg, "Adipose triglyceride lipase regulates basal lipolysis and lipid droplet size in adipocytes," *Journal of Cellular Biochemistry*, vol. 105, no. 6, pp. 1430–1436, 2008.

[50] Y. Zhang, H. Yu, P. Gao et al., "The effect of growth hormone on lipid accumulation or maturation in adipocytes," *Cellular Physiology and Biochemistry*, vol. 39, no. 6, pp. 2135–2148, 2016.

[51] W. A. Bauman, M. F. La Fountaine, C. M. Cirnigliaro, S. C. Kirshblum, and A. M. Spungen, "Lean tissue mass and energy expenditure are retained in hypogonadal men with spinal cord injury after discontinuation of testosterone replacement therapy," *The Journal of Spinal Cord Medicine*, vol. 38, no. 1, pp. 38–47, 2015.

[52] S. Santosa, S. Khosla, L. K. McCready, and M. D. Jensen, "Effects of estrogen and testosterone on resting energy expenditure in older men," *Obesity*, vol. 18, no. 12, pp. 2392–2394, 2010.

[53] A. Santillo, R. Monteforte, P. De Lange, A. Lanni, P. Farina, and G. C. Baccari, "Dimorphic expression of uncoupling protein-3 in golden hamster harderian gland: effects of castration and testosterone administration," *Journal of Cellular Physiology*, vol. 215, no. 2, pp. 481–487, 2008.

[54] Y. Greenman, V. Rouach, R. Limor, S. Gilad, and N. Stern, "Testosterone is a strong correlate of ghrelin levels in men and postmenopausal women," *Neuroendocrinology*, vol. 89, no. 1, pp. 79–85, 2009.

[55] J. Kozakowski, M. Rabijewski, and W. Zgliczyński, "Ghrelin—growth hormone releasing and orexigenic hormone in men declines with age, insulin and with a decrease in testosterone concentration," *Neuro Endocrinology Letters*, vol. 29, no. 1, pp. 100–106, 2008.

[56] H. M. Abou Heif, M. M. Deif, and H. K. Abdel Aziz, "Effect of food restriction on ghrelin in adult male rats and its relation to male reproductive hormones," *Andrologia*, vol. 42, no. 2, pp. 97–105, 2010.

[57] D. V. Skarra, A. Hernández-Carretero, A. J. Rivera, A. R. Anvar, and V. G. Thackray, "Hyperandrogenemia induced by letrozole treatment of pubertal female mice results in hyperinsulinemia prior to weight gain and insulin resistance," *Endocrinology*, vol. 158, no. 9, pp. 2988–3003, 2017.

[58] T. Iwasa, T. Matsuzaki, K. Yano et al., "The effects of chronic testosterone administration on body weight, food intake, and adipose tissue are changed by estrogen treatment in female rats," *Hormones and Behavior*, vol. 93, pp. 53–61, 2017.

[59] N. I. Cataldi, V. A. Lux-Lantos, and C. Libertun, "Perinatal programming of the orexinergic (hypocretinergic) system in hypothalamus and anterior pituitary by testosterone," *Peptides*, vol. 99, pp. 117–127, 2018 Jan.

[60] S. E. Lynn, A. M. Houtman, W. W. Weathers, E. D. Ketterson, and V. Nolan, "Testosterone increases activity but not daily energy expenditure in captive male dark-eyed juncos, Junco hyemalis," *Animal Behaviour*, vol. 60, no. 5, pp. 581–587, 2000.

[61] B. Braun, L. Gerson, T. Hagobian, D. Grow, and S. R. Chipkin, "No effect of short-term testosterone manipulation on exercise substrate metabolism in men," *Journal of Applied Physiology*, vol. 99, no. 5, pp. 1930–1937, 2005.

[62] J. L. Keller, P. R. Casson, and M. J. Toth, "Relationship of androgens to body composition, energy and substrate metabolism and aerobic capacity in healthy, young women," *Steroids*, vol. 76, no. 12, pp. 1247–1251, 2011.

[63] R. B. S. Harris, "Direct and indirect effects of leptin on adipocyte metabolism," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1842, no. 3, pp. 414–423, 2014.

[64] M. Schweiger, R. Schreiber, G. Haemmerle et al., "Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism," *Journal of Biological Chemistry*, vol. 281, no. 52, pp. 40236–40241, 2006.

[65] M. Morak, H. Schmidinger, G. Riesenhuber et al., "Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) deficiencies affect expression of lipolytic activities in mouse adipose tissues," *Molecular & Cellular Proteomics*, vol. 11, no. 12, pp. 1777–1789, 2012.

[66] G. Schoiswohl, M. Stefanovic-Racic, M. N. Menke et al., "Impact of reduced ATGL-mediated adipocyte lipolysis on obesity-associated insulin resistance and inflammation in male mice," *Endocrinology*, vol. 156, no. 10, pp. 3610–3624, 2015.

Hindawi
International Journal of Endocrinology
Volume 2019, Article ID 6718761, 7 pages
https://doi.org/10.1155/2019/6718761

Research Article

Causes and Metabolic Consequences of Gynecomastia in Adult Patients

Ralitsa Robeva, Atanaska Elenkova, and Sabina Zacharieva

USHATE "Acad. Iv. Penchev", Department of Endocrinology, Faculty of Medicine, Medical University-Sofia, Sofia, Bulgaria

Correspondence should be addressed to Ralitsa Robeva; rali_robeva@yahoo.com

Received 7 May 2019; Revised 21 August 2019; Accepted 6 September 2019; Published 3 October 2019

Guest Editor: Antonio Mancini

Background. Gynecomastia (GM) is a benign enlargement of male breast due to glandular tissue proliferation. GM is a symptom of systemic or local hormonal disturbances, which could be associated with functional changes or pathological conditions. However, the long-lasting steroid imbalance in men with GM might exert negative influence on their metabolic health. *Methods*. A total of 110 adult men with symptomatic GM were included in the present retrospective cross-sectional study. Anthropometric, metabolic, and hormonal data of the patients were collected. *Results*. In almost 64% of GM patients, the underlying pathological condition was identified. Moreover, the development of GM was among the primary symptoms leading to the proper diagnosis in more than 40% of hypogonadal patients. The prevalence of metabolic syndrome (MS) was 53%; the highest prevalence of MS was found in patients with medication-induced GM and in the hypogonadal patients, whereas the lowest prevalence was observed in men with idiopathic postpubertal GM despite the similar degree of obesity. The lower testosterone levels were associated with more unfavorable lipid profile in the GM patients. *Conclusion*. The development of GM in adults might be an important symptom of an underlying gonadal disease. Moreover, it could be associated with an increased risk of metabolic disturbances. Our results support the need of detailed laboratory and hormonal investigations in patients with GM including targeted screening for metabolic disturbances. Further longitudinal studies are needed to evaluate the long-term consequences of sex hormones imbalance on cardiovascular morbidity and mortality in adults with GM.

1. Introduction

Gynecomastia (GM) is a benign unilateral or bilateral enlargement of male breast, which results from glandular tissue proliferation [1, 2]. The histology and growth potential of mammary gland is similar in both sexes during early development [3]. After the onset of puberty, synergetic effects of growth hormone, insulin-like growth factor 1, and estrogens ensure complete breast development in females, whereas increased androgens suppress breast development in males [4, 5]. GM might develop at any age in case of a hormonal imbalance [3]. Its prevalence peaks during mid puberty and decreases thereafter, in parallel with the completion of sexual maturation and normalization of estrogen to androgen ratio [6, 7]. The temporary breast enlargement in adolescent boys is usually benign, but a thorough physical examination is required to exclude additional signs and symptoms suggesting of an underlying disorder [8].

In the adulthood, the newly developed GM is often a symptom of a pathological condition [3]. Increased estrogens, decreased androgens, changes in sex hormone-binding globulin levels, and steroid receptor defects might be involved in the pathogenesis of GM associated with different diseases, such as hormone-secreting tumors, hypogonadism, hyperprolactinemia, hyperthyroidism, chronic liver impairment, and androgen-resistance syndromes [9]. Approximately one-quarter of the adult men develop idiopathic GM in the absence of apparent hormonal disturbances, probably, because of local hormonal changes or tissue receptor alterations [3, 10].

Because GM is a symptom of systemic or local hormonal disturbances, the detailed endocrine investigation of the patients seems reasonable despite the existing controversy on this topic [11]. Moreover, the long-lasting steroid imbalance in men with GM might exert negative influence on their metabolic profile, which could be related to increased

cardiovascular risk. Men with overt atherosclerotic disease and lower testosterone to estradiol ratio have shown increased risk for major cardiovascular events in comparison to male individuals with normal sex hormone ratio [12]. However, the possible associations between the presence of GM and the development of metabolic disturbances have not been thoroughly investigated. Therefore, the present retrospective study aims to investigate the causes for GM in adult patients attending endocrinology clinic and to estimate the prevalence of metabolic syndrome in GM of pathological or idiopathic origin.

2. Methods

2.1. Participants and Study Protocol. Medical records of all adult Caucasian men referred to Endocrinology Department, Medical University-Sofia, because of symptomatic GM or diagnosed with GM during clinical evaluation in the period 2008–2018 were initially selected ($n = 144$). A total of 22 men were excluded because of pseudogynecomastia, whereas another 12 men were excluded because of GM spontaneous regression. Thus, 110 patients were finally included in the presented retrospective cross-sectional study. Data concerning GM type (unilateral or bilateral) and duration, anthropometric (height, weight, waist circumference/WC/) and biochemical characteristics (blood count, fasting glucose/Glu/, creatinine, liver enzymes, high-density lipoprotein cholesterol/HDL/, triglycerides/TG/, and total cholesterol), blood pressure, use of medication, anabolic steroids and nutritional supplements, concomitant diseases, and hormonal values (testosterone/T/, luteinizing hormone/LH/, follicle-stimulating hormone/FSH/, estradiol/E2/, thyroid-stimulating hormone/TSH/, and prolactin/Prol/) were collected retrospectively.

A presence of firm subareolar glandular tissue determined by palpation was considered GM, as in other studies [6, 11]. In case of uncertainty or suspicion of mammary gland carcinoma, an ultrasound and/or radiological investigation was accomplished additionally ($n = 64$). Alpha-fetoprotein and human chorionic gonadotropin were also investigated, if tumor origin of GM was suspected. Testicular volume was determined by palpation; however, in case of hypogonadism, hyperestrogenemia, or suspected testicular tumor, testicular ultrasound was also performed ($n = 47$).

The biochemical parameters were measured enzymatically by an automatic analyzer (Cobas Mira Plus; Hoffmann La Roche). Low-density lipoprotein cholesterol (LDL) was calculated according to the Friedewald equation. TSH ($n = 91$), T ($n = 96$), LH ($n = 74$), FSH ($n = 74$), E2 ($n = 46$), and prolactin ($n = 92$) levels were determined in the participants. Testosterone and estradiol levels were measured with commercially available DELFIA kits (DELFIA; Perkin Elmer, Wallac Oy, Turku, Finland). Analytical sensitivity for the testosterone was 0.3 nmol/l, and for estradiol, it was 0.05 nmol/l; the intra-assay variations of the kits were 5.6% and 5.9%, respectively, whereas the interassay variations were 6.8% for testosterone and 5.1% for estradiol. Gonadotropins, prolactin, and TSH were measured with IRMA kits (Beckman-Coulter Inc., France/Czech). For FSH and LH, the intra-assay variations were 4.05% and 7.33%, and interassay variations were 8.2% and 8.42%, respectively, whereas the analytical sensitivity was 0.17 mIU/ml for FSH and 0.16 mIU/ml for LH. For the serum prolactin and TSH, the analytical sensitivity was 15.2 mIU/l and 0.04 mIU/l, intra-assay CV ≤ 2.8% and CV ≤ 3.7%, and interassay CV ≤ 8% and CV ≤ 8.6%, respectively.

In case of consistent symptoms and repeatedly low testosterone levels (<11 nmol/l), additional tests were made to prove the hypogonadism and to found out the possible reasons (free testosterone calculation, gonadotropins to discriminate primary/LH, FSH—above the upper reference range/or secondary hypogonadism/LH, FSH—below the upper reference range/, MRT of the pituitary, karyotype etc.) according to the published guidelines [13–15]. In case of persistent hyperprolactinemia, the diagnostic approach was focused on exclusion of secondary causes such as medication or nutritional supplements, hypothyroidism, liver or kidney dysfunction, other stress factors, and pituitary mass (MRT) [16, 17].

2.2. Obesity and Metabolic Syndrome Criteria. Body mass index (BMI) was calculated according to the well-known formula: BMI = weight (kg)/height (m^2). Metabolic syndrome was diagnosed in the presence of any three of the following five criteria: (1) elevated waist circumference of ≥94 cm; (2) elevated triglycerides of ≥1.7 mmol/l or drug treatment for elevated triglycerides; (3) decreased HDL-ch of <1.03 mmol/l or drug treatment for decreased HDL-ch; (4) elevated fasting glucose of ≥5.6 mmol/l or drug treatment for elevated glucose; and (5) systolic blood pressure of ≥130 mmHg and/or diastolic blood pressure of ≥85 mmHg and/or antihypertensive therapy [18].

2.3. Missing Data. In case of missing waist circumference values, a BMI ≥ 30 were used as a marker of obesity (http://www.idf.org). If the available data did not allow a definitive MS positive or negative classification, then the patients ($n = 7$) were excluded from analysis as in other studies [19]. In patients with clear medication-induced GM, testosterone and prolactin levels were rarely measured, and thus, testosterone and prolactin values are available in 96 and 92 of the patients, respectively. Estradiol levels were measured usually in case of suspected gonadal tumor and were available in only 46 patients.

2.4. Statistics. Descriptive statistics and frequency analysis were used where appropriate. Comparisons between subjects were performed using chi-square or Fisher's exact test for categorical variables. Correlation analyses were performed with Spearman's rank correlation test. Comparisons between groups were made through Mann–Whitney or Kruskal–Wallis test. p values of ≤0.05 were accepted as statistically significant. The data were analyzed by MedCalc Software for Windows (version 15.0; MedCalc Software, Ostend, Belgium).

3. Results

A total of 110 males (18–91 years; median, 32.5 years) with GM were included in the study. In 14.5% of the patients, the GM was unilateral, whereas in the other 85.5%, it was bilateral. The duration of the GM varied between one month and 20 years (median, 2 years). A total of 44 of men (40%) were with hypogonadism. Hypergonadotropic hypogonadism was found in 14 patients; 6 of them being with mosaic or complete Klinefelter syndrome. Hypogonadotropic hypogonadism was established in 30 patients; in 12 of them, the gonadotropin and testosterone decrease was caused by prolactinoma, and in 5 of them, the decrease was caused by other pituitary formations. A total of 4 patients were with Kallmann syndrome, other 5 patients were with idiopathic hypogonadotropic hypogonadism, and 1 patient was diagnosed with Bardet–Biedl syndrome. A total of 22 patients (20.0%) were with medication-induced GM due to the use of spironolactone, bicalutamide, dutasteride, chemotherapy, ACE-inhibitors, allopurinol, statins, anabolic steroids, and alcohol, but in some patients on multitherapy, the specific provoking drug could not be identified. In four patients, the GM results from other reasons: leidigoma/$n = 2$/, hepatic injury/$n = 1$/, and thyroid dysfunction/$n = 1$/. In the other 40 patients (36.4%), the cause for GM was not identified. In 18 of these patients, GM appeared during puberty/persistent pubertal GM/, whereas in the other 22 patients, the GM developed in the adulthood/idiopathic postpubertal GM/. Hyperestrogenemia was found in 20 of these patients (50%), whereas mild hyperprolactinemia (prolactin less than twice above the upper referent limit) was found in 10 of them (25%); however, no cause for these alterations had been established. A total of 6 patients (5.5%) admitted use of anabolic steroids, and/or nutritional supplements, but a clear temporal relationship with the development of GM was proven in only two of them. Only four patients admitted alcohol use, whereas 24.5% of the men were current smokers or exsmokers. In 16.4% of patients, more than one cause for GM might be suspected; thus, the most likely cause according to the temporal relationships was accepted as the leading etiological factor. For instance, a patient with a congenital secondary hypogonadism on androgen replacement therapy started to use additionally anabolic steroids to increase his muscle mass and developed symptomatic GM. The prevalence of different GM causes and hormonal parameters of the patients are shown on Table 1.

Only five of the hypogonadal patients (11.4%) were on testosterone treatment, whereas others did not receive hormone replacement therapy because of newly diagnosed disease or noncompliance to the therapy. In 20 of the hypogonadal patients (45.5%), GM was among the primary symptoms leading to the proper diagnosis. A history of cryptorchidism was present in 10 patients (9.1%) with hypogonadism, but in none of the eugonadal patients ($p < 0.001$). All men with history of cryptorchidism were with bilateral GM.

Medication-induced GM was a rare reason for GM in patients younger than 40 years (2.8%) but turned out to be the most frequent cause for the complaints after the same age (55.6%).

The prevalence of family history for diabetes and hypertension among GM patients was 16.4% and 23.6%, respectively; no statistically significant differences between eugonadal and hypogonadal groups were established ($p > 0.05$ for both). In eugonadal idiopathic GM patients and hypogonadal GM patients, the HDL-cholesterol and LDL-cholesterol levels were similar ($p > 0.05$ for both), but the triglyceride concentrations were significantly increased in hypogonadal compared with eugonadal men (1.36 [0.42–4.06] vs. 0.94 [0.42–4.11]; $p = 0.013$). The prevalence of hypertension, metabolic syndrome, and diabetes type 2 differed significantly among different groups of patients with GM, whereas the prevalence of obesity was similar among them (Table 2). In patients younger than 40 years, the prevalence of MS was 20% in the idiopathic GM group, 43.8% in men with persistent pubertal GM, 47.6% in patients with a secondary hypogonadism, and 50% in patients with primary hypogonadism. The clinical and hormonal characteristics of patients with GM and MS are shown on Table 3.

In the whole GM group, the levels of testosterone were positively related to the hemoglobin ($r = 0.423$; $p < 0.001$) and hematocrit ($r = 0.471$; $p < 0.001$) levels, as well as negatively associated with the total cholesterol ($r = -0.225$; $p = 0.032$), LDL cholesterol ($r = -0.252$; $p = 0.029$), and triglyceride concentrations ($r = -0.226$; $p = 0.032$). The testosterone levels were not associated with the age, BMI, waist circumference, blood pressure levels, or other metabolic parameters of the patients ($p > 0.05$ for all). Estradiol to testosterone ratio was positively related to leucocytes ($r = 0.500$; $p < 0.001$), waist circumference ($r = 0.365$; $p = 0.028$), and triglycerides ($r = 0.500$; $p = 0.001$). In males with idiopathic GM, testosterone levels were significantly associated with hemoglobin ($r = 0.360$; $p = 0.031$), BMI ($r = -0.335$; $p = 0.046$), and HDL levels ($r = 0.601$; $p = 0.001$), whereas in other GM patients, the androgen concentration was related to the total ($r = -0.317$; $p = 0.015$) and LDL ($r = -0.353$; $p = 0.015$) cholesterol as well as to hemoglobin ($r = 0.357$; $p = 0.005$).

4. Discussion

The present study was focused on the causes of GM in adult individuals and on the metabolic disturbances in that specific group of patients. Results showed that in almost two-third of patients, GM was of pathological origin. Moreover, the development of GM was among the primary symptoms leading to the proper diagnosis in more than 40% of hypogonadal patients. These results supported the conclusions of the largest Danish study on GM which emphasized on the need of structured and detailed investigations of the adults with GM [11].

The relatively higher percentage of pathological findings including hyperprolactinemia and hypogonadism in our group was related to the specific centralized organization of medical services in the country. Most of the patients were referred from general practitioners or endocrinologists, and thus, the patients were admitted to our tertiary endocrine

Table 1: Causes for gynecomastia in different patients and hormonal characteristics in the investigated group. Data are presented as median (*n*), min–max. *low prolactin levels in patients already treated with dopamine agonists or transsphenoidal surgery. The minimal prolactin level at the time of prolactinoma diagnosis was 1088 mIU/l. One of the patients was with somatoprolactinoma. **indicates normal testosterone levels in patients with already started testosterone therapy. All hypogonadal patients had initial testosterone levels under 11 nmol/l and additional tests to prove hypogonadism. In one patient with panhypopituitarism, the testosterone level was very low but measured in other laboratory/not included/.

Causes for GM	*N*	%	Categories	Age (years)	Testosterone (nmol/l)	LH (IU/l)	FSH (IU/l)	Prolactin (mIU/l)	TSH (mIU/l)	Estradiol (pmol/l)
Persistent pubertal GM	18	16.4	Persistent pubertal GM	22.00 (18) 18–40	20.90 (15) 9.70–30.60	2.85 (12) 0.60–9.70	2.30 (13) 1.40–7.60	308.00 (14) 91.00–652.00	1.80 (15) 0.87–26.80	394.00 (11) 112.00–633.00
Idiopathic postpubertal GM	22	20.0	Idiopathic postpubertal GM	28.00 (22) 19–66	18.60 (21) 11.40–40.20	2.55 (16) 0.47–7.70	4.04 (15) 0.53–8.30	214.00 (21) 82.00–430.00	2.45 (20) 0.62–6.90	282.00 (14) 23.00–960.00
Secondary hypogonadism	18	16.4	Secondary hypogonadism	28.00 (18) 18–69	2.60 (17) 0.60–17.90**	1.02 (14) 0.47–3.50	1.45 (14) 0.60–3.40	160.00 (15) 108.00–559.00	2.30 (15) 1.00–15.80	304.00 (6) 178.00–761.00
Prolactinoma	12	10.9	Prolactinoma	31.50 (12) 23–53	12.50 (11) 3.20–35.10	0.80 (5) 0.60–2.10	2.40 (5) 1.20–6.00	474.00 (12) 82.00*–10561.00	2.00 (8) 0.51–4.20	248.00 (1)
Primary hypogonadism	14	12.7	Primary hypogonadism	33.00 (14) 21–67	5.05 (14) 0.60–10.80	12.65 (14) 6.70–29.00	25.71 (14) 13.50–56.50	191.00 (14) 72.00–667.00	1.80 (13) 0.95–4.40	467.50 (6) 110.20–631.00
Medication-induced GM	20	18.2	Medication-induced GM	60.00 (20) 33–91	14.30 (13) 4.50–44.20	3.30 (9) 2.00–29.90	6.70 (9) 2.00–47.00	286.00 (11) 137.00–602.00	2.10 (16) 0.90–4.80	332.50 (6) 129.00–1171.00
Testicular tumor	2	1.8	Other causes	34.00 (6) 18–44	8.50 (5) 1.40–22.90	2.10 (4) 0.80–3.50	4.35 (4) 1.30–7.40	309.00 (5) 110.00–1030.00	0.39 (4) 0.03–1.40	997.00 (2) 747.00–1247.00
Hepatic injury	1	0.9								
Thyreotoxicosis	1	0.9								
Anabolic steroid abuse	2	1.8								
All	110	100.0	Referent ranges		8.5–42	2–8	3–12	<350	0.3–4.0	<180

Table 2: Prevalence of obesity, hypertension, prediabetes (impaired fasting glucose and/or impaired glucose tolerance), metabolic syndrome (MS), and diabetes mellitus type 2 (DM2) in patients with GM due to different causes. The prevalence of obesity, hypertension, prediabetes, MS, and DM2 was compared among the different GM groups through Pearson χ^2 test, $p < 0.05$ considered statistically significant. Data are presented as % (n). The presence of MS in 6 patients and the presence of obesity in 3 patients were not established due to missing metabolic or anthropometric data.

GM cause	Obesity % ($N = 103$)	Hypertension % ($N = 106$)	Prediabetes % ($N = 106$)	DM2 % ($N = 106$)	MS % ($N = 100$)
GM group ($N = 106$)	**37.9 (39)**	**34.9 [37]**	**13.2 [14]**	**15.1 [16]**	**53 (53)**
Persistent pubertal GM ($N = 18$)	38.9 (7)	38.9 (7)	11.1 (2)	0 (0)	47.1 (8)
Idiopathic postpubertal GM ($N = 22$)	31.8 (7)	22.7 (5)	18.2 (4)	4.5 (1)	35.0 (7)
Secondary hypogonadism ($N = 30$)	37.9 (11)	23.3 (7)	10.0 (3)	10.0 (3)	46.7 (14)
Primary hypogonadism ($N = 14$)	35.7 (5)	21.4 (3)	21.4 (3)	7.1 (1)	57.1 (8)
Medication and AAS-induced GM ($N = 22$)	45 (9)	68.2 (15)	9.1 (2)	50 (11)	84.2 (16)
p	0.937	**0.004**	0.742	**<0.001**	**0.028**

Table 3: Clinical and hormonal characteristics of GM patients with and without metabolic syndrome (patients with medication-induced gynecomastia were excluded from analyses). Data are presented as median [min–max] or percentage (n). Differences between groups were established through Mann–Whitney test or Fisher's exact test, $p < 0.05$ considered statistically significant (*).

	Metabolic healthy ($N = 47$)	Metabolic syndrome ($N = 38$)	p
Age (years) $n = 85$	27 [18–69] ($n = 47$)	32 [18–67] ($n = 38$)	0.078
Family history for DM2 (%) ($n = 85$)	8.5% (4) ($n = 47$)	21.1% (8) ($n = 38$)	0.124
Family history for AH (%) ($n = 85$)	21.3% (10) ($n = 47$)	28.9% (11) ($n = 38$)	0.456
Obesity (%) ($n = 84$)	25.5% (12) ($n = 47$)	45.9% (17) ($n = 37$)	0.066
Testosterone (nmol/l) ($n = 78$)	13.00 [0.60–35.10] ($n = 44$)	9.25 [0.60–30.60] ($n = 34$)	0.148
Estradiol (pmol/l) ($n = 37$)	275.00 [23.00–585.00] ($n = 19$)	357.50 [107.00–960.00] ($n = 18$)	0.081
Prolactin (mIU/l) ($n = 76$)	227.50 [82.00–5240.00] ($n = 44$)	191.50 [72.00–10561.00] ($n = 32$)	0.245
TSH (mIU/l) ($n = 72$)	1.85 [0.03–6.9] ($n = 38$)	2.20 [0.62–26.80] ($n = 34$)	0.119
LH (IU/l) ($n = 61$)	2.30 [0.47–22.40] ($n = 35$)	2.55 [0.47–29.00] ($n = 26$)	0.924
FSH (IU/l) ($n = 60$)	3.0 [0.53–38.90] ($n = 35$)	2.8 [0.77–56.50] ($n = 25$)	0.514
Estradiol (pmol/l) to testosterone (nmol/l) ratio *($n = 36$)	13.14 [1.65–90.00] ($n = 18$)	56.03 [7.23–691.82] ($n = 18$)	0.001

center in case of a suspected endocrine disease or lack of other obvious reasons requiring investigations by other specialists (e.g. drug abuse, renal or hepatic insufficiency, breast cancer, antiandrogen use in prostate cancer patients etc). On the other hand, some young men with idiopathic GM considered the condition as a primarily cosmetic problem and sought consultation with a plastic surgeon only. Therefore, some pathologic GM causes and the percentage of idiopathic GM might be underrepresented in our GM group.

Most of the investigated adult patients presented with bilateral GM, as in a former study on adolescent boys in the same population [6]. In opposite, the prevalence of unilateral and bilateral GM in other ethnic GM groups was almost similar [20].

Interestingly, only 5.5% of our patients recognized anabolic steroid use, whereas no one admitted use of marihuana. The reported prevalence of anabolic steroids use in other adult GM groups was significantly higher (12.9%–13.9%) [11, 20]. Thus, the unexplained hyperestrogenemia in some of our patients might be related to unreported anabolic or drug abuse. Other studies have shown that more than a half of anabolic steroid users would not disclose their substance abuse to any physician, which coincides with our impression [21]. Other possible explanations for the unexplained hyperestrogenemia might be an idiopathic increase of aromatase activity in some patients, obesity and an intake of unknown endocrine disruptors.

The local imbalance between the free estrogens and androgens in the breast is paramount for the development of GM [2]. At the same time, the estradiol to testosterone ratio in the circulation might modulate the risk for the metabolic syndrome development in men [22]. Therefore, it is interesting to find out if the hormonal imbalance in GM individuals might be related to metabolic disturbances. The prevalence of metabolic syndrome among the investigated GM patients was 53%, which was higher than the estimated prevalence of metabolic syndrome in the common male Bulgarian population at similar age (40.9%) [23]. The prevalence of metabolic syndrome was increased in the patients with medication-induced GM and in the hypogonadal patients, whereas it was lowest in the group of men with idiopathic postpubertal GM despite the similar degree of obesity. Even in patients younger than 40 years, the prevalence of MS in hypogonadal GM men was twice as high as that in those with idiopathic GM.

The increased prevalence of MS in patients taking different drugs has been expected because many medications with known side effects on male breast have been used for the prevention or treatment of metabolic and cardiovascular

complications. However, the increased prevalence of MS in hypogonadal GM patients and in men with persistent pubertal GM needs further research. The prevalence of metabolic syndrome was strongly increased (44% vs. 10%), whereas insulin sensitivity was decreased in men with Klinefelter syndrome (KS) compared with healthy controls [24]. On the other hand, the prevalence of MS was slightly (but not significantly) increased in KS patients in comparison to 46XY men with nonobstructive azoospermia and men with obstructive azoospermia (34.3%, 23.3%, and 22.2%, respectively) [25]. The presence of metabolic syndrome was also increased in patients with congenital hypogonadotropic hypogonadism compared with healthy men (1.5% vs. 0.25%). However, the prevalence of MS in that group of hypogonadal patients was very low in comparison to the published data for men with primary hypogonadism [26]. In opposite, our data showed increased prevalence of MS in young hypogonadal men with GM, irrespective of the cause of hypogonadism. Further studies are needed to reveal whether the metabolic risk is increased in hypogonadal patients with GM in comparison to those without GM.

Our data showed a strongly increased estradiol to testosterone ratio in GM patients with MS in comparison to those without the syndrome, which correlated to visceral obesity and hypertriglyceridemia in the patients. Moreover, the higher testosterone levels in the patients with GM were associated with a better lipid profile, whereas the androgen influence on other MS components was inconclusive. Some authors reported a negative correlation between testosterone levels and BMI, suggesting increased testosterone to estradiol conversion in the fat tissue of patients with GM [27]. However, other authors did not support such relationships [28]. The positive associations between the testosterone levels and lipid profile were already shown in the common male population [29], in ageing males with andropausal symptoms [30] and in patients with coronary artery disease [31]. On the other hand, increased estradiol levels in men were associated with increased risk of obesity, metabolic syndrome, and diabetes type 2 [32–34]. Moreover, elderly men with prevalent coronary heart disease, heart failure, or stroke had significantly increased estradiol to testosterone ratio in comparison to those without cardiovascular diseases [35]. According to our results, the hormonal imbalance in GM patients might be associated with metabolic disturbances. The increased GM prevalence in hospitalized men with diabetes mellitus supported indirectly this conclusion [36]. However, further longitudinal studies are needed to evaluate the long-term consequences of sex hormones changes on the cardiovascular morbidity and mortality of adults with GM.

To the best of our knowledge, this is the first study focused on metabolic disturbances in adult men with GM due to different causes. However, some limitations should be mentioned. GM established by palpation was not confirmed by ultrasonography or X-ray mammography in all men. Additionally, patients with incomplete laboratory and hormonal data were included in the study to avoid preselection bias. The analysis of the missing data showed that standardized approach with detailed laboratory investigations and imaging studies was commonly applied to patients with newly developed GM and in case of concomitant symptoms suggesting pathologic condition (e.g. hypogonadism, hyperprolactinemia, systemic disease etc). On the contrary, patients with long-lasting pubertal GM without additional symptoms and those with a plausible explanation for GM (e.g. use of spironolactone) were rarely an object of further investigations. However, the recently published guideline for the evaluation and treatment of GM emphasizes on the need of thorough investigations, even in case of an apparent reason for GM in adults [37]. The implementation of that principle in the clinical practice might help early diagnosis of some endocrine and oncological diseases.

In conclusion, our study showed that the development of GM in adults might be an important symptom of an underlying gonadal disease. Moreover, it could be associated with an increased risk of metabolic disturbances. Our results support the need of detailed laboratory and hormonal investigations in patients with GM. Moreover, the targeted screening for lipid and carbohydrate disturbances in males with GM might reduce the potential cardiovascular risk related to the hormonal imbalance in these patients.

Data Availability

Datasets are available from the corresponding author on reasonable request after permission from the local authorities.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] A. C. S. D. de Barros and M. D. C. M. Sampaio, "Gynecomastia: physiopathology, evaluation and treatment," *Sao Paulo Medical Journal*, vol. 130, no. 3, pp. 187–197, 2012.

[2] G. D. Braunstein, "Gynecomastia," *New England Journal of Medicine*, vol. 357, no. 12, pp. 1229–1237, 2007.

[3] A. A. A. Ismail and J. H. Barth, "Endocrinology of gynaecomastia," *Annals of Clinical Biochemistry*, vol. 38, no. 6, pp. 596–607, 2001.

[4] H. Macias and L. Hinck, "Mammary gland development," *Wiley Interdisciplinary Reviews: Developmental Biology*, vol. 1, no. 4, pp. 533–557, 2012.

[5] P. Kumanov, "Pubertal gynecomastia," in *Puberty: Physiology and Abnormalities*, P. Kumanov and A. Agarwal, Eds., pp. 183–196, Springer International Publishing, Cham, Switzerland, 2016.

[6] P. Kumanov, F. Deepinder, R. Robeva, A. Tomova, J. Li, and A. Agarwal, "Relationship of adolescent gynecomastia with varicocele and somatometric parameters: a cross-sectional study in 6200 healthy boys," *Journal of Adolescent Health*, vol. 41, no. 2, pp. 126–131, 2007.

[7] C. Lazala and P. Saenger, "Pubertal gynecomastia," *Journal of Pediatric Endocrinology and Metabolism*, vol. 15, no. 5, pp. 553–560, 2002.

[8] V. Lemaine, C. Cayci, P. S. Simmons, and P. Petty, "Gynecomastia in adolescent males," *Seminars in Plastic Surgery*, vol. 27, no. 1, pp. 56–61, 2013.

[9] R. E. Johnson and M. H. Murad, "Gynecomastia: pathophysiology, evaluation, and management," *Mayo Clinic Proceedings*, vol. 84, no. 11, pp. 1010–1015, 2009.

[10] Jane F. Desforges and G. D. Braunstein, "Gynecomastia," *New England Journal of Medicine*, vol. 328, no. 7, pp. 490–495, 1993.

[11] M. G. Mieritz, P. Christiansen, M. B. Jensen et al., "Gynaecomastia in 786 adult men: clinical and biochemical findings," *European Journal of Endocrinology*, vol. 176, no. 5, pp. 555–566, 2017.

[12] I. D. van Koeverden, M. de Bakker, S. Haitjema et al., "Testosterone to oestradiol ratio reflects systemic and plaque inflammation and predicts future cardiovascular events in men with severe atherosclerosis," *Cardiovascular Research*, vol. 115, no. 2, pp. 453–462, 2019.

[13] S. Bhasin, G. R. Cunningham, F. J. Hayes et al., "Testosterone therapy in men with androgen deficiency syndromes: an endocrine society clinical practice guideline," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 6, pp. 2536–2559, 2010.

[14] F. C. W. Wu, A. Tajar, J. M. Beynon et al., "Identification of late-onset hypogonadism in middle-aged and elderly men," *New England Journal of Medicine*, vol. 363, no. 2, pp. 123–135, 2010.

[15] G. R. Dohle, S. Arver, C. Bettocchi, T. Jones, S. Kliesch, and M. Punab, *Guidelines on Male Hypogonadism*, European Association of Urology, Arnhem, Netherlands, 2016, https://uroweb.org.

[16] F. F. Casanueva, M. E. Molitch, J. A. Schlechte et al., "Guidelines of the pituitary society for the diagnosis and management of prolactinomas," *Clinical Endocrinology*, vol. 65, no. 2, pp. 265–273, 2006.

[17] S. Melmed, F. F. Casanueva, A. R. Hoffman et al., "Diagnosis and treatment of hyperprolactinemia: an endocrine society clinical practice guideline," *The Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 2, pp. 273–288, 2011.

[18] K. G. M. M. Alberti, R. H. Eckel, S. M. Grundy et al., "Harmonizing the metabolic syndrome," *Circulation*, vol. 120, no. 16, pp. 1640–1645, 2009.

[19] R. K. Bhattacharya, M. Khera, G. Blick, H. Kushner, D. Nguyen, and M. M. Miner, "Effect of 12 months of testosterone replacement therapy on metabolic syndrome components in hypogonadal men: data from the testim registry in the US (TRiUS)," *BMC Endocrine Disorders*, vol. 11, no. 1, p. 18, 2011.

[20] P. R. Costanzo, N. A. Pacenza, S. M. Aszpis et al., "Clinical and etiological aspects of gynecomastia in adult males: a multicenter study," *BioMed Research International*, vol. 2018, Article ID 8364824, 7 pages, 2018.

[21] H. G. Pope, G. Kanayama, M. Ionescu-Pioggia, and J. I. Hudson, "Anabolic steroid users' attitudes towards physicians," *Addiction*, vol. 99, no. 9, pp. 1189–1194, 2004.

[22] L. Antonio, F. C. W. Wu, T. W. O'Neill et al., "Associations between sex steroids and the development of metabolic syndrome: a longitudinal study in European men," *The Journal of Clinical Endocrinology & Metabolism*, vol. 100, no. 4, pp. 1396–1404, 2015.

[23] A.-M. Borissova, A. Shinkov, J. Vlahov, L. Dakovska, and T. Todorov, "Metabolic syndrome in Bulgarian population in 2012 year," *Endocrinología*, vol. 20, no. 3, pp. 144–152, 2015.

[24] A. Bojesen, K. Kristensen, N. H. Birkebaek et al., "The metabolic syndrome is frequent in Klinefelter's syndrome and is associated with abdominal obesity and hypogonadism," *Diabetes Care*, vol. 29, no. 7, pp. 1591–1598, 2006.

[25] T. Ishikawa, K. Yamaguchi, Y. Kondo, A. Takenaka, and M. Fujisawa, "Metabolic syndrome in men with Klinefelter's syndrome," *Urology*, vol. 71, no. 6, pp. 1109–1113, 2008.

[26] A. Sonmez, C. Haymana, E. Bolu et al., "Metabolic syndrome and the effect of testosterone treatment in young men with congenital hypogonadotropic hypogonadism," *European Journal of Endocrinology*, vol. 164, no. 5, pp. 759–764, 2011.

[27] H. O. Ersoz, M. E. Onde, H. Terekeci, S. Kurtoglu, and H. Tor, "Causes of gynaecomastia in young adult males and factors associated with idiopathic gynaecomastia," *International Journal of Andrology*, vol. 25, no. 5, pp. 312–316, 2002.

[28] M. Yazici, M. Sahin, E. Bolu et al., "Evaluation of breast enlargement in young males and factors associated with gynecomastia and pseudogynecomastia," *Irish Journal of Medical Science*, vol. 179, no. 4, pp. 575–583, 2010.

[29] N. Zhang, H. Zhang, X. Zhang et al., "The relationship between endogenous testosterone and lipid profile in middle-aged and elderly Chinese men," *European Journal of Endocrinology*, vol. 170, no. 4, pp. 487–494, 2014.

[30] J. I. Mäkinen, A. Perheentupa, K. Irjala et al., "Endogenous testosterone and serum lipids in middle-aged men," *Atherosclerosis*, vol. 197, no. 2, pp. 688–693, 2008.

[31] C. Wickramatilake, M. Mohideen, and C. Pathirana, "Association of serum testosterone with lipid abnormalities in patients with angiographically proven coronary artery disease," *Indian Journal of Endocrinology and Metabolism*, vol. 17, no. 6, pp. 1061–1065, 2013.

[32] M. Muller, I. den Tonkelaar, J. H. Thijssen, D. E. Grobbee, and Y. T. van der Schouw, "Endogenous sex hormones in men aged 40–80 years," *European Journal of Endocrinology*, vol. 149, no. 6, pp. 583–589, 2003.

[33] T. Vikan, H. Schirmer, I. Njølstad, and J. Svartberg, "Low testosterone and sex hormone-binding globulin levels and high estradiol levels are independent predictors of type 2 diabetes in men," *European Journal of Endocrinology*, vol. 162, no. 4, pp. 747–754, 2010.

[34] M. Maggio, F. Lauretani, G. P. Ceda et al., "Estradiol and metabolic syndrome in older Italian men: the InCHIANTI Study," *Journal of Andrology*, vol. 31, no. 2, pp. 155–162, 2008.

[35] T. Naessen, U. Sjogren, J. Bergquist, M. Larsson, L. Lind, and M. M. Kushnir, "Endogenous steroids measured by high-specificity liquid chromatography-tandem mass spectrometry and prevalent cardiovascular disease in 70-year-old men and women," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 4, pp. 1889–1897, 2010.

[36] C. B. Niewoehner and F. Q. Nuttall, "Gynecomastia in a hospitalized male population," *The American Journal of Medicine*, vol. 77, no. 4, pp. 633–638, 1984.

[37] G. A. Kanakis, L. Nordkap, A. K. Bang et al., "EAA clinical practice guidelines-gynecomastia evaluation and management," *Andrology*, 2019.

Hindawi
International Journal of Endocrinology
Volume 2019, Article ID 1041760, 18 pages
https://doi.org/10.1155/2019/1041760

Research Article

Effects of Osteocalcin on Synthesis of Testosterone and INSL3 during Adult Leydig Cell Differentiation

Gulfidan Coskun,[1] Leman Sencar,[1] Abdullah Tuli,[2] Dilek Saker,[1] Mustafa Muhlis Alparslan,[2] and Sait Polat[1]

[1]*Department of Histology and Embryology, Faculty of Medicine, Cukurova University, Adana TR01330, Turkey*
[2]*Department of Biochemistry, Faculty of Medicine, Cukurova University, Adana TR01330, Turkey*

Correspondence should be addressed to Gulfidan Coskun; gcoskun@cu.edu.tr

Received 12 March 2019; Revised 30 May 2019; Accepted 20 June 2019; Published 28 August 2019

Guest Editor: Andrea Silvestrini

Proliferation and differentiation of adult Leydig cells are mainly completed in puberty. In many studies, apart from normal postnatal development process, it is widely indicated that, through administrating EDS, Leydig cell population is eliminated and regenerated. It is believed that osteocalcin released from osteoblasts, which is responsible for modulating bone metabolism, induces testosterone production in Leydig cells, independent of the HPG axis. In addition, INSL3 produced by Leydig cells, such as testosterone, plays a critical role in bone metabolism and is known to reflect the development process and functional capacities of Leydig cells. This study is aimed at investigating OC-mediated testosterone regulation and INSL3 synthesis during differentiation of adult Leydig cells that are independent of LH. For this purpose, male rats were divided into 2 groups: prepubertal normal rats and adult EDS-injected rats. Each group was divided into 4 subgroups in which GnRH antagonist or OC was applied. After adult Leydig cells completed their development, testicular tissue samples obtained from the sacrificed rats were examined by light-electron microscopic, immunohistochemical, and biochemical methods. Slight upregulation in 3βHSD, INSL3, and GPRC6A expressions along with the increase in serum testosterone levels was observed in groups treated with osteocalcin against GnRH antagonist. In addition, biochemical and microscopic findings in osteocalcin treated groups were similar to those in control groups. While there was no significant difference in the number of Leydig cells reported, the presence of a significant upregulation in INSL3 and GPRC6A expressions and the increase in serum testosterone and ucOC levels were observed. After evaluation of findings altogether, it is put forward that, for the first time in this study, although osteocalcin treatment made no significant difference in the number of Leydig cells, it increased the level of testosterone through improving the function of existing adult Leydig cells during normal postnatal development process and post-EDS regeneration. This positive correlation between osteocalcin-testosterone and osteocalcin-INSL3 is concluded to be independent of LH at in vivo conditions.

1. Introduction

Sex steroid hormones are extensively known to be necessary for skeletal development and bone health throughout adult life, except for reproductive functions [1–3], which could set new hypotheses related to the effects of the bone on the synthesis and secretion of steroid hormones. The most common noncollagenous protein in the bone is osteocalcin in 2 forms: Gla-OC and Glu-OC, according to carboxylated (cOC) and uncarboxylated (ucOC) osteocalcin, respectively. While the active form of Glu-OC (ucOC) is used as hormone, the Gla-OC (cOC) contained in the bone matrix is biologically inactive. In in vivo and in vitro research studies, ucOC is shown to regulate male fertility by increasing testosterone production in Leydig cells that are independent of LH and inhibiting germ cell apoptosis. The specific ucOC receptor in Leydig cells is GPRC6A which also exists in the brain, heart, lung, spleen, kidney, skeletal muscle, adipose tissue, and pancreatic B cells, but not in the ovaries. In order to activate the steroidogenesis process, ucOC bypasses LHR and is connected to GPRC6A [4–9]. In addition, ucOC has been reported to induce pancreatic B-cell proliferation and

insulin secretion, resulting in insulin sensitivity in the liver, muscle, and white fat [10–15]. Also, the orally administered Gla-OC form is reported to be decarboxylated in stomach acidity and converted into Glu-OC, resulting in increased serum ucOC and insulin levels [16].

Another hormone that plays a critical role in bone metabolism is INSL3. INSL3 is produced by Leydig cells in the prenatal and the postnatal period in rodents. It has also been reported that INSL3, known to be regulated by long-term LH secretions during adulthood, is used as a marker of Leydig cell development and functions [17–23]. Besides, the reason of synthesis and regulation of INSL3 in the testis is still unclear.

The populations of Leydig cells that differentiate prenatally and postnatally are identified as fetal and adult Leydig cells, respectively. In the fetus of rodents and humans, fetal Leydig cells produce testosterone and INSL3, which are needed for normal development of male external genitals and testis descent into the scrotum [24–30]. In contrast to fetal Leydig cells, adult Leydig cell differentiation is LH dependent. In rodents, Leydig cells complete their development in 4 distinct cellular stages which have different steroidogenic enzymes and mitotic activities. These steps are stem Leydig cells, progenitor Leydig cells, immature Leydig cells, and adult Leydig cells. Stem Leydig cells give rise to a progenitor cell type that further differentiates into immature Leydig cells followed by their maturation into adult Leydig cells. Adult Leydig cells produce androgens during the entire adult life span. Testosterone production by mature adult Leydig cells is required for the onset of spermatogenesis. Moreover, by expressing INSL3, adult Leydig cells play a role in germ cell viability and bone metabolism [25, 31–36].

The development process of the adult Leydig cell population can also be mimicked in adulthood after EDS injection. EDS is an alkylating agent that specifically kills adult Leydig cells without affecting other testicular cell types. After one dose of intraperitoneal injection of 70 mg/kg of EDS, all adult Leydig cells were reported to have been destroyed within 4–7 days where within 7- to 10-week-old new adult Leydig cells proliferate from stem Leydig cells [37–41].

Revealing paracrine factors and signal pathways that take part in differentiation of the adult Leydig cell population provides new approaches towards pharmacological and/or nutritional therapy, in impaired Leydig cell functions and in reduction in Leydig cell numbers for any reason [42]. In experimental studies, it is thought that ucOC could be a new therapeutic agent in regulating male fertility through increasing Leydig cell numbers and functions [4, 5]. Although the relationship between INSL3, testosterone, and osteocalcin has been reported in the previous study [43] in patients with Klinefelter syndrome, this is the first study in which the relationship between osteocalcin, testosterone, and INSL3 in Leydig cell development is reported.

Therefore, this study is aimed at investigating the effects of orally administered Gla-OC on testosterone and INSL3 synthesis in adult Leydig cells during normal development and after EDS injection. In addition, the obtained light-electron microscopic, immunohistochemical, and biochemical findings are compared for LH levels.

2. Materials and Methods

Seventy-two Sprague Dawley male rats were divided into two groups, namely, Group 1 and Group 2. While 35-day-old rats were chosen for normal adult Leydig cell development, 12- to 13-week-old rats were preferred for post-EDS Leydig cell regeneration. In addition, 4 subgroups were formed within each group. Subjects were obtained from the Medical Sciences and Experimental Research and Application Center of Cukurova University (Adana, Turkey) and housed in well-ventilated polypropylene cages with food and tap water ad libitum. They were kept under controlled laboratory conditions of a normal light/dark cycle and normal temperature ($25 \pm 2^{\circ}$C) and were allowed to acclimatize for one week. After receiving the approval of Cukurova University's Experimental Animal Ethics Committee (dated 28.04.2014), all experimental procedures were carried out according to the Universal Declaration of International Animal Rights.

2.1. Chemicals. In our study, cetrorelix acetate (450 μg/kg/day) (Sigma C5249, USA) with less side effects as a GnRH antagonist was preferred [44], and osteocalcin (Gla-OC) protein (10 μg/kg/day) (Fitzgerald 30R-3286, USA) was administered to the subjects orally [16]. Furthermore, EDS, used for adult Leydig cell elimination in postpubertal subjects, was synthesized at the Chemistry Department of Science Faculty in Cukurova University (Adana-Turkey) as specified by Jackson and Jackson (1984) [45]. Firstly, 75 mg of EDS was dissolved in 0.5 ml of dimethylsulfoxide (DMSO) where 1.5 ml of distilled water was added to prepare 2 ml of EDS solution injected intraperitoneally into the rats as 2 ml per kg.

2.2. Surgical Procedure. To investigate the development of normal adult Leydig cells in the postnatal period, 18 adult female rats induced with 50 IU hCG and 30 IU PMSG for ovulation [46] were divided into 6 separate cages containing 3 female and 1 male rats. After 48 hours, 15 female rats, which had vitelline plug formation, were taken into separate cages to complete the 21-day gestation period. The born pups were kept in the same cages with their mothers without any application for 35 days. Thirty-two male pups of the 67 offsprings were allocated to 4 subgroups under the application period until postnatal day 63, as summarized in Figure 1:

Group 1A: control group ($n = 8$). No application procedure was applied.

Group 1B: cetrorelix treated group ($n = 8$).

Group 1C: cetrorelix [44] and Gla-OC treated group ($n = 8$).

Group 1D: Gla-OC treated group ($n = 8$).

To investigate the development of adult Leydig cells in EDS-injected animal models, from the 35th day to the 63rd day, after a single dose of EDS injection, 40 adult male rats were divided into 4 subgroups, as summarized in Figure 2:

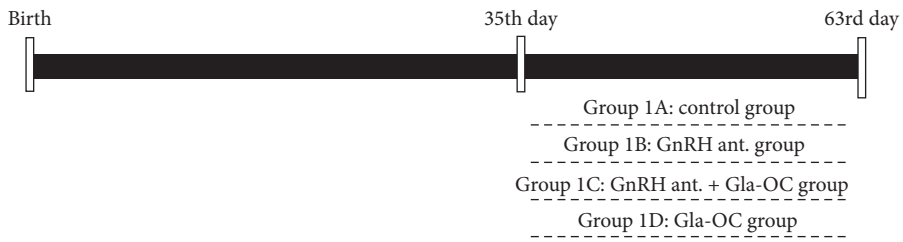


Figure 1: Group 1: postnatal normal adult Leydig cell development surgical procedure.

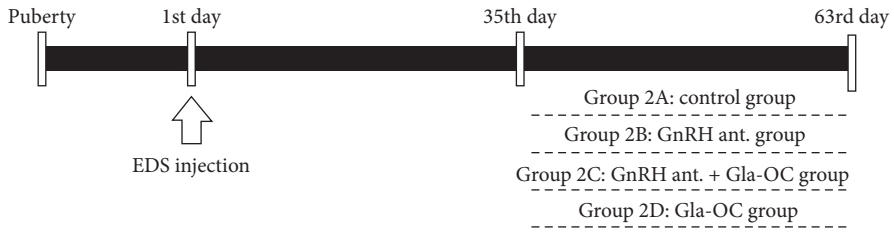


Figure 2: Post-EDS regeneration of the adult Leydig cell surgical procedure.

Group 2A: EDS treated group ($n = 16$). Four days after EDS application, 8 rats in this group were sacrificed to research whether EDS eliminated Leydig cells. The remaining 8 rats survived for 63 days with no application except for a single dose of EDS.

Group 2B: EDS and cetrorelix treated group ($n = 8$).

Group 2C: EDS, cetrorelix, and Gla-OC treated group ($n = 8$).

Group 2D: EDS and Gla-OC treated group ($n = 8$).

On the 63rd day of the postnatal normal developmental period and post-EDS application period, the differentiation of adult Leydig cells in all subjects was completed, and therefore, the 63rd day was selected as the sacrifice time of the subjects [31–33, 35].

Testicular tissue samples obtained from the sacrificed experimental animals were taken into Bouin's solution for light microscopic examination, 5% glutaraldehyde solution for electron microscopic examination, and 4% paraformaldehyde for immunohistochemical examination.

LH, testosterone, and ucOC levels were measured by the blood serum samples, and testicular tissues were processed by light-electron microscopic examination and immunohistochemical staining for 3βHSD, INSL3, and GPRC6A. The data obtained were evaluated with appropriate statistical methods.

2.3. Light Microscopic Methods. In order to perform light microscopic examinations, one of each animal's testes was fixed in Bouin's solution for 8 hours and dehydrated for 6 hours by 50% alcohol and then by 70% alcohol. Tissue samples were washed under tap water and then dehydrated with an alcohol series (70%, 80%, 90%, 96%, and 100%). Thereafter, the tissues were kept in xylene for an hour in three separate containers at the same concentration. After xylene treatment, tissues were kept in molten paraffin at 60°C which was allowed to solidify at room temperature overnight. Paraffin-embedded tissue sections were cut by a microtome (Shandon Finesse 325, Thermo Scientific) to a thickness of 5 μm. After staining with hematoxylin and eosin, tissue sections were examined under light microscope (Olympus BX53, Tokyo, Japan) [46].

2.4. Immunohistochemical Methods. The most commonly used marker to identify adult Leydig cells is 3βHSD [47–49]. INSL3 is an indicator of adult Leydig cell activity [17–19]. GPRC6A acts as an osteocalcin receptor in the testis [4, 5]. For 3βHSD, INSL3, and GPRC6A immunoreactivity analysis, testicular tissue specimens obtained from rats sacrificed on day 63 were fixed in 4% paraformaldehyde for 24 hours at room temperature. After routine tissue attachments with a Leica TP 1020 autotechnical device, paraffin sections with a thickness of 4 μm were taken from the tissue specimens embedded in paraffin. After being dewaxed in xylene and rehydrated in graded alcohol, 4 μm thick paraffin sections were treated with heat-induced epitope retrieval solution and put into a microwave irradiation for 10 min. Then, the sections were cooled off, washed in PBS, and incubated with 3% H_2O_2 to block endogenous peroxidase for 15 min at room temperature. After washing three times in PBS, the sections were blocked with blocking (IHC Kit ab93705, Abcam, MA, USA) for 15 minutes. The primary antibodies including anti-HSD3B1 (1 : 2000; anti-rat rabbit monoclonal antibody, ab150384, Abcam, MA, USA), anti-INSL3 (1 : 2000; anti-rat rabbit polyclonal antibody, ab65981, Abcam, MA, USA), and anti-GPRC6A (1 : 1000; anti-rabbit polyclonal antibody, NLS2576, Novus, Littleton, USA) were added before the samples were incubated overnight at 4°C. Negative control slides were prepared by omitting the primary antibodies. After washing them on the next day, the secondary antibody (IHC Kit, ab93705, Abcam, MA, USA) was added which was followed by staining with 3-amino-9-ethylcarbazole (AEC IHC Kit ab93705, Abcam, MA, USA) for 10 minutes and hematoxylin reagent for 3 minutes before

dehydration. Images of the stained sections were analysed and photographed by light microscopy (Olympus BX53, Tokyo, Japan).

2.5. Scoring Assessment. For H-score assessment of each antibody, 10 fields were chosen randomly at ×400 magnification while the staining intensity in Leydig cells was scored as 0, 1, 2, or 3 corresponding to, respectively, the presence of negative, weak, intermediate, and strong staining. The total number of cells in each field and the number of cells stained at each intensity were counted. The average percentage of cells showing positive expression was calculated before the following formula was applied: IHC H-score = (% of cells stained at intensity category 1×1) + (% of cells stained at intensity category 2×2) + (% of cells stained at intensity category 3×3) [50]. The number of 3βHSD-positive Leydig cells was calculated regardless of the staining differences in each of the 10 random fields at ×400 magnification [51]. All evaluations were performed by two blinded histologists.

2.6. Electron Microscopic Methods. For electron microscopy, the other testis of each animal was fixed in 5% glutaraldehyde in a phosphate buffer (pH 7.2) for 24 hours before tissue pieces were postfixed in 1% osmium tetroxide. Thereafter, the tissue was dehydrated in graded ethanol, embedded in Araldite, and processed for electron microscopy. The stained sections were examined with a JEOL-JEM 1400 transmission electron microscope [46].

2.7. Serum Assays. Intracardiac blood samples were centrifuged at 3600 rpm for 5 minutes to obtain serum. Serum was kept in Eppendorf tubes at −20°C. Serum LH, testosterone, and Glu-OC levels were, respectively, measured through LH (CSB-E12654r, Cusabio), testosterone (CSB-E05100r, Cusabio), and Glu-OC (E-EL-R2477, Elabscience) ELISA kits in the ELISA Reader (Epoch) at the Medical Biochemistry Department of Cukurova University.

2.8. Statistical Analysis. The GraphPad Prism 5 (GraphPad Software Inc., USA) program is used to assess immunohistochemical scoring and biochemical data. Using the D'Agostino and Pearson omnibus normality test, data's distribution normality was checked. One-way ANOVA by Tukey's test was used to assess the differences between experimental and control groups based on immunoreactivity scores, Leydig cell numbers, and biochemical data. An unpaired *t*-test was used to compare the biochemical data of subjects that were sacrificed 4 days after EDS administration with the control groups. Moreover, P values less than 0.05 were considered significant.

3. Results

3.1. Light Microscopic Results. It was observed that seminiferous tubules and interstitium were in normal structure in the light microscopic examination of the testicular tissue sections of the control groups (Figures 3(a) and 3(e)). In the testicular tissue sections of GnRH-antagonist-administered groups, spermatogenic arrest that occurred from spillage in the tubular epithelium and accumulation of immature spermatogenic cells in the tubule lumen was observed. Along with vacuolization, hyalinization was observed in the seminiferous tubule epithelium. The membrane propria was seen to be thick and irregular. In addition, large spaces were observed in the interstitial area because of shrinkage in the tubules and decrease in the number of Leydig cells (Figures 3(b) and 3(f)). Subjects administered with osteocalcin and GnRH antagonist showed less vacuolization in their testicular tissue sections. However, an immature spermatogenic cell accumulation in the tubule lumens, a decrease in the number of Leydig cells, and an edema in the interstitial area were in progress in spite of many tubules preserving the integrity (Figures 3(c) and 3(g)). Testicular tissue sections of subjects only administered with osteocalcin had a similar appearance to seminiferous tubules and interstitium of control groups. In addition to this, it was observed that the Leydig cells in the interstitial area were in normal structure and distribution (Figures 3(d) and 3(h)).

3.2. Immunochemical Results. As a Leydig cell marker, 3βHSD expression was observed to be high in adult Leydig cells located in the interstitium of testicular tissues of control group subjects (Figures 4(a) and 5(a)). However, 3βHSD expression significantly decreased in testicular tissue sections of the GnRH antagonist treated group (Figures 4(b) and 5(b)). Testicular tissue sections of subjects administered with osteocalcin together with GnRH antagonist showed a slight increase in 3βHSD immunoreactivity compared to those of subjects of only-GnRH-antagonist-administered groups (Figures 4(c) and 5(c)). Immunohistochemical examinations of testicular tissue sections of only osteocalcin treated subjects have shown similarity to the expression of 3βHSD Leydig cells of the control groups (Figures 4(d) and 5(d)).

As a functional indicator of adult Leydig cells, INSL3 expression was observed to be high in Leydig cells of the testicular tissue sections of control group subjects (Figures 4(e) and 5(e)). Because of continued Leydig cell dysfunction, a decrease in INSL3 expression in the testicular tissue sections of subjects administered with GnRH antagonist together with osteocalcin (Figures 4(f) and 5(f)) was similar to that of only-GnRH-antagonist-administered groups (Figures 4(g) and 5(g)). Expression of INSL3 also showed an increase under the effect of osteocalcin on testicular tissue sections of subjects administered with only osteocalcin (Figures 4(h) and 5(h)).

Osteocalcin receptor GPRC6A expression showed high expression in adult Leydig cells located in the interstitium of testicular tissues of control group subjects (Figures 4(i) and 5(i)). Because of the decrease in the number of Leydig cells, a decrease in GPRC6A expression was observed in subjects administered with GnRH antagonist (Figures 4(j) and 5(j)). The expression of GPRC6A was observed to have moderately increased in testicular tissue sections of subjects

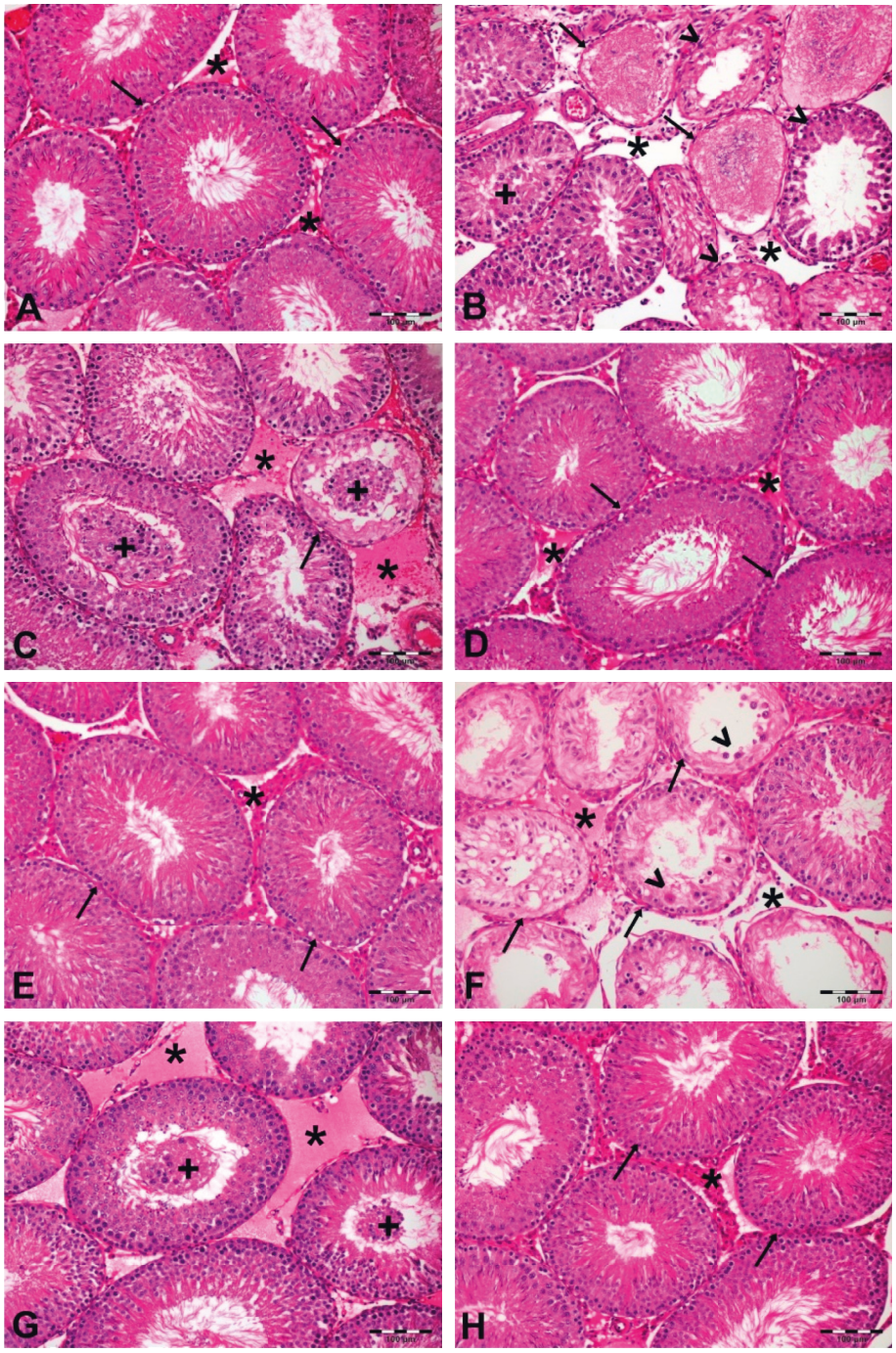


Figure 3: Light microscopic view of the testicular tissue samples of postnatal subjects (Group 1) and EDS-administered subjects (Group 2) (H&E). (a) Seminiferous tubules (arrows) and interstitial area (*) were observed to be normal in control subjects of Group 1 (Group 1A). (b) Vacuolization (arrowheads) in the seminiferous tubule epithelium, immature cell accumulation (+) in the tubule lumen, empty seminiferous tubules (arrows), and interstitial edema (*) were seen in GnRH-antagonist-administered subjects of Group 1 (Group 1B). (c) Vacuolization (arrows) in seminiferous tubules, immature cell accumulation (+) in the tubule lumen, and interstitial edema (*) were seen to continue in GnRH-antagonist-and-osteocalcin-administered subjects of Group 1 (Group 1C). (d) Seminiferous tubules (arrows) and the interstitium (*) were normal in only-osteocalcin-administered subjects of Group 1 (Group 1D). (e) In control subjects of Group 2 (Group 2A), the seminiferous tubules (arrows) and the interstitium (*) were shown to be normal. (f) Vacuolization (arrows) and multinucleated giant spermatogenic cells (arrowheads) in the seminiferous tubule epithelium and interstitial edema (*) were observed in GnRH-antagonist-administered subjects of Group 2 (Group 2B). (g) In GnRH-antagonist-and-osteocalcin-administered subjects of Group 2 (Group 2C), immature cell accumulation (+) and interstitial edema (*) were seen to continue in some seminiferous tubule lumens. (h) Seminiferous tubules (arrows) and interstitium (*) were observed as normal in only-osteocalcin-administered subjects of Group 2 (Group 2D). Bar = 100 μm.

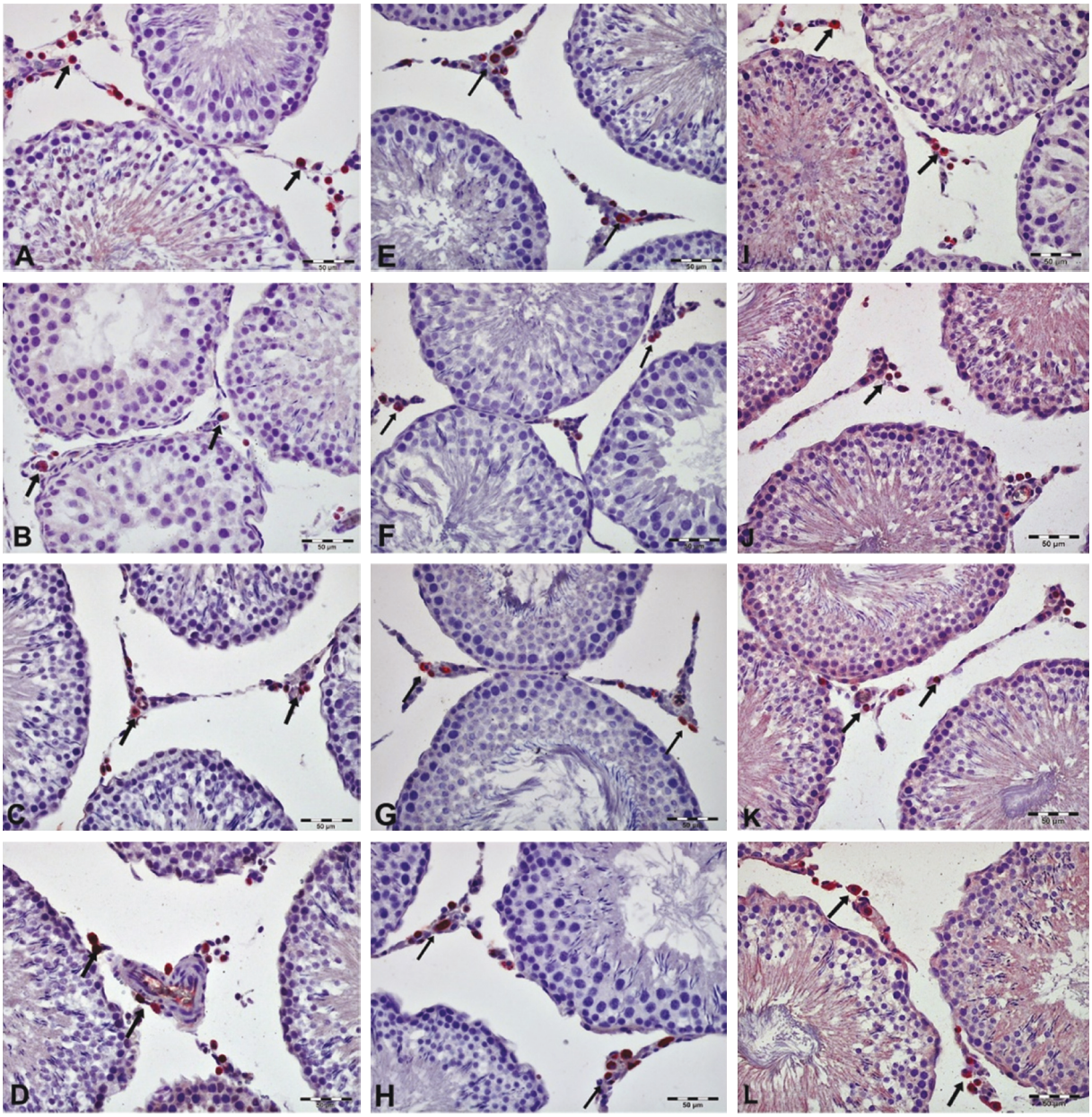


Figure 4: 3βHSD, INSL3, and GPRC6A immunoreactivity in testicular tissue samples of postnatal subjects (Group 1). (a) High 3βHSD expression was observed in Leydig cells of control subjects (Group 1A). (b) 3βHSD expression decreased in Leydig cells of GnRH-antagonist-administered subjects (Group 1B). (c) A slight increase in 3βHSD expression was seen in Leydig cells of GnRH-antagonist-and-osteocalcin-administered subjects (Group 1C). (d) Intensive 3βHSD expression was observed in Leydig cells of only-osteocalcin-administered subjects (Group 1D). (e) High INSL3 expression was seen in Leydig cells of control subjects (Group 1A). (f) INSL3 expression decreased in Leydig cells of GnRH antagonist subjects (Group 1B). (g) A decrease in INSL3 expression seemed to continue in Leydig cells of GnRH-antagonist-and-osteocalcin-administered subjects (Group 1C). (h) High INSL3 expression was noticed in Leydig cells of only-osteocalcin-administered subjects (Group 1D). (i) High INSL3 expression was noted in Leydig cells of control subjects (Group 1A). (j) GPRC6A expression decreased in Leydig cells of GnRH-antagonist-administered subjects (Group 1B). (k) A decrease in GPRC6A expression seemed to continue in Leydig cells of GnRH-antagonist-and-osteocalcin-administered subjects

administered with GnRH antagonist together with osteocalcin (Figures 4(k) and 5(k)). On the contrary, in subjects administered with only osteocalcin, a significant rise in GPRC6A expression was observed in comparison with control groups (Figures 4(l) and 5(l)).

In order to show whether adult Leydig cells were eliminated as a result of single-dose EDS administration or not, expressions of 3βHSD, INSL3, and GPRC6A were monitored in the testicular tissue sections of the subjects sacrificed 4 days after EDS, and none was found. That is, the

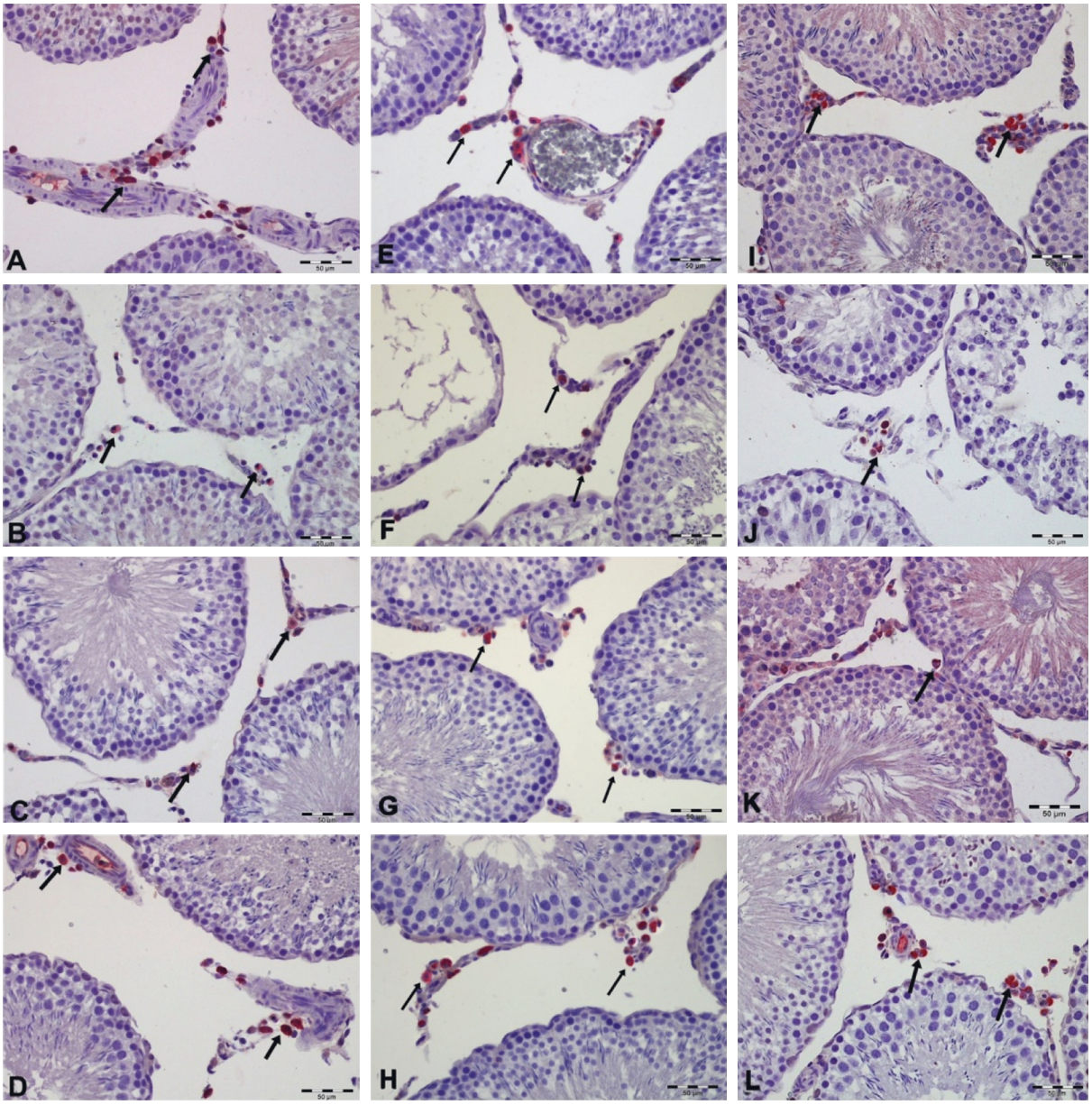
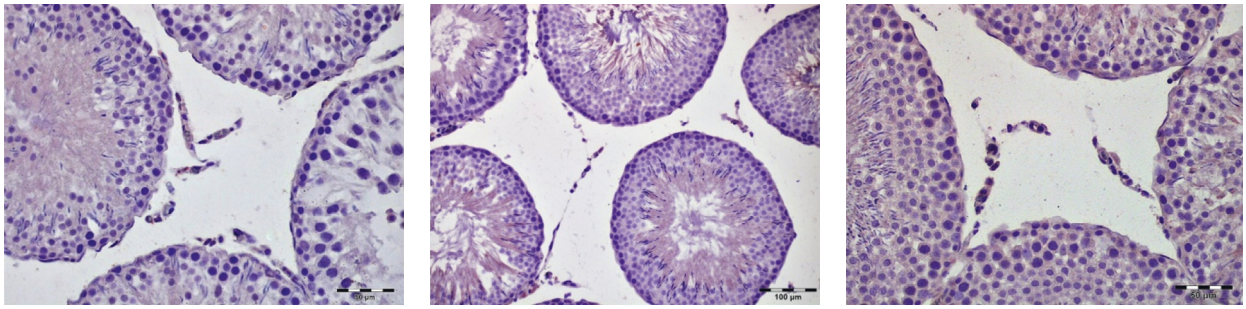


Figure 5: 3βHSD, INSL3, and GPRC6A immunoreactivity in testicular tissue samples of EDS-administered subjects (Group 2). (a) High 3βHSD expression was reported in Leydig cells of control subjects (Group 2A). (b) 3βHSD expression decreased in Leydig cells of GnRH-antagonist-administered subjects (Group 2B). (c) A decrease in 3βHSD expression was seen to continue in Leydig cells of GnRH-antagonist-and-osteocalcin-administered subjects (Group 2C). (d) Intensive 3βHSD expression was observed in Leydig cells of only-osteocalcin-administered subjects (Group 2D). (e) High INSL3 expression was detected in Leydig cells of control subjects (Group 2A). (f) INSL3 expression decreased in Leydig cells of GnRH-antagonist-administered subjects (Group 2B). (g) A decrease in INSL3 expression was reported to continue in Leydig cells of GnRH-antagonist-and-osteocalcin-administered subjects (Group 2C). (h) High INSL3 expression was observed in Leydig cells of only-osteocalcin-administered subjects (Group 2D). (i) High INSL3 expression was seen in Leydig cells of control subjects (Group 2A). (j) GPRC6A expression decreased in Leydig cells of GnRH-antagonist-administered subjects (Group 2B). (k) A decrease in GPRC6A expression was seen to continue in Leydig cells of GnRH-antagonist-and-osteocalcin-administered subjects (Group 2C). (l) High GPRC6A expression was observed in only-osteocalcin-administered subjects (Group 2D). Bar = 50 μm.

absence of any expression in the interstitium was interpreted as that EDS effectively eliminates Leydig cells (Figure 6).

3.3. Scoring Results. According to the results of immunohistochemical scoring, 3βHSD, INSL3, and GPRC6A immunoreactivity was significantly lower in testicular tissue sections of Group 1B, Group 1C, Group 2B, and Group 2C subjects administered with GnRH antagonist and GnRH antagonist together with osteocalcin when compared to control groups. Results of 3βHSD, INSL3, and GPRC6A immunohistochemical scoring of testicular tissue sections of subjects administered with GnRH antagonist and osteocalcin did not show any statistically significant increase when



(a) (b) (c)

Figure 6: No (a) 3βHSD, (b) INSL3, and (c) GPRC6A immunoreactivity was observed in the testicular tissue sections of the subjects sacrificed on the 4th day after EDS injection. Bar = 50 μm.

compared to only-GnRH-antagonist-administered subjects. However, 3βHSD immunohistochemical scoring results of only-osteocalcin-administered subjects did not show any significant difference from the testicular tissues of subjects in control groups, whereas INSL3 and GPRC6A immunoreactivity scores of the same groups showed a significant increase when compared to the control groups. When ucOC levels of the GnRH-antagonist-administered and GnRH-antagonist-and-osteocalcin-administered groups were compared with those of osteocalcin-administered groups, a significant increase was found in GnRH-antagonist-and-osteocalcin-administered groups (Figure 7). In consideration of the number of 3βHSD-positive Leydig cells, it was seen that the number of Leydig cells markedly decreased in testicular tissue sections of subjects administered with GnRH antagonist. However, a slight increase in the number of 3βHSD-positive Leydig cells was found to be statistically insignificant in testicular tissue sections of subjects administered with GnRH antagonist and osteocalcin. It was noted that 3βHSD-positive Leydig cell numbers in testicular tissue sections of only-osteocalcin-administered subjects did not show any significant difference when compared to control groups (Figure 8).

3.4. Electron Microscopic Results. Testicular tissue samples of the control group subjects maintained under normal laboratory conditions showed normal seminiferous tubules and interstitium, at the electron microscopic level (Figures 9(a) and 10(a)).

Electron microscopic examination of testicular tissue samples of GnRH-antagonist-administered subjects showed thickening and irregularity in the membrane propria. Spermatogenic cells were not observed in many seminiferous tubules because of tight junction disruption between Sertoli cells. In addition, excessive SER vacuolization was observed in the Sertoli cell cytoplasm. Nuclei of some Sertoli cells showed an increase in electron density (Figures 9(b) and 10(b)). The nucleus of Leydig cells in the interstitium was observed to have become pyknotic and displayed peripheral heterochromatin accumulations. In some Leydig cells, a decrease in cytoplasmic volume along with an increase in electron density was observed. On the contrary, in some of them, low electron density in the cytoplasm and intensive SER vacuolization were noted. All in all, in the cytoplasm of all Leydig cells, the integrity of the mitochondria was preserved and a significant reduction in lipid droplets was reported (Figures 9(c), 10(c), and 10(d)).

In electron microscopic examination of testicular tissue samples of osteocalcin-and-GnRH-antagonist-administered subjects, it was seen that the thickening and irregularity of the membrane propria went relatively down in comparison with the subjects administered with only GnRH antagonist. In contrast to only-GnRH-antagonist-administered groups, there were not any empty seminiferous tubules. On the contrary, giant vacuoles in the cytoplasm of Sertoli cells were observed to be maintained (Figures 9(d) and 10(e)). Although the number of indentations in the nuclei of the Leydig cells indicated an increase, the heterochromatin content of these nuclei decreased when compared to Group 1B. While mitochondria in the Leydig cell cytoplasm were in normal structure, vacuolization in SER cisternae was noted. In addition, it was determined that the cytoplasmic volumes of many Leydig cells declined and contained a few lipid droplets. However, in the Leydig cell cytoplasm, it was shown that SER and mitochondria retained their normal structure (Figures 9(e) and 10(f)).

In the electron microscopic examination of testicular tissue samples of osteocalcin-and-GnRH-antagonist-administered subjects, it was seen that seminiferous tubules and interstitium were similar to those in the control groups. Sertoli and spermatogenic cells forming the seminiferous tubule epithelium were observed as normal. The tight junctions between the Sertoli cells were intact (Figures 9(f) and 10(g)). In contrast to Group 1B and Group 1C, Leydig cell nuclei indicated less heterochromatin and indentation, while cytoplasmic volumes were in normal size and contained a large number of lipid droplets. As a result, Leydig cells were observed to preserve normal structure in terms of nuclei and cytoplasmic organelles (Figures 9(g) and 10(h)).

3.5. Biochemical Results. In order to determine whether adult Leydig cells were eliminated as a result of single-dose EDS administration or not, serum samples of the subjects sacrificed on the 4th day after EDS administration were compared with the serum samples of the untreated adult subjects. In the EDS group, serum testosterone and LH levels were found to be significantly reduced. However, a

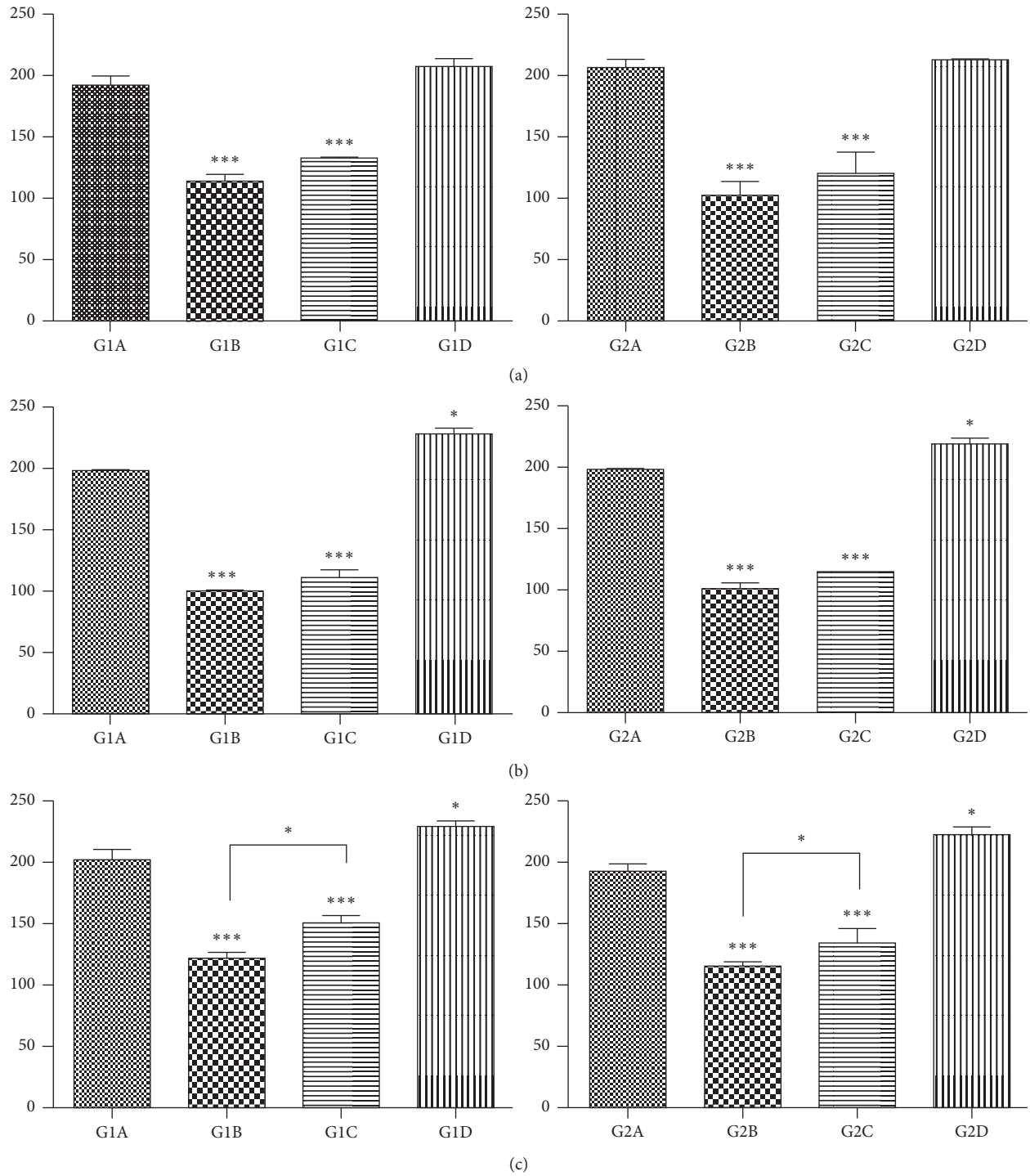


Figure 7: Statistical evaluation of 3βHSD (a), INSL3 (b), and GPRC6A (c) immunoreactivity scores of Leydig cells in testicular tissue sections of Group 1 and Group 2 subjects. $^{***}p < 0.0001$; $^{**}p < 0.001$; $^{*}p < 0.01$.

significant difference was not seen in the ucOC level of these subjects sacrificed on the 4th day after EDS when compared to the control group (Figure 11).

According to biochemical data, subjects administered with GnRH antagonist were found to have significantly low testosterone, LH, and ucOC levels in comparison to not only control groups but also osteocalcin-administered groups. In addition to this, testosterone and LH levels were significantly less in GnRH-antagonist-and-osteocalcin-administered groups when compared to the control groups and osteocalcin groups. On the contrary, a slight and statistically insignificant increase in testosterone levels was observed when compared to only-GnRH antagonist-administered groups, while LH levels of GnRH-antagonist-and-

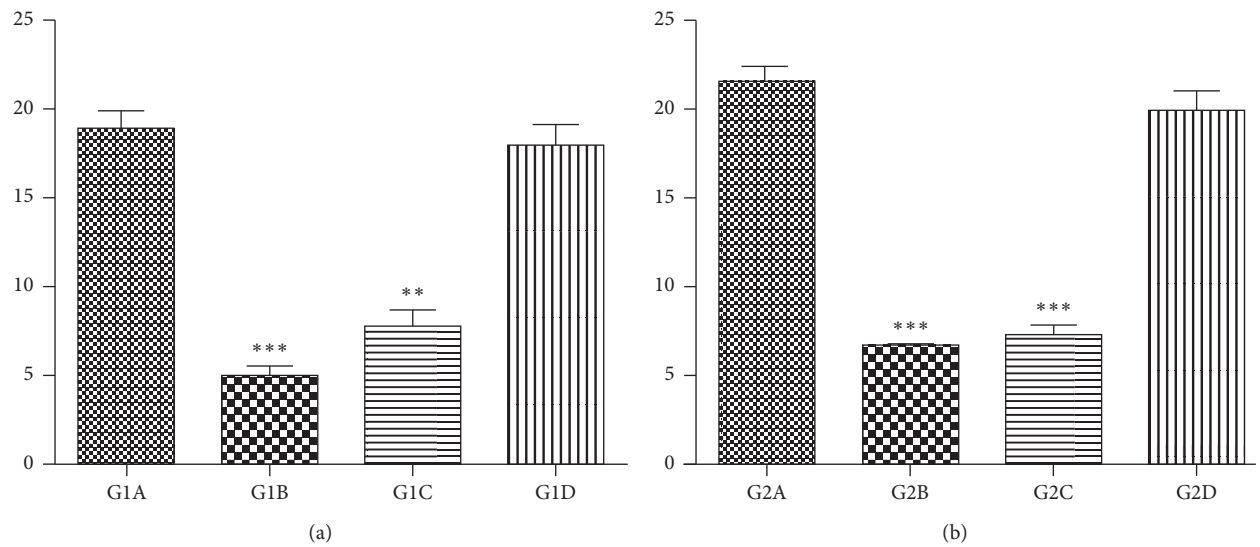


Figure 8: Statistical evaluation of 3βHSD-positive Leydig cell numbers in testicular tissue sections of Group 1 (a) and Group 2 (b) subjects. $^{***}p < 0.0001$; $^{**}p < 0.001$; $^{*}p < 0.01$.

osteocalcin-administered groups were not found to be significantly different from those of only-GnRH-administered groups. No significant difference was found between ucOC levels of Group 1A and Group 1C. However, subjects in Group 1B had significantly lower ucOC levels than Group 1C subjects. Moreover, ucOC levels of each subgroup of Group 1 were found to be higher than those of Group 2. Although LH levels of only-osteocalcin-administered groups did not significantly differ from those of the control groups, the testosterone and ucOC levels of these groups were significantly higher than those of the control groups (Figure 12).

4. Discussion

In this study, it is determined that, during postnatal normal adult Leydig cell development or new adult Leydig cell regeneration after postpubertal EDS treatment, the positive effects of osteocalcin on testosterone and INSL3 synthesis are not completely independent of LH. Within 3-4 days following a single dose of EDS injection in adult rats, elimination in adult Leydig cells, decrease in testosterone production, and reduction in weights of the testis, epididymis, prostate gland, and seminal vesicle have been reported [37–39, 52–54]. In our study, 4 days after a single dose of 75 mg/kg EDS, no Leydig cell marker 3βHSD expression in the interstitium of testicular tissue samples obtained from injected postpubertal subjects was found. Furthermore, serum LH and testosterone levels of the same subjects were shown to decrease.

As a medical hypophysectomy model, there are numerous studies about chronic GnRH antagonist applications that only inhibit GnRH release [55–61]. So, GnRH antagonist administration was preferred to determine whether the osteocalcin effects are LH dependent or not. In this research, LH suppression through administering a high dose of GnRH antagonist caused a significant decrease in serum LH and testosterone levels together with Leydig cell numbers when compared to normal subjects. In addition, there were significant degenerative changes in seminiferous tubules and interstitium due to impaired HPG axis. LH-controlled sex steroid hormone secretion has been reported to have a role in bone metabolism besides sexual maturation and reproductive functions in many studies [62–64]. Also, bone tissue has been found to regulate the synthesis and secretions of steroid hormones such as an endocrine organ as a result of clinical research studies and observations carried out within the last 10 years [4–6, 9]. In in vitro and in vivo studies, it has been put forward that LH and OC do not regulate each other; therefore, OC stimulates testosterone synthesis in Leydig cells via a second endocrine axis between the bone and the testis independently of the HPG axis [4]. This steroidogenesis process is activated by osteocalcin binding to GPRC6A, a specific receptor in Leydig cells. In our study, low levels of ucOC in subjects after GnRH antagonist application have shown that osteocalcin was adversely affected by decreasing testosterone levels because of suppression of LH. Moreover, this significant relationship between osteocalcin and testosterone has been shown not to be completely independent of LH in in vivo conditions. In this study, serum LH and testosterone levels of subjects administered with GnRH antagonist together with osteocalcin were reported to be significantly lower than those of control groups and only-osteocalcin-administered groups. However, there was no significant difference between LH levels compared to the GnRH antagonist groups, whereas serum testosterone and ucOC levels indicated a small increase. This non-significant increase in testosterone levels of subjects administered with GnRH antagonist together with osteocalcin showed that osteocalcin cannot fully tolerate decreased testosterone levels because of LH suppression by GnRH antagonist. Serum LH levels of subjects administered with only osteocalcin were similar to those of the control groups, while testosterone and ucOC levels were significantly higher

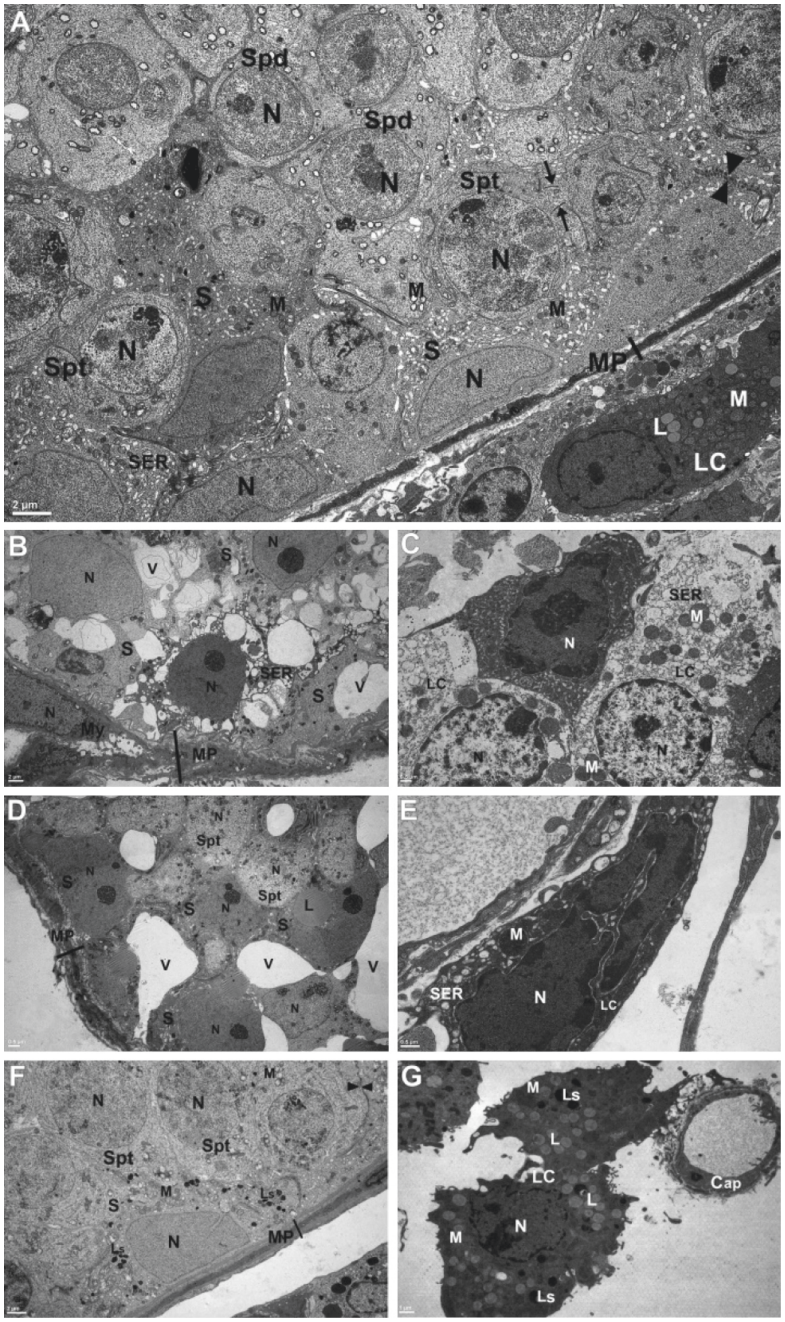


Figure 9: Electron microscopic view of testicular tissue samples of postnatal subjects (Group 1). (a) Membrane propria (MP), Sertoli cells (S), spermatocytes (Spt), spermatids (Spd), and nucleus (N) were seen to be normal in control subjects (Group 1A). Tight connections between the Sertoli cells (arrowhead) were observed as normal like the cytoplasmic bridges (arrows) between the spermatogenic cells. Leydig cells (LC) were seen to have normal tubular-type mitochondria (M) and abundant lipid droplets (L) in their cytoplasm. SER: agranular endoplasmic reticulum; bar = 2 μm. (b) Thick and curved membrane propria (MP) and Sertoli cells (S) containing excessive and abnormal vacuolization (V) were indicated in GnRH-antagonist-administered subjects of Group 1 (Group 1B). My: myoid cells. (c) A decrease in the cytoplasmic volumes of some Leydig cells (LC) and an increase in the peripheral heterochromatin patches in the nucleus (N) were seen in testicular tissue samples of Group 1B. In the cytoplasm of some Leydig cells (LC), low electron density, dense agranular endoplasmic reticulum (SER) vacuolization, and intact mitochondria (M) were noted. Bar = 0.5 μm. (d) Relatively reduced curvature and thickness in the membrane propria (MP) were observed. Giant vacuoles (V) between Sertoli cells (S) and relatively normal spermatocytes (Spt) were seen in GnRH-antagonist-and-osteocalcin-administered subjects of Group 1 (Group 1C). (e) Decreased cytoplasmic volume in Leydig cells (LC) showed an increase in indentation and a decrease in heterochromatin content of the nucleus (N) in testicular tissue samples of Group 1C. In the cytoplasm, it was noted that agranular endoplasmic reticulum (SER) seemed to have vacuolization, but mitochondria (M) remained intact. Bar = 0.5 μm. (f) Sertoli cells (S) and spermatocytes (Spt) were also found to be normal in seminiferous tubules surrounded by normal membrane propria (MP) in only-osteocalcin-administered subjects of Group 1 (Group 1D). The tight junctions between the Sertoli cells (arrowhead) were normal. Ls: lysosome; bar = 1 μm. (g) Increased lipid droplets (L) were observed in the cytoplasm of the Leydig cells (LC), which have decreased heterochromatin content and indentation in the nuclei (N) of testicular tissue samples of Group 1D. Cap: capillary; bar = 1 μm.

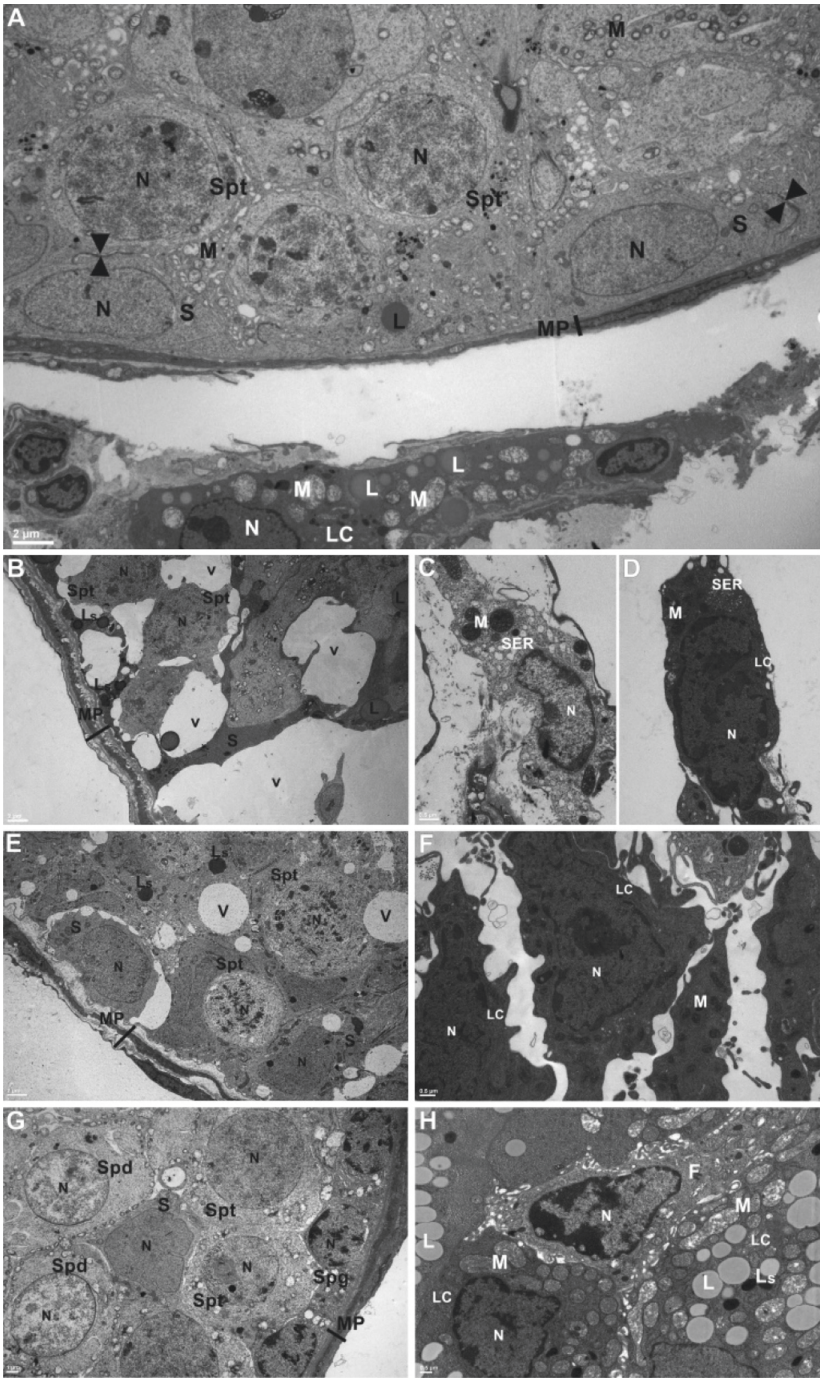


Figure 10: Electron microscopic view of testicular tissue samples of EDS-administered subjects (Group 2). (a) Membrane propria (MP), Sertoli cells (S), spermatocytes (Spt), and spermatids (Spd) were in normal structure in control subjects of Group 2 (Group 2A). Tight connections between the Sertoli cells (arrowhead) were seen in normal structure. Tubular-type mitochondria (M) and abundant lipid droplets (L) were observed in the cytoplasm of Leydig cells (LC). N: nucleus; bar = 2 μm. (b) An increase in membrane propria (MP) total thickness and giant vacuoles (V) between Sertoli cells (S) and spermatocytes (Spt) was observed in GnRH-antagonist-administered subjects of Group 2 (Group 2B). Ls: lysosome; bar = 2 μm. (c) Low electron density and agranular endoplasmic reticulum (SER) vacuolization were seen in some Leydig cells (LC) in testicular tissue samples of Group 2B. Mitochondria (M) were intact. Bar = 0.5 μm. (d) In some Leydig cells, a decrease in the cytoplasmic volume and an increase in peripheral heterochromatin patches in the nucleus (N) were also observed in testicular tissue samples of Group 2B. Bar = 0.5 μm. (e) Thickening and irregularity in the membrane propria (MP) were seen to continue. A relative decrease in the volume of the vacuoles between Sertoli cells (S) and spermatocytes (Spt) was observed in GnRH-antagonist-and-osteocalcin-administered subjects of Group 2 (Group 2C). Bar = 2 μm. (f) A decrease in the cytoplasmic volumes of Leydig cells (LC) and a decrease in the amount of indentation in the nucleus (N) and in the content of heterochromatin were observed in testicular tissue samples of Group 2C. Bar = 0.5 μm. (g) Sertoli cells (S), spermatogonia (Spg), and spermatocytes (Spt) surrounded by membrane propria (MP) were seen to be normal in only-osteocalcin-administered subjects of Group 2 (Group 2D). Bar = 1 μm. (h) Numerous lipid droplets (L) were observed in the cytoplasm of Leydig cells (LC) containing the nucleus (N) in normal structure in testicular tissue samples of Group 2D. F: fibroblast; bar = 0.5 μm.

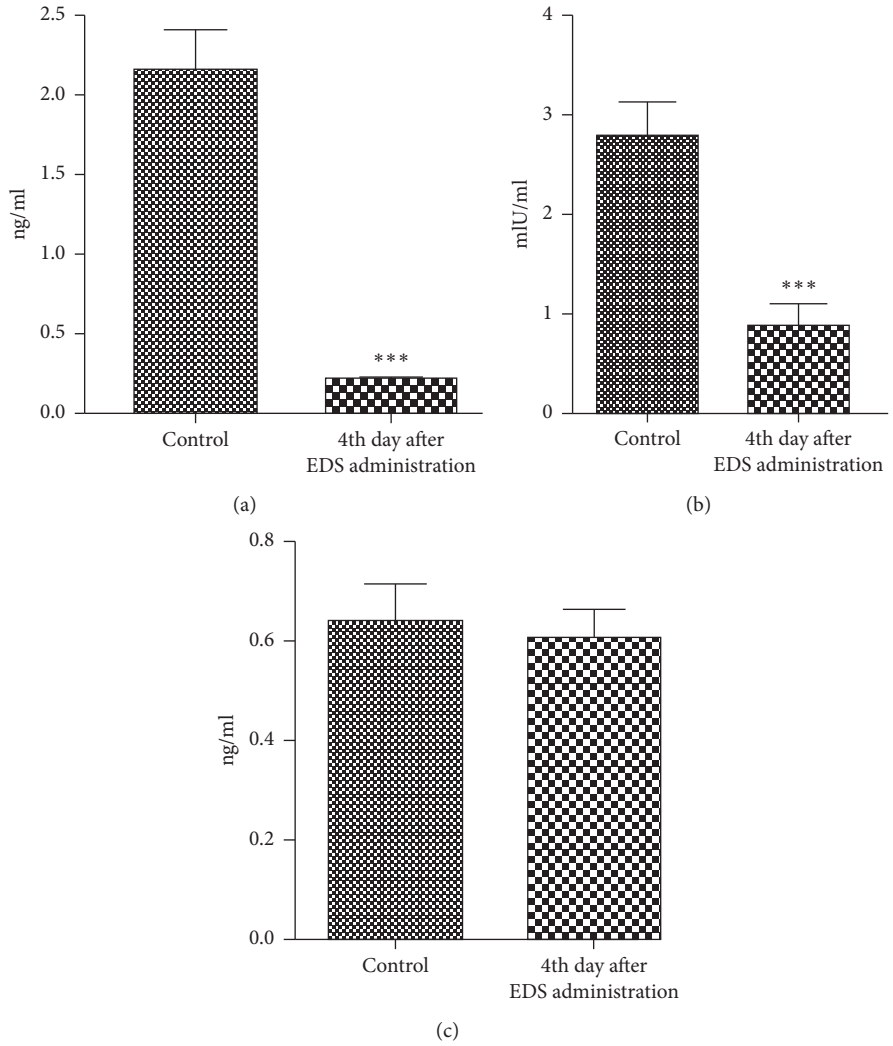


Figure 11: Statistical evaluation of serum testosterone (a), LH (b), and ucOC (c) levels of subjects sacrificed on the 4th day after EDS administration. $^{***}p < 0.0001$; $^{**}p < 0.001$; $^{*}p < 0.01$.

than those of control groups. Whilst no significant difference was observed in terms of 3βHSD expression when testicular tissue sections were compared with control groups, it was observed that the expression of INSL3 and GPRC6A showed a significant increase because of exogenous osteocalcin administration. Immunohistochemical and biochemical findings suggest that osteocalcin does not cause considerable change in Leydig cell number; however, it may increase the function of current Leydig cells in only-osteocalcin-administered groups. In addition to the biochemical and immunohistochemical findings of only-osteocalcin-administered groups, light-electron microscopic examination showed seminiferous tubules and interstitium structure were found to be normal in testicular tissue samples.

In parallel with LH secretions at puberty, the level of testosterone is known to increase, together with the rise in osteocalcin levels, because of the skeletal development [56, 65, 66]. So in this study, it was seen that the ucOC levels of the prepubertal subjects in all subgroups were higher than those of the postpubertal EDS-injected subjects. As reported by Mizokami and colleagues [16] and in consistent with this research, after Gla-OC oral administration, it was converted into the Glu-OC form and an increased serum ucOC level which is a hormone form. Comparing GnRH-antagonist-administered and GnRH-antagonist-and-osteocalcin-administered groups, a remarkable increase in ucOC levels was found in the Gla-OC-administered groups.

INSL3 released from Leydig cells under LH control during adulthood regulates the functions of germ cells and Leydig cells by performing autocrine and paracrine effects as well as systemic effects [17, 21]. The positive relationship between LH and INSL3 has been shown in many studies in the literature over the last 15 years. In these studies, reduction in Leydig cell numbers and functions was shown to cause a decrease in INSL3 synthesis [67–72]. The INSL3 level is more sensitive than testosterone in determining Leydig cell damage and can be used clinically to determine androgen suppression [21]. Considering all these studies, the INSL3 concentration is thought to affect the terminal function capacities of the Leydig cells through creating a

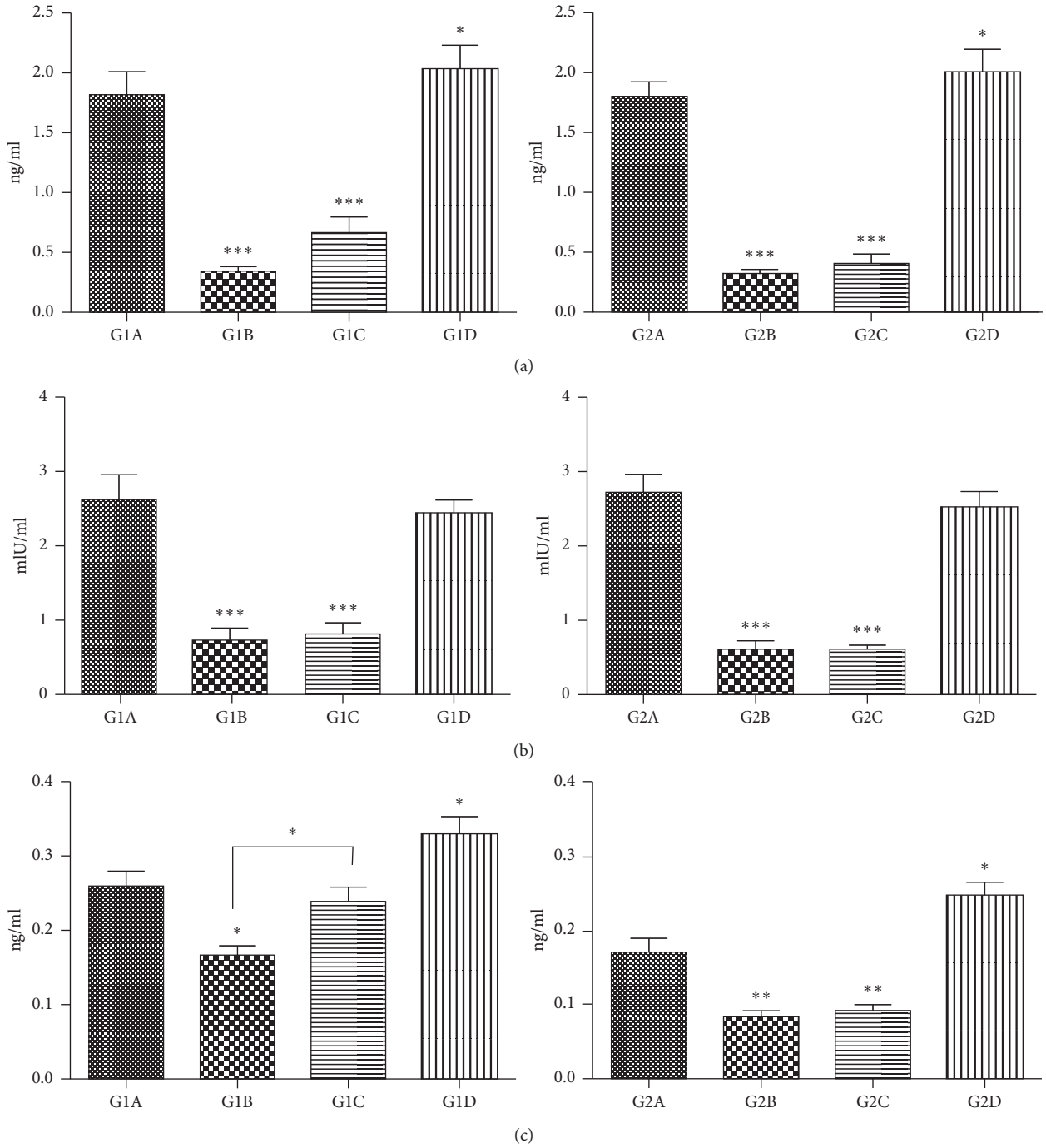


Figure 12: Statistical evaluation of serum testosterone (a), LH (b), and ucOC (c) levels in Group 1 and Group 2 subjects. $^{***}p < 0.0001$; $^{**}p < 0.001$; $^{*}p < 0.01$.

damage memory in cells during or after the development of the Leydig cells [17]. Although there are numerous studies showing a positive relationship between INSL3 and LH, only one study has been found in the literature investigating the association of INSL3 with osteocalcin. In the study published by Overvad et al. in 2014, researchers reported a positive correlation between INSL3 and osteocalcin by associating the decrease in bone mineral density with low INSL3 levels in patients with Klinefelter syndrome [43]. The findings of this study support the positive relation between LH, osteocalcin, and INSL3. In our study, because of LH suppression in the subjects administered with GnRH antagonist, an outstanding decrease in the expression of INSL3 and GPRC6A was observed in parallel with the decrease in ucOC and testosterone levels. In addition, in subjects administered with GnRH antagonist and osteocalcin, ucOC, INSL3, and GPRC6A expressions were reported to be significantly lower than those in control groups and only-osteocalcin-

administered groups. It can be said that decreasing testosterone levels as a result of LH suppression decreases the level of ucOC in these groups, and therefore, there is a decrease in the expression of osteocalcin receptors GPRC6A and INSL3 which are positively associated with osteocalcin. On the contrary, it is determined that osteocalcin against severe loss of function observed in Leydig cells due to LH suppression does not cause any significant increase in the expression of INSL3, which is used as a function marker for Leydig cells. However, further studies are needed to answer the question of whether the decrease in INSL3 expression is due to LH suppression or indirectly sourced from the decrease in the osteocalcin level. Besides, the level of ucOC in subjects administered with GnRH antagonist together with osteocalcin showed a statistically insignificant increase when compared to only-GnRH-antagonist-administered subjects. This finding shows that INSL3 expression in LH control is also stimulated by osteocalcin such as testosterone when the LH release is suppressed. It was noted that osteocalcin caused a significant increase in the expression of INSL3 and GPRC6A by inducing the Leydig cell function in testicular tissue sections of the subjects administered with only osteocalcin. This increase in GPRC6A activity in only-osteocalcin-administered groups describes the significant increase in testosterone synthesis mediated by GPRC6A in the same groups. Furthermore, an increase in GPRC6A immunoreactivity may occur due to the fact that osteocalcin as a ligand increases its own receptor GPRC6A expression density or the number of receptors by using different signal pathways. On the contrary, it is a matter of discussion whether this increase in the testosterone level in the same groups stems from the effect of osteocalcin on increasing the number or activity of GPRC6A receptors or the increase in expression of INSL3.

Although OC has been shown to stimulate testosterone synthesis independently of LH in many studies in in vitro conditions or using LHR−/− mice, no study has investigated the relationship of OC-testosterone in subjects undergoing LH suppression in in vivo conditions. In this study, whether OC was able to stimulate testosterone synthesis in patients who had an LH insensitivity problem or those who were diagnosed with hypogonadism due to LH deficiency was investigated. Based on these findings, although it was determined that OC stimulates testosterone synthesis moderately, the main effect was shown only in the subjects who had osteocalcin treatment without any LH suppression. According to the 3βHSD immunoreactivity scores and Leydig cell number analysis, osteocalcin is believed to increase the current Leydig cell function, which resulted in an increase in the level of testosterone, without any increase in the number of Leydig cells. Our study was the first to evaluate the relationship between osteocalcin and testosterone according to the Leydig cell numbers and Leydig cell functions during the development of adult Leydig cells. Additionally, in our study, the matter of whether OC could induce INSL3 synthesis in LH-suppressed subjects in the development process of adult Leydig cells like testosterone or not was investigated. It was determined that the expression of INSL3 such as testosterone increased by OC independently of LH, whereas in the subjects that did not undergo LH suppression, OC raised the Leydig cell function and INSL3 expression above the normal limits. Thus, this study is the first study to investigate the relationship between osteocalcin and INSL3 in the development process of adult Leydig cells.

The most important advantage of this reciprocal functional relationship between osteocalcin and Leydig cell functions is bone health protection. However, in these conditions, unexpected side effects may occur in other systems. Therefore, there is a need for further studies to reveal the physiological relation between multiple tissues and organs such as nutrition, skeletal health, and reproduction. In recent years, except for environmental and genetic factors, the use of exogenous testosterone in men diagnosed with hypogonadism due to aging or testicular damage has been known to reduce long-term LH secretion and endogenous testosterone production. In addition to osteoporosis resulting from the decrease in bone mineral density, various side effects such as loss of muscle strength and sexual function, cardiovascular diseases, kidney failure, and prostate cancer have been reported [1, 2]. In order to eliminate all these side effects, it has been concluded that exogenous osteocalcin may be a new therapeutic route rather than testosterone replacement therapies, especially against primary hypogonadism or for improving existing fertility conditions.

5. Conclusion

When all the findings were evaluated together in this research, after oral administration of Gla-OC, a positive correlation between serum ucOC, testosterone, and INSL3 levels during the differentiation of adult Leydig cells has been put forward. However, this correlation was not identified completely independent of LH since it was observed to be indirectly affected by LH suppression. During the development of adult Leydig cells, even though ucOC has shown to have no change in the number of Leydig cells in the presence of LH, it had a positive effect on increasing the functional capacity of the current Leydig cells.

Abbreviations

EDS:	Ethane dimethane sulphonate
OC:	Osteocalcin
HPG axis:	Hypothalamic-pituitary-gonadal axis
INSL3:	Insulin-like factor-3
LH:	Luteinizing hormone
GnRH:	Gonadotrophin-releasing hormone
3βHSD:	3β-Hydroxysteroid dehydrogenase
GPRC6A:	G protein-coupled receptor family C group 6 member alpha
ucOC:	Uncarboxylated osteocalcin
cOC:	Carboxylated osteocalcin
LHR:	Luteinizing hormone receptor
hCG:	Human chorionic gonadotropin
PMSG:	Pregnant mare serum gonadotropin.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

This study was supported by the "Education Membership Program" of Council of Higher Education, Turkey.

References

[1] F. M. Köhn, "Testosterone and body functions," *The Aging Male*, vol. 9, no. 4, pp. 183–188, 2006.

[2] C. Wang, G. Cunningham, A. Dobs, A. Iranmanesh, A. M. Matsumoto et al., "Long-term testosterone gel (AndroGel) treatment maintains beneficial effects on sexual function and mood, lean and fat mass, and bone mineral density in hypogonadal men," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 5, pp. 2085–2098, 2004.

[3] L. Katznelson, J. S. Finkelstein, D. A. Schoenfeld, D. I. Rosenthal, E. J. Anderson, and A. Klibanski, "Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism," *Journal of Clinical Endocrinology & Metabolism*, vol. 81, no. 12, pp. 4358–4365, 1996.

[4] F. Oury, M. Ferron, W. Huizhen, C. Confavreux, L. Xu et al., "Osteocalcin regulates murine and human fertility through a pancreas-bone-testis axis," *Journal of Clinical Investigation*, vol. 123, no. 6, pp. 2421–2433, 2013.

[5] F. Oury, G. Sumara, O. Sumara et al., "Endocrine regulation of male fertility by the skeleton," *Cell*, vol. 144, no. 5, pp. 796–809, 2011.

[6] G. Karsenty, "The mutual dependence between bone and gonads," *Journal of Endocrinology*, vol. 213, no. 2, pp. 107–114, 2012.

[7] V. Schwetz, T. Pieber, and B. Obermayer-Pietsch, "Mechanisms in endocrinology: the endocrine role of the skeleton: background and clinical evidence," *European Journal of Endocrinology*, vol. 166, no. 6, pp. 959–967, 2012.

[8] A. Patti, L. Gennari, D. Merlotti, F. Dotta, and R. Nuti, "Endocrine actions of osteocalcin," *International Journal of Endocrinology*, vol. 2013, Article ID 846480, 10 pages, 2013.

[9] L. De Toni, A. Di Nisio, M. S. Rocca, M. De Rocco Ponce, A. Ferlin, and C. Foresta, "Osteocalcin, a bone-derived hormone with important andrological implications," *Andrology*, vol. 5, no. 4, pp. 664–670, 2017.

[10] N. K. Lee, H. Sowa, E. Hinoi et al., "Endocrine regulation of energy metabolism by the skeleton," *Cell*, vol. 130, no. 3, pp. 456–469, 2007.

[11] M. Ferron, J. Wei, T. Yoshizawa et al., "Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism," *Cell*, vol. 142, no. 2, pp. 296–308, 2010.

[12] J. Shao, Z. Wang, T. Yang, H. Ying, Y. Zhang, and S. Liu, "Bone regulates glucose metabolism as an endocrine organ through osteocalcin," *International Journal of Endocrinology*, vol. 2015, Article ID 967673, 9 pages, 2015.

[13] S. L. Booth, A. Centi, S. R. Smith, and C. Gundberg, "The role of osteocalcin in human glucose metabolism: marker or mediator?," *Nature Reviews Endocrinology*, vol. 9, no. 1, pp. 43–55, 2013.

[14] M. Ferron, E. Hinoi, G. Karsenty, and P. Ducy, "Osteocalcin differentially regulates β cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice," *Proceedings of the National Academy of Sciences*, vol. 105, no. 13, pp. 5266–5270, 2008.

[15] M. Ferron, M. D. McKee, R. L. Levine, P. Ducy, and G. Karsenty, "Intermittent injections of osteocalcin improve glucose metabolism and prevent type 2 diabetes in mice," *Bone*, vol. 50, no. 2, pp. 568–575, 2012.

[16] A. Mizokami, Y. Yasutake, J. Gao et al., "Osteocalcin induces release of glucagon-like peptide-1 and thereby stimulates insulin secretion in mice," *PLoS One*, vol. 8, no. 2, Article ID e57375, 2013.

[17] R. Ivell, K. Heng, and R. Anand-Ivell, "Insulin-like factor 3 and the HPG axis in the male," *Frontiers in Endocrinology (Lausanne)*, vol. 5, no. 6, 2014.

[18] R. Ivell, J. D. Wade, and R. Anand-Ivell, "INSL3 as a biomarker of Leydig cell functionality," *Biology of Reproduction*, vol. 88, no. 6, p. 147, 2013.

[19] R. Anand-Ivell and R. Ivell, "Insulin-like factor 3 as a monitor of endocrine disruption," *Reproduction*, vol. 147, no. 4, pp. R87–R95, 2014.

[20] R. Ivell, M. Balvers, R. Domagalski, H. Ungefroren, N. Hunt, and W. Schulze, "Relaxin-like factor: a highly specific and constitutive new marker for Leydig cells in the human testis," *Molecular Human Reproduction*, vol. 3, no. 6, pp. 459–466, 1997.

[21] A. Ferlin and C. Foresta, "Insulin-like factor 3: a novel circulating hormone of testicular origin in humans," *Annals of the New York Academy of Sciences*, vol. 1041, no. 1, pp. 497–505, 2005.

[22] A. Ferlin, A. Pepe, L. Gianesello et al., "New roles for INSL3 in adults," *Annals of the New York Academy of Sciences*, vol. 1160, no. 1, pp. 215–218, 2009.

[23] A. Ferlin, A. Garolla, F. Rigon, L. Rasi Caldogno, A. Lenzi, and C. Foresta, "Changes in serum insulin-like factor 3 during normal male puberty," *The Journal of Clinical Endocrinology & Metabolism*, vol. 91, no. 9, pp. 3426–3431, 2006.

[24] P. J. O'Shaughnessy, P. J. Baker, and H. Johnston, "The foetal Leydig cell—differentiation, function and regulation," *International Journal of Andrology*, vol. 29, no. 1, pp. 90–95, 2006.

[25] K. Svechnikov, L. Landreh, J. Weisser et al., "Origin, development and regulation of human Leydig cells," *Hormone Research in Paediatrics*, vol. 73, no. 2, pp. 93–101, 2010.

[26] I. B. Barsoum and H. H.-C. Yao, "Fetal Leydig cells: progenitor cell maintenance and differentiation," *Journal of Andrology*, vol. 31, no. 1, pp. 11–15, 2010.

[27] K. J. Teerds and I. T. Huhtaniemi, "Morphological and functional maturation of Leydig cells: from rodent models to primates," *Human Reproduction Update*, vol. 21, no. 3, pp. 310–328, 2015.

[28] B. Gondos, D. C. Paup, J. Ross, and R. A. Gorski, "Ultrastructural differentiation of Leydig cells in the fetal and postnatal hamster testis," *The Anatomical Record*, vol. 178, no. 3, pp. 551–565, 1974.

[29] H. M. Scott, J. I. Mason, and R. M. Sharpe, "Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds," *Endocrine Reviews*, vol. 30, no. 7, pp. 883–925, 2009.

[30] T. Klonisch, P. A. Fowler, and S. Hombach-Klonisch, "Molecular and genetic regulation of testis descent and external

genitalia development," *Developmental Biology*, vol. 270, no. 1, pp. 1–18, 2004.

[31] R. Habert, H. Lejeune, and J. M. Saez, "Origin, differentiation and regulation of fetal and adult Leydig cells," *Molecular and Cellular Endocrinology*, vol. 179, no. 1-2, pp. 47–74, 2001.

[32] H. Chen, R. S. Ge, and B. R. Zirkin, "Leydig cells: from stem cells to aging," *Molecular and Cellular Endocrinology*, vol. 306, no. 1-2, pp. 9–16, 2009.

[33] R.-S. Ge, Q. Dong, C. M. Sottas, V. Papadopoulos, B. R. Zirkin, and M. P. Hardy, "In search of rat stem Leydig cells: identification, isolation, and lineage-specific development," *Proceedings of the National Academy of Sciences*, vol. 103, no. 8, pp. 2719–2724, 2006.

[34] L. Benton, L. X. Shan, and M. P. Hardy, "Differentiation of adult Leydig cells," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 53, no. 1–6, pp. 61–68, 1995.

[35] Y. Zhang, R. Ge, and M. P. Hardy, "Androgen-forming stem Leydig cells: identification, function and therapeutic potential," *Disease Markers*, vol. 24, no. 4-5, pp. 277–286, 2008.

[36] R. Ivell and R. Anand-Ivell, "Biology of insulin-like factor 3 in human reproduction," *Human Reproduction Update*, vol. 15, no. 4, pp. 463–476, 2009.

[37] Yu-F. Zhang, K.-M. Yuan, Y. Liang et al., "Alterations of gene profiles in Leydig-cell-regenerating adult rat testis after ethane dimethane sulfonate-treatment," *Asian Journal of Andrology*, vol. 17, no. 2, pp. 253–260, 2015.

[38] K. J. Teerds, M. de Boer-Brouwer, J. H. Dorrington, M. Balvers, and R. Ivell, "Identification of markers for precursor and Leydig cell differentiation in the adult rat testis following ethane dimethyl sulphonate Administration," *Biology of Reproduction*, vol. 60, no. 6, pp. 1437–1445, 1999.

[39] S. Ariyaratne, I. Kim, N. Mills, I. Mason, and C. Mendis-Handagama, "Effects of ethane dimethane sulfonate on the functional structure of the adult rat testis," *Archives of Andrology*, vol. 49, no. 4, pp. 313–326, 2003.

[40] J. T. M. Vreeburg, M. P. Ooms, F. F. G. Rommerts, and K. J. Teerds, "Functional properties of developing rat Leydig cells after treatment with ethylene dimethanesulphonate (EDS)," *Reproduction*, vol. 84, no. 1, pp. 63–69, 1988.

[41] J. Guo, H. Zhou, Z. Su et al., "Comparison of cell types in the rat Leydig cell lineage after ethane dimethanesulfonate treatment," *Reproduction*, vol. 145, no. 4, pp. 371–380, 2013.

[42] E. Nieschlag, H. M. Behre, and S. Nieschlag, *Andrology; Male Reproductive Health and Dysfunction*, Springer, Berlin, Germany, 3rd edition, 2010.

[43] S. Overvad, K. Bay, A. Bojesen, and C. H. Gravholt, "Low INSL3 in Klinefelter syndrome is related to osteocalcin, testosterone treatment and body composition, as well as measures of the hypothalamic-pituitary-gonadal axis," *Andrology*, vol. 2, no. 3, pp. 421–427, 2014.

[44] G. F. Weinbauer, A. Limberger, H. M. Behre, and E. Nieschlag, "Can testosterone alone maintain the gonadotrophin-releasing hormone antagonist-induced suppression of spermatogenesis in the non-human primate?," *Journal of Endocrinology*, vol. 142, no. 3, pp. 485–495, 1994.

[45] C. M. Jackson and H. Jackson, "Comparative protective actions of gonadotrophins and testosterone against the antispermatogenic action of ethane dimethanesulphonate," *Reproduction*, vol. 71, no. 2, pp. 393–401, 1984.

[46] G. Coşkun, H. Özgür, Ş. Doran, and S. Polat, "Ameliorating effects of curcumin on nicotine-induced mice testes," *Turkish Journal of Medical Sciences*, vol. 46, no. 2, pp. 549–560, 2016.

[47] S. M. L. Chamindrani Mendis-Handagama and H. B. Siril Ariyaratne, "Differentiation of the adult Leydig cell population in the postnatal testis," *Biology of Reproduction*, vol. 65, no. 3, pp. 660–671, 2001.

[48] K. J. Teerds, E. Rijntjes, M. B. Veldhuizen-Tsoerkan, F. F. G. Rommerts, and M. de Boer-Brouwer, "The development of rat Leydig cell progenitors in vitro: how essential is luteinising hormone?," *Journal of Endocrinology*, vol. 194, no. 3, pp. 579–593, 2007.

[49] P. J. Baker, H. Johnston, M. Abel, H. M. Charlton, and P. J. O'Shaughnessy, "Differentiation of adult-type Leydig cells occurs in gonadotrophin-deficient mice," *Reproductive Biology and Endocrinology*, vol. 1, no. 1, p. 4, 2003.

[50] D. Bolat, F. Oltulu, A. Uysal et al., "Effects of losartan on experimental varicocele-induced testicular germ cell apoptosis," *Andrologia*, vol. 48, no. 7, pp. 840–846, 2016.

[51] P. M. Petersen and B. Pakkenberg, "Stereological quantitation of Leydig and Sertoli cells in the testis from young and old men," *Image Analysis and Stereology*, vol. 19, no. 3, pp. 215–218, 2000.

[52] M. A. Cornejo-Cortés, C. Sánchez-Torres, J. C. Vázquez-Chagoyán, H. M. Suárez-Gómez, G. Garrido-Fariña, and M. A. Meraz-Ríos, "Rat embryo quality and production efficiency are dependent on gonadotrophin dose in superovulatory treatments," *Laboratory Animals*, vol. 40, no. 1, pp. 87–95, 2006.

[53] N. Atanassova, Y. Koeva, M. Bakalska, E. Pavlova, B. Nikolov, and M. Davidoff, "Loss and recovery of androgen receptor protein expression in the adult rat testis following androgen withdrawal by ethane dimethanesulfonate," *Folia Histochemica et Cytobiologica*, vol. 44, no. 2, pp. 81–86, 2006.

[54] K. Heng, R. Anand-Ivell, K. Teerds, and R. Ivell, "The endocrine disruptors dibutyl phthalate (DBP) and diethylstilbestrol (DES) influence Leydig cell regeneration following ethane dimethane sulphonate treatment of adult male rats," *International Journal of Andrology*, vol. 35, no. 3, pp. 353–363, 2012.

[55] N. Sukcharoen, "GnRH antagonists: an update," *Thai Journal of Obstetrics and Gynaecology*, vol. 12, pp. 71–76, 2000.

[56] S. P. Bliss, A. M. Navratil, J. Xie, and M. S. Roberson, "GnRH signaling, the gonadotrope and endocrine control of fertility," *Frontiers in Neuroendocrinology*, vol. 31, no. 3, pp. 322–340, 2010.

[57] C. Gobello, "Effects of GnRH antagonists vs agonists in domestic carnivores, a review," *Reproduction in Domestic Animals*, vol. 47, no. 6, pp. 373–376, 2012.

[58] M. M. Misro, A. Ganguly, and R. P. Das, "GnRH antagonist treatment affects nuclear size and membrane associated indentations in rat Leydig cells," *Archives of Andrology*, vol. 27, no. 1, pp. 25–33, 1991.

[59] A. Ganguly, M. M. Misro, and R. P. Das, "Roles of FSH and testosterone in the initiation of spermatogenesis in prepubertal rats medically hypophysectomized by a GnRH antagonist," *Archives of Andrology*, vol. 32, no. 2, pp. 111–120, 1994.

[60] M. A. Hannan, N. Kawate, Y. Fukami et al., "Effects of long-acting GnRH antagonist, degarelix acetate, on plasma insulin-like peptide 3, testosterone and luteinizing hormone concentrations, and scrotal circumference in male goats," *Theriogenology*, vol. 88, pp. 228–235, 2017.

[61] M. M. Misro, A. Ganguly, and R. P. Das, "Adverse effects of chronic GnRH antagonist administration on seminiferous epithelium in adult rats," *Archives of Andrology*, vol. 29, no. 1, pp. 69–78, 1992.

[62] T. Nakamura, Y. Imai, T. Matsumoto et al., "Estrogen prevents bone loss via estrogen receptor α and induction of fas ligand in osteoclasts," *Cell*, vol. 130, no. 5, pp. 811–823, 2007.
[63] B. L. Riggs, S. Khosla, and L. J. Melton III, "Sex steroids and the construction and conservation of the adult skeleton," *Endocrine Reviews*, vol. 23, no. 3, pp. 279–302, 2002.
[64] D. Vanderschueren, L. Vandenput, S. Boonen, M. K. Lindberg, R. Bouillon, and C. Ohlsson, "Androgens and bone," *Endocrine Reviews*, vol. 25, no. 3, pp. 389–425, 2004.
[65] S. Kirmani, E. J. Atkinson, L. J. Melton III, B. L. Riggs, S. Amin, and S. Khosla, "Relationship of testosterone and osteocalcin levels during growth," *Journal of Bone and Mineral Research*, vol. 26, no. 9, pp. 2212–2216, 2011.
[66] A. Ferlin, R. Selice, U. Carraro, and C. Foresta, "Testicular function and bone metabolism-beyond testosterone," *Nature Reviews Endocrinology*, vol. 9, no. 9, pp. 548–554, 2013.
[67] R. Anand-Ivell, J. Wohlgemuth, M. T. Haren et al., "Peripheral INSL3 concentrations decline with age in a large population of Australian men," *International Journal of Andrology*, vol. 29, no. 6, pp. 618–626, 2006.
[68] K. Bay, S. Hartung, R. Ivell et al., "Insulin-like factor 3 serum levels in 135 normal men and 85 men with testicular disorders: relationship to the luteinizing hormone-testosterone axis," *The Journal of Clinical Endocrinology & Metabolism*, vol. 90, no. 6, pp. 3410–3418, 2005.
[69] K. Bay, H. E. Virtanen, S. Hartung et al., "Insulin-like factor 3 levels in cord blood and serum from children: effects of age, postnatal hypothalamic-pituitary-gonadal axis activation, and cryptorchidism," *The Journal of Clinical Endocrinology & Metabolism*, vol. 92, no. 10, pp. 4020–4027, 2007.
[70] K. Bay, K. L. Matthiesson, R. I. McLachlan, and A.-M. Andersson, "The effects of gonadotropin suppression and selective replacement on insulin-like factor 3 secretion in normal adult men," *The Journal of Clinical Endocrinology & Metabolism*, vol. 91, no. 3, pp. 1108–1111, 2006.
[71] S. Zimmermann, G. Steding, J. M. A. Emmen et al., "Targeted disruption of the *Insl3* gene causes bilateral cryptorchidism," *Molecular Endocrinology*, vol. 13, no. 5, pp. 681–691, 1999.
[72] S. Zimmermann, P. Schöttler, W. Engel, and I. M. Adham, "Mouse Leydig insulin-like (Ley I-L) gene: structure and expression during testis and ovary development," *Molecular Reproduction and Development*, vol. 47, no. 1, pp. 30–38, 1997.