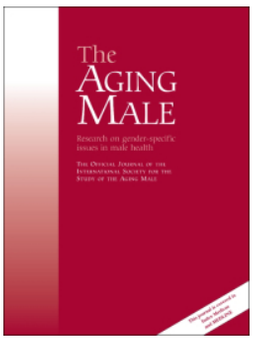

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RESEARCH ARTICLE

Primary, secondary and compensated male biochemical hypogonadism in people living with HIV (PLWH): relevance of sex hormone-binding globulin (SHBG) measurement and comparison between liquid chromatography-tandem mass spectrometry (LC-MS/MS) and chemiluminescent immunoassay for sex steroids assay

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ABSTRACT

Background: Data about classification of hypogonadism and estrogen deficiency in male people living with HIV (PLWH) are scanty.

Aim: To investigate the prevalence and characterization of biochemical hypogonadism and relative estrogen deficiency in male PLWH aged < 50 comparing liquid chromatography-tandem mass spectrometry (LC-MS/MS) with chemiluminescent immunoassay (CI), and combining gonadotropin, sex hormone-binding globulin (SHBG) and serum estradiol (E2) measurements.

Methods: Prospective, cross-sectional, observational study. Serum total testosterone (TT), E2, gonadotropins, SHBG were measured by CI. TT and E2 were also assessed by LC-MS/MS. Free testosterone (cFT) was calculated by Vermeulen equation.

Results: A total of 316 PLWH (45.3 ± 5.3 years) were enrolled. TT and cFT by LC-MS/MS were lower compared to CI ($p < 0.0001$). The prevalence of biochemical hypogonadism was higher with LC-MS/MS than CI, both for TT (5.1% vs 3.2%, $p < 0.0001$) or cFT (9.5% vs 7%, $p < 0.0001$). The prevalence of hypogonadism (overt + compensated) was 17.1% for cFT using LC-MS/MS. Secondary form of hypogonadism was more prevalent than primary. The prevalence of relative estrogen deficiency was of 30.0% among hypogonadal patients and 15.5% among eugonadal.

Conclusions: The prevalence of male hypogonadism results underestimated by CI compared to LC-MS/MS in PLWH, both for TT and cFT. SHBG and gonadotropins are essential for detecting T deficiency.

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Introduction

Hypogonadism is a frequent finding among young to middle-aged men with Human Immunodeficiency Virus (HIV) infection and seems to occur earlier in comparison with HIV-uninfected men [1–3]. Although the prevalence of male hypogonadism among people living with HIV (PLWH) has significantly lowered after the introduction of antiretroviral therapy (ART) [4], it remains high if compared with age-matched HIV-uninfected men, ranging from 13% to 40% in the age group of 20–60 years [3,5,6]. Accordingly, in a recent meta-analysis we fixed the prevalence of male hypogonadism among PLWH to about 26% [4]. Male hypogonadism in PLWH has multi-factorial nature and it is linked to the virus *per se*, to the therapeutic management, to HIV-related comorbidities and to changes in body fat distribution [1–3,7,8]. Signs and symptoms of low serum testosterone (T) are non-specific, of

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mild-to-moderate degree, and often overlapping with those of infection *per se* in PLWH. For these reasons, hypogonadism can be underestimated in the absence of targeted laboratory blood examinations [1,3].

Immunoassay-based techniques are generally used in clinical practice for the assay of serum total T (TT), but they lack adequate accuracy and sensitivity in particular for values in the lowest normal range for men or below it [9–11]. Similarly, immunoassay-based techniques show poor accuracy also in the assessment of serum estradiol (E2), being quite unreliable especially at the low serum E2 concentration typical of men [12–14]; for this reason, liquid chromatography tandem mass spectrometry (LC-MS/MS) represents the recommended methodology for the assessment of TT, E2 and, in general, of adrenal and sexual steroids [9,10,12,15–22]. Moreover, serum T circulates mainly as protein-bound to sex hormone binding globulin (SHBG) and albumin, but only the small fraction of non-protein bound or free T (FT) is responsible for the biological activity of T [9,12,23,24]. Abnormalities in SHBG levels can influence the serum TT reading [23,25], especially in systemic diseases associated to SHBG alterations [26]. Thus, increased levels of SHBG that are typical of the HIV clinical setting may lead to normal levels of TT despite actually low FT levels, causing biochemical hypogonadism [3,7,17]. The importance of SHBG measurement in male PLWH has been largely emphasized by the guidelines on male hypogonadism of the Endocrine Society [27] as well as by recent reviews [3,28], original studies [29], and meta-analysis [4]. At present, however, data on the prevalence of hypogonadism based on the combination of serum sex steroids measured by LC-MS/MS, SHBG and gonadotropins are lacking. In fact, the measurement of serum TT by the recommended methodology has been performed only in a few studies, and none of them considered also E2, SHBG, and gonadotropins levels in order to obtain a detailed and accurate evaluation of the pituitary-gonadal axis; this does not allow a precise esteem of prevalence and a full characterization of male hypogonadism [6,30,31]. Furthermore, serum E2 levels in male PLWH have been evaluated by LC-MS/MS only in two studies, and neither aimed to investigate trends of serum E2 [8,32], thus leaving the issue of relative estrogen deficiency unexplored in this specific population.

The aim of this study was to investigate the prevalence of male biochemical hypogonadism in a cohort of young to middle-aged PLWH by comparing the values obtained using the recommended methodology LC-MS/MS with those of a chemiluminescent immunoassay (CI) for serum TT assessment, and by calculating FT levels according to the validated Vermeulen formula [24,33]. Furthermore, we investigated relative estrogen deficiency and used the measurement of gonadotropins in order to differentiate primary from secondary and compensated hypogonadism and to provide assessment of the hypothalamus-pituitary-gonadal axis function among young to middle-aged male PLWH.

Materials and methods

Study design and participants

A prospective, cross-sectional, observational study was carried out involving male HIV-infected outpatients attending the Modena HIV Metabolic Clinic from May 2013 to December 2017. Three hundred nineteen consecutive male PLWH were assessed for suitability before enrolment in the study by the Unit of Endocrinology of the University of Modena and Reggio Emilia.

Inclusion and exclusion criteria have been previously detailed [8]. Inclusion criteria were men aged 18–50 years, with documented HIV-infection and ongoing HAART treatment.

Exclusion criteria were prior treatment (referred or documented) with androgens, sex steroids, antiandrogens, anabolic agents, GnRH agonists, psychotropic agents; documented hypothyroidism, known pituitary, testicular or adrenal diseases, a previous conventional pituitary surgery or radiotherapy; documented Acquired Immunodeficiency Syndrome (AIDS), active cancer, severe liver insufficiency or severe chronic renal failure (estimated glomerular filtration rate $<$30 ml/min).

Only young to middle-aged (18–50 years) men were included to avoid the effects of aging on gonadal status and to exclude late onset hypogonadism (LOH) as a confounding factor [12,34,35].

At enrolment visit each patient was evaluated for inclusion and exclusion criteria through medical interview and adequate clinical work-up. Finally, 316 male PLWH m*et al*l inclusion criteria and were enrolled in the study.

Main outcome measures

Laboratory analyses

After an overnight fasting an intravenous cannula was inserted into an antecubital vein at 8.00 am to collect a blood sample for serum and plasma assays. The

blood samples were centrifuged, and the serum was stored at −20° C until assayed.

Serum TT [16], serum dihydrotestosterone and serum E2 [36] were measured by two validated LC-MS/MS methods. Sensitivity was 19, 39.1 and 9.8 pg/mL, intra-assay coefficient of variation (CV) was <4%, <6% and <6%, inter-assay CV was <7%, <9% and <7% and accuracy ranged 97–100%, 81–112% and 92–108% for T, DHT and E2, respectively. T and E2 accuracy were verified through certified quality controls and multicenter comparison studies [16,36,37]. Of the 316 enrolled patients, 71 participants were missing LC-MS/MS assay E2 data.

Serum TT and E2 were also assayed by CI assays: serum TT was measured by Architect 2nd Generation Testosterone (Abbott, USA), with an intra-assay CV <10% and serum E2 was measured by Chemiluminescent Microparticle Immunoassay on the ARCHITECT platform (Abbott Laboratories). The sensitivity was 0.6 pg/mL with the lowest standard at 1.5 pg/mL, linearity to 150 pg/mL, and an ED50 of 20 pg/mL. The cross-reactivity with estrone and with less potent estrogens was less than 7 and 0.45%, respectively.

Serum SHBG was assayed by CI (Architect, Abbott GmbH &Co, Germany) with a sensitivity of 0.02 nmol/L. Inter- and intra-assay coefficients of variation of ≤10.0%, being lower in the mean normal range (around 5%) and higher for lowest and highest values (around 9%). SHBG levels were not available for 1 patient.

FT was calculated (cFT) by using the validated Vermeulen equation from both serum TT assessed with LC-MS/MS and serum TT assessed with CI [24,33].

According to the current Endocrine Society guidelines, diagnosis of biochemical hypogonadism was made when serum TT was below 320 ng/ml and/or cFT was below 64 pg/ml [27].

Serum LH and follicle stimulating hormone (FSH) were assayed by CI (Architect, Abbott GmbH & Co, Germany). The inter- and intra-assay CV were ≤10%.

Clinical parameters

At the time of the blood collection, demographic and anthropometric parameters (weight, height, body mass index [BMI]), together with the duration of HIV-infection and ART treatment and other risk factors and comorbidities (including the frailty index) were recorded, as previously described [8].

Ethics

The Institutional review board of Modena approved the protocol study (protocol n. 1446/15). This trial was registered in ClinicalTrials.gov (Identifier: NCT03747003).

Written informed consent has been obtained from each subject.

Statistical analysis

The non-parametric Mann-Whitney *U* test followed by the Dunn's multiple comparison *post hoc* test was used for comparisons of continuous variables since they resulted not normally distributed at the Kolmogorov-Smirnov test. Categorical variables were compared with Chi-square test.

Linear regression was used to examine the association between continuous variables; results were expressed through β and R^2 coefficients. Stepwise, linear, multiple regression analysis using a backward elimination method was applied to the data with $p < 0.1$ as the criterion for a variable to enter the model.

The relationship and the agreement of the estimations between the two analytical techniques were investigated by calculating the concordance correlation coefficient (pc), the Passing and Bablok regression (PB) [38] and by the Bland and Altman plot (BA) [39], without exclusion of potential outliers. Comparisons were made assuming the LC-MS/MS assay as the reference method.

Statistical analyses were performed using the Statistical Package for the Social Sciences' (SPSS) software for Windows (version 26.0; SPSS Inc, Chicago, IL). Pc, PB, and BA analyses were performed using the MedCalc Software Version 15, 8 (©1993–2015 MedCalc Software bvba, Belgium) [40]. For all comparisons, $p < 0.05$ was considered statistically significant.

Results

A total of 316 consecutive PLWH were enrolled, with a median age of 47 years (range 25.2–50.5 years) and a median duration of HIV-infection of 16.16 years (range 1.13–35.4 years). For comprehensive clinical characteristics of the 316 patients and hormonal outcomes, see our previous publication [8]. Hormonal outcomes, grouped according to patients' gonadal status, are summarized in Table 1.

Table 1. Differences between eugonadal and hypogonadal PLWH considering serum TT and/or cFT assessed by LC-MS/MS. Data are expressed as median (minimum–maximum).

	n.v.	Eugonadal	Hypogonadal	*p* Value
n 316		281 (88.9%)	35 (11.1%)	
Clinical characteristics				
Age (years)	–	46.75 (25.22–50.47)	49.63 (38.39–50.19)	**0.002**
BMI (Kg/m^2)	18.5–25	23.59 (16.33–38.74)	23.71 (18.29–36.52)	0.291
Years of HIV	–	14.64 (1.13–35.40)	22.85 (4.60–33.95)	**<0.0001**
ART Years	–	12.86 (0.00–33.78)	17.88 (1.94–30.78)	**<0.0001**
Hormonal measurements by CI (Architect 2nd generation, Abbott)				
LH (mIU/mL)	1–9	4.7 (1.1–43.0)	6.0 (0.0–35.6)	0.057
FSH (mIU/mL)	1–12	5.5 (1.2–42.0)	7.1 (0.0–58.5)	**0.030**
PRL (ng/mL)	2.1–17.7	7.4 (1.9–62.0)	7.4 (2.7–62.4)	0.304
TT (ng/dL)	>320	740.0 (350.0–1870.0)	410.0 (170.0–850.0)	<**0.0001**
cFT (pg/mL)	>64	125.6 (66.0–338.3)	59.8 (15.4–93.2)	<**0.0001**
E2 (pg/mL)	<50	32.0 (2.9–92.0)	25.5 (1.0–76.0)	**0.001**
SHBG (nmol/L)	13.5–71.4	47.3 (14.4–161.0)	60.5 (11.9–165.5)	0.160
Hormonal measurements by LC-MS/MS				
TT (ng/dL)	>320	662.65 (335.0–1675.0)	339.45 (178.3–765.5)	<**0.0001**
cFT (pg/mL)	>64	111.0 (64.3–271.9)	54.6 (12.7–73.2)	<**0.0001**
E2 (pg/mL)	12.4–43.5	25.1 (8.5–70.3)	20.1 (9.8–57.7)	0.137
DHT (pg/mL)	165–679	382 (39–1804)	304 (39–672)	0.064

PLWH: people living with HIV; n.v.: normal values; BMI: Body Mass Index; HIV: Human Immunodeficiency Virus; ART: Antiretroviral Therapy; LH: Luteinizing Hormone; FSH: Follicle-Stimulating Hormone; PRL: Prolactin; E2: Estradiol; TT: Total Testosterone; SHBG: Sex Hormone-Binding Globulin; cFT: Calculated Free Testosterone; CI: Chemiluminescent Immunoassay; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry.

Comparison between CI and LC-MS/MS for T assessment

Serum TT and FT assessed with LC-MS/MS were directly related to serum TT (R^2=0.928, $p < 0.0001$) and FT (R^2=0.896, $p < 0.0001$) assessed with CI. For serum TT the PB regression did not exclude a linear relationship ($p = 0.380$), notwithstanding proportional (slope: 1.173, 95% CI 1.138–1.213) and systematic (intercept: −35.995; 95% CI −58.657 to −15.948) errors were significantly present (Figure 1(a)). Similar results were obtained for cFT (slope: 1.226, 95% CI 1.179–1.276; intercept: −0.863; 95% CI −1.337 to −0.437) (Figure 1(b)).

However, an overestimation by CI was observed compared to LC-MS/MS. Both serum TT and cFT assessed by LC-MS/MS were significantly lower compared to serum TT (639 ng/ml vs 720 ng/ml, $p < 0.0001$) and cFT (105.7 pg/ml vs 118.6 pg/ml, $p < 0.0001$) assessed by CI. The BA plot highlighted the presence of a significant mean difference between the two methods of 12.8% for TT (Figure 1(c)) and of 15.7% for cFT (Figure 1(d)).

In particular, 6 patients (1.9%) presented serum TT assessed by CI $\geq$ 320 ng/mL, but they resulted hypogonadal when serum TT was assessed by LC-MS/MS. Similarly, 9 patients (2.8%) presented cFT calculated from CI assessment $\geq$ 64 pg/mL, but they resulted hypogonadal when cFT was calculated from LC-MS/MS assessment.

Accordingly, by establishing LC-MS/MS as the reference method for TT assessment and the calculation of FT, CI presented high specificity for the diagnosis of hypogonadism considering both serum TT and cFT (100% and 99.6%, respectively), but lacked high sensitivity considering both serum TT and cFT (62.5% and 70%, respectively) (Table 2).

Prevalence of hypogonadism

Prevalence of hypogonadism, comprised overt (primary and secondary) and compensated hypogonadism, according to serum TT or FT and laboratory assay, is reported in Table 3 and Figure 2. By using cFT on the basis of serum TT obtained by LC-MS/MS, the prevalence of hypogonadism (overt + compensated) was 17.1% (Table 3, Figure 2). The distribution of patients in relation to cFT and LH is represented in Figure 3. The prevalence of laboratory assessed (biochemical) hypogonadism was significantly higher when serum TT was measured by LC-MS/MS than by CI, both for hypogonadism defined considering serum TT (5.1% vs 3.2%, $p < 0.0001$) and cFT (9.5% vs 7%, $p < 0.0001$) (Table 3).

Prevalence of biochemical hypogonadism detected by cFT was higher compared to serum TT ($p < 0.0001$), regardless of the hormonal assay used (Table 3).

Differences between eugonadal and hypogonadal patients

We classified patients in eugonadal and hypogonadal considering serum TT and/or cFT assessed by LC-MS/MS (Table 1). Comparing the 2 subgroups, hypogonadal patients were significantly older ($p = 0.002$) than eugonadal patients. Moreover, the duration of

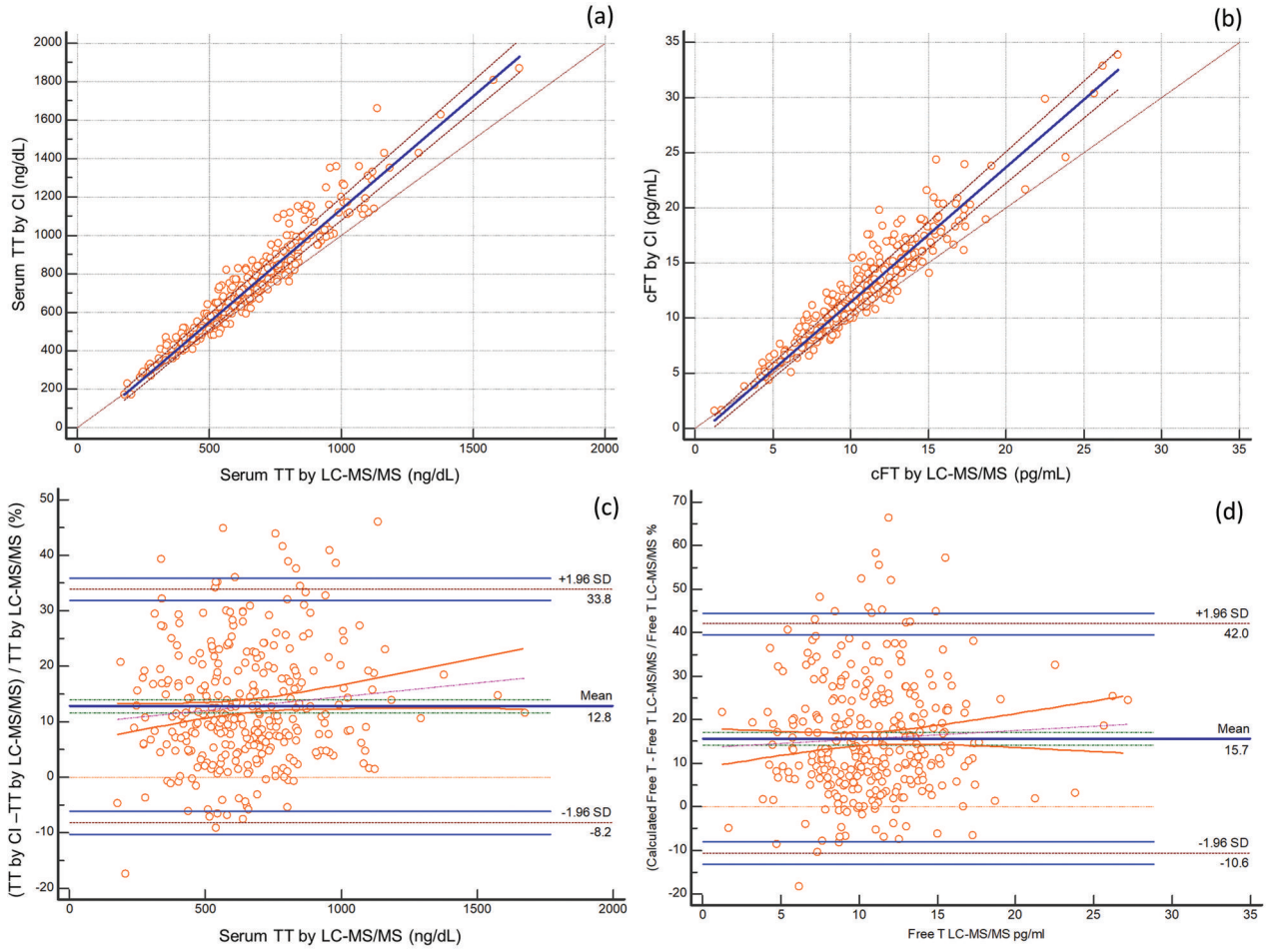

Figure 1. Passing and Bablok regression between the CI and LC-MS/MS assays for serum TT (a) and cFT (b); Bland–Altman plot of serum TT (c) and cFT (d).

Table 2. Diagnostic value of different methods used for the measurement of serum TT and cFT, taken LC-MS/MS as the reference method.

	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
TT (CI)	320 ng/mL	62.5	100	100	98	98.1
cFT (CI)	64 pg/mL	70	99.6	95.4	99.6	96.8

TT: Total Testosterone; cFT: Calculated Free Testosterone; CI: Chemiluminescent Immunoassay; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; PPV: Positive Predictive Value; NPV: Negative Predictive Value.

HIV infection was significantly longer in the hypogonadal subgroup ($p < 0.0001$), as well as the duration of ART therapy ($p < 0.0001$) (Table 1). Considering other hormonal parameters of the pituitary-gonadal axis, FSH levels ($p = 0.030$) were significantly higher in the hypogonadal patients, while LH levels did not significantly differ between the 2 groups ($p = 0.057$), although the mean levels were higher among hypogonadal patients; as expected, E2 was significantly lower in hypogonadal patients ($p < 0.0001$) (Table 1). Finally, no significant differences were found for SHBG, DHT, and prolactin levels (Table 1).

Except for SHBG, similar differences were found also when patients were classified into eugonadism, primary hypogonadism, secondary hypogonadism and compensated hypogonadism on the basis of cFT (threshold 64 pg/mL) and serum LH (threshold 9 mIU/L) (Table 4).

Serum DHT did not change between hypogonadal and eugonadal PLWH (Table 1), also when different types of hypogonadism (primary, secondary, and compensated) were considered (Table 4).

Serum SHBG in HIV-infected patients

Serum SHBG did not differ between eugonadal and hypogonadal PLWH (Table 2). SHBG was significantly different among PLWH group according to different subtype of biochemical hypogonadism (compensated,

Table 3. Prevalence (expressed as number and percentage) of eugonadism, compensated hypogonadism, primary hypogonadism and secondary hypogonadism according to serum TT assessed by CI, serum TT assessed by LC-MS/MS, FT calculated by using TT assessed by CI and FT calculated by using TT assessed by LC-MS/MS.

	TT *n* (%)		cFT *n* (%)	
	CI	LC-MS/MS	CI	LC-MS/MS
Eugonadism	274 (86.7%)	268 (84.8%)	266 (84.4%)	261 (82.9%)
Impaired function of HPG axis	42 (13.3%)	48 (15.2%)	49 (15.6%)	54 (17.1%)
Compensated Hypogonadism	32 (10.1%)	32 (10.1%)	27 (8.6%)	24 (7.6%)
Overt Hypogonadism	10 (3.2%)*	16 (5.1%)*	22 (7.0%)**	30 (9.5%)**
Primary Hypogonadism	3 (1.0%)	3 (1.0%)	8 (2.5%)	11 (3.5%)
Secondary Hypogonadism	7 (2.2%)	13 (4.1%)	14 (4.4%)	19 (6.0%)

TT: Total Testosterone; CI: Chemiluminescent Immunoassay; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; cFT: Calculated Free Testosterone; HPG: Hypothalamic-Pituitary-Gonadal. *$p < 0.0001$; **$p < 0.0001$.

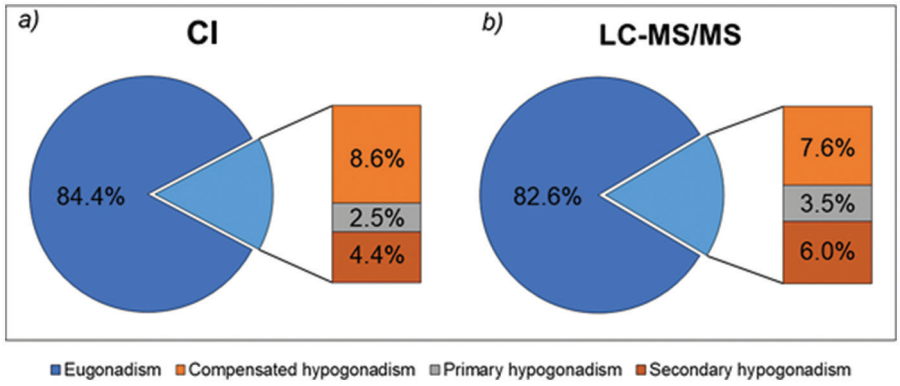

Figure 2. Prevalence of each category of gonadal status according to cFT levels calculated by using serum TT assessed by CI (a) and by LC-MS/MS (b).

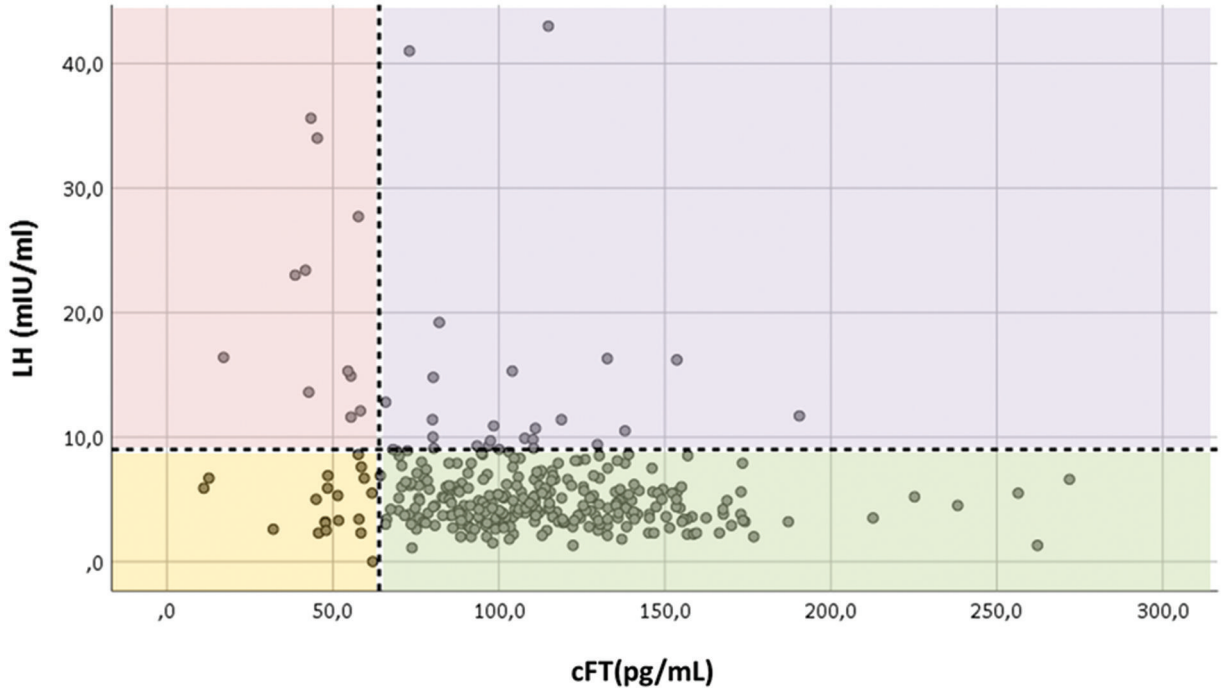

Figure 3. Gonadal status of the entire cohort of PLWH according to cFT calculated from LC-MS/MS and serum LH levels. Green: eugonadism; Purple: compensated hypogonadism; Yellow: secondary hypogonadism; Pink: primary hypogonadism.

Table 4. Characteristics according to cFT in tandem with serum LH. Data are expressed as median (minimum-maximum).

	cFT ≥ 64 pg/mL		cFT < 64 pg/mL		
	E	CH	SH	PH	
n 316	**261 (82.9%)**	**24 (7.6%)**	**19 (6.0%)**	**11 (3.5%)**	
Clinical characteristics					***p* Value**
Age (years)	46.7 (25.2–50.5)	48.2 (36.5–50.3)	49.5 (38.6–50.0)	49.6 (43.7–50.0)	**0.004**
BMI (Kg/m^2)	23.6 (16.9–38.7)	22.8 (16.3–32.1)	25.8 (18.3–36.5)	22.8 (20.5–34.3)	0.210
Years of HIV	14.1 (1.1–35.4)	18.8 (3.4–34.0)	25.9 (10.2–34.0)	22.4 (15.5 (26.5)	**<0.0001**
ART Years	11.9 (0.0–33.8)	16.8 (1.3–29.9)	17.8 (1.9–30.5)	18.5 (13.4–24.5)	**0.001**
Hormonal measurements by CI (Architect 2nd generation, Abbott)					
LH (mIU/mL)	4.5 (1.1–9.0)	11.1 (9.1–43.0)	4.2 (0.0–8.6)	16.4 (11.6–35.6)	**<0.0001**
FSH (mIU/mL)	5.2 (1.2–37.9)	10.5 (3.1–42.0)	5.4 (0.0–12.3)	18.9 (6.6–58.5)	**<0.0001**
PRL (ng/mL)	7.4 (1.9–62.0)	6.6 (3.7–10.6)	8.1 (2.8–62.4)	6.7 (3.2–20.7)	0.355
TT (ng/dL)	730 (270–1810)	755 (410–1870)	440 (170–780)	440 (170–850)	**<0.0001**
cFT (pg/mL)	126.1 (63.2–338.3)	111.8 (80.7–237.6)	56.4 (15.4–70.4)	59.0 (16.2–76.7)	**<0.0001**
E2 (pg/mL)	32.0 (2.9–92.0)	31.5 (10.0–59.0)	26.0 (1.0–76.0)	22.0 (1.0–34.0)	**0.010**
SHBG (nmol/L)	46.5 (14.4–161.0)	62.1 (22.7–131.2)	75.5 (11.9 (165.5)	65.0 (31.0–129.7)	**<0.0001**
Hormonal measurements by LC-MS/MS					
TT (ng/dL)	655 (277–1578)	717 (392–1675)	399 (191–760)	342 (178–767)	**<0.0001**
cFT (pg/mL)	111.0 (64.3–271.9)	101.2 (65.9–190.5)	50.0 (12.7–62.0)	45.3 (17.1–58.3)	**<0.0001**
E2 (pg/mL)	25.1 (8.5–70.3)	24.3 (11.2–49.1)	20.3(9.8–57.7)	–	0.364
DHT (pg/mL)	376 (39–1804)	420 (196–893)	371 (39–672)	287 (94–416)	0.220

BMI: Body Mass Index; HIV: Human Immunodeficiency Virus; ART: Antiretroviral Therapy; LH: Luteinizing Hormone; FSH: Follicle-Stimulating Hormone; PRL: Prolactin; E2: Estradiol; TT: Total Testosterone; SHBG: Sex Hormone-Binding Globulin; cFT: Calculated Free Testosterone; CI: chemiluminescent Immunoassay; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry.

primary, and secondary) ($p < 0.0001$) (Table 4). At the post-hoc test, in fact, eugonadal PLWH had significanly lower levels of serum SHBG than PLWH with secondary hypogonadism ($p = 0.001$), and PLWH with compensated hypogonadism ($p = 0.002$); in addition SHBG was higher in PLWH with secondary hypogonadism than in those with compensated hypogonadism ($p < 0.0001$).

At linear regression analysis, serum SHBG was directly related to serum E2 ($\beta = 0.330$, R^2=0.109; $p < 0.0001$) (Figure 4(a)), serum TT ($\beta = 0.272$, R^2=0.521; $p < 0.001$) (Figure 4(b)), and serum LH ($\beta = 0.223$, R^2=0.050; $p < 0.001$) (Figure 4(c)). In addition, also cFT resulted inversely related to LH (β=-0.242, R^2=0.058; $p < 0.0001$) (Figure 4(d)).

The stepwise multivariate regression analysis showed that SHBG was associated with serum TT, viral liver co-infection and duration of HIV according to the following 3 different models: ***model 1***: only serum TT enter the model as a predictive variable ($\beta = 0.527$, R^2:0.278, $p < 0.0001$); ***model 2***: serum TT ($\beta = 0.493$) and viral liver co-infection ($\beta = 0.351$) entered the model (R^2:0.400, $p < 0.0001$); ***model 3***: serum TT ($\beta = 0.518$), viral liver co-infection ($\beta = 0.286$), and duration of HIV ($\beta = 0.159$) entered the model (β=, R^2:0.421, $p < 0.0001$). On the other hand, serum E2, LH, BMI, total alkaline phosphatase, smoke, alcohol and frailty index did not enter any model.

Serum E2 trends according to CI and LC-MS/MS

Serum E2 was significantly lower in hypogonadal than eugonadal male PLWH when assayed by CI, while did not differ when it was measured by LC-MS/MS (Table 1). The same results were obtained when different types of hypogonadism (primary, secondary, and compensated) were considered (Table 4).

Serum E2 assessed by LC-MS/MS was available for 204 patients out of 316. According to the lower reference limit of 12.4 pg/mL estimated with theranges of LC-MS/MS assay in a cohort of healthy unmedicated men, 10 patients (4.9%) elevated serum E2. According to the reference range of CI assay (n.v. < 50 pg/ml), 292 patients (92.7%) had normal serum E2 and 23 patients (7.3%) had serum E2 above the upper limit of normal range.

The prevalence of relative estrogen deficiency, defined as serum E2 below 18 pg/ml, was of 8.5% (27 patients out of 316) with CI and of 16.1% (33 patients out of 204) with LC-MS/MS.

Among hypogonadal PLWH, 8 out of 30 patients had relative estrogen deficiency, defined as serum E2 measured by CI below18 pg/ml, with a prevalence of 26.7%, while 19 out of 286 eugondal PLWH had relative estrogen deficiency with a prevalence of 6.6%.

Among PLWH with serum E2 assessed by LC-MS/MS available, 3 out of 10 hypogonadal patients had relative estrogen deficiency, defined as serum E2 measured by LC-MS/MS below18 pg/ml, with a prevalence of 30.0%, while 30 out of 194 eugonadal PLWH had relative estrogen deficiency with a prevalence of 15.5%.

Serum E2 was significantly related to serum TT ($p < 0.0001$, beta = 0.461, R^2=0.213), cFT ($p = 0.002$, beta = 0.219, R^2=0.048) by LC-MS/MS, and SHBG ($p < 0.0001$, beta = 0.346, R^2=0.120).

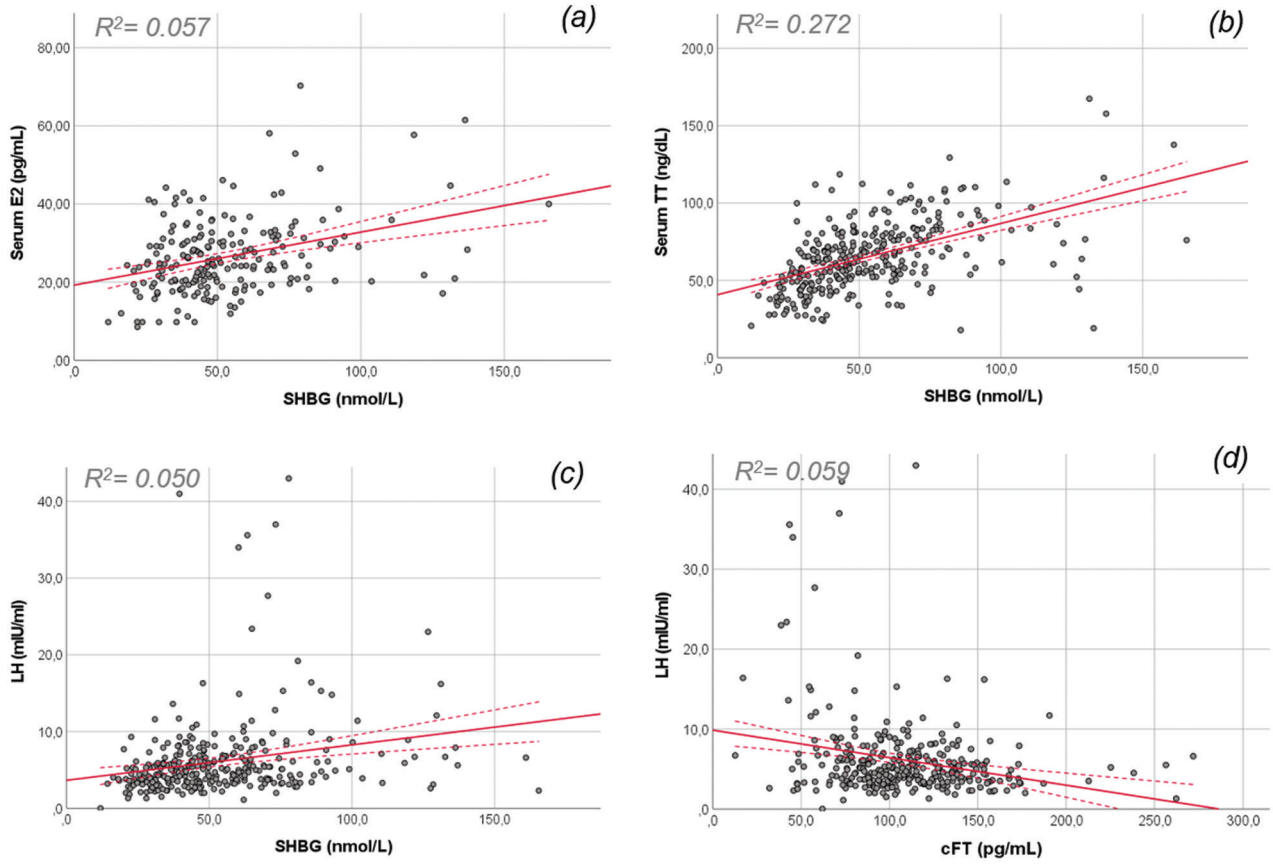

Figure 4. Linear regression between serum SHBG and serum E2 (a), serum TT (b), and serum LH (c). In (d) is reported the correlation between cFT and serum LH.

Serum E2 assessed with LC-MS/MS was directly related to E2 assessed with CI (R^2=0.498, $p < 0.0001$). Accordingly, the PB regression did not exclude a linear relationship ($p = 0.500$), notwithstanding proportional (slope: 1.237, 95% CI 1.102–1.391) and systematic (intercept: 2.814; 95% CI −1.617 to 6.732) errors were significantly present (Figure 5(a)). As expected, E2 assessed by LC-MS/MS was significantly lower compared to CI (24.7 pg/ml vs 31 pg/ml, $p < 0.0001$). The BA plot highlighted the presence of a significant mean difference between the two methods of 42.4% as well as a trend for increasing overestimation by the CI at lowering E2 levels (Figure 5(b)).

Discussion

This study demonstrates that the measurement of serum TT by LC-MS/MS coupled with SHBG improves biochemical hypogonadism diagnosis in male PLWH. Besides, these results confirm that SHBG is a key diagnostic tool useful to identify biochemical hypogonadism in the context of HIV since prevalence of hypogonadism is about 1.9-fold increased using serum cFT rather than TT, regardless of the method used for TT measurement (CI or LC-MS/MS). This is in line with literature showing that cFT raises on average of 1.7 times the prevalence of biochemical hypogonadism in male PLWH [4,6,29,30] and fit with the evidence of significantly lower cFT in PLWH than HIV-uninfected men [6,30], despite no difference in serum TT assessed by LC-MS/MS [30]. The importance of SHBG measurement in PLWH has been already largely emphasized [27,29], but it has never been explored in combination with gonadotropins and serum TT measured by LC-MS/MS [6,30,31]. Serum SHBG is known to be altered in HIV infection and may interfere with the amount of unbound circulating sex steroids [3]. As in other studies [29], the SHBG increase found in our study highly influences serum TT and E2 since it leads to compensatory LH increase in order to leave unchanged the unbound quote of circulating sex steroids as in compensated hypogonadism. Accordingly, SHBG was significantly higher in PLWH with secondary and compensated hypogonadism compared to eugonadal PLWH and was directly correlated with serum LH. In PLWH with secondary hypogonadism, SHBG was significantly higher than in those with compensated hypogonadism indicating that the hypothalamic-

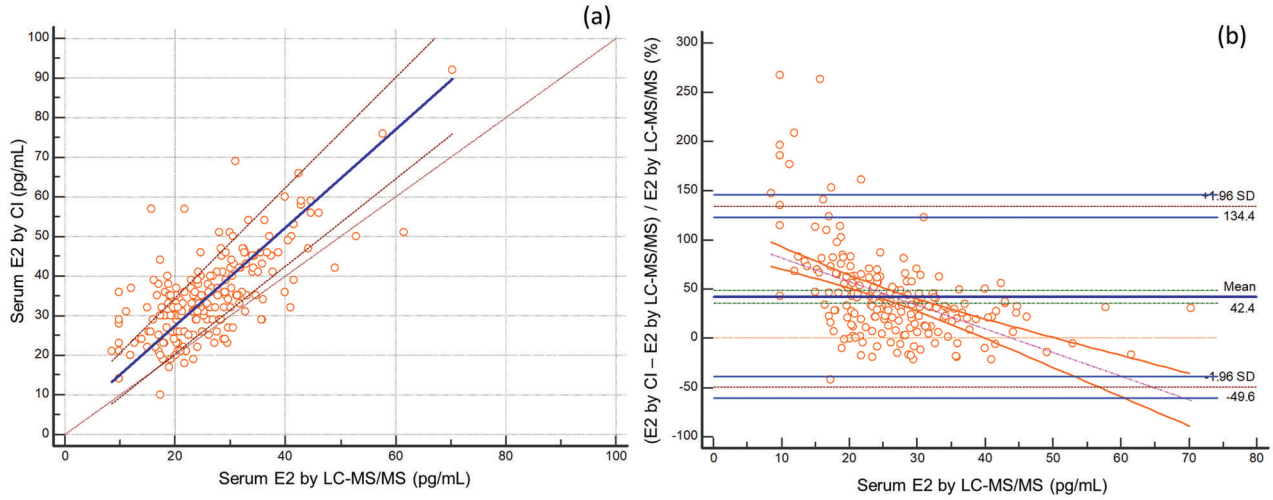

Figure 5. Passing and Bablok regression (a) and Bland–Altman plot (b) between the CI and LC-MS/MS assay for serum E2.

pituitary-gonadal axis was no more able to increase LH production for counteract T deficiency. In this study, serum TT, viral liver co-infection and duration of HIV infection resulted to be stronger predictor of the SHBG increase confirming that liver dysfunction and HIV infection may alter SHBG in these patients [3,41].

The prevalence of overt biochemical hypogonadism in our cohort is higher with LC-MS/MS than with CI, both for serum TT (5.1% vs 3.2%) and cFT (9.5% vs 7.0%), thus confirming the superiority of LC-MS/MS over CI in unraveling mild forms of biochemical hypogonadism in HIV [6,30] as in men without HIV infection [16,27]. Serum TT in young to middle-aged male PLWH has been measured by LC-MS/MS only in two studies, the first showing a percentage of hypogonadism (24% for serum TT and 19% for cFT with cut-offs of <300 ng/ml and <50 pg/ml, respectively) higher than our study) [31], the second showing a similar prevalence (9.3%) based on the cut-off of cFT <50 pg/ml [30].

The novelty of this study lies in the comprehensive evaluation of pituitary-gonadal axis in PLWH <50 years based on the measurement of serum TT by LC-MS/MS coupled with the measurement of both gonadotropins and SHBG, which allows classifying biochemical hypogonadism as: secondary (6.0%), primary (3.5%), and compensated (7.6%), with hypogonadotropic hypogonadism as the most common form of overt hypogonadism as previously described [1–3,7,42–44]. Moreover, the pre-clinical condition of compensated hypogonadism, defined as normal serum T together with increased LH [45], seems to occur as frequently as secondary hypogonadism in PLWH [1,3], similarly to what happens in older men (9.5% in men 40–79 years) where it is common and is considered a precursor of overt primary hypogonadism [1,45]. Taken together overt and compensated forms biochemical hypogonadism is 17.1% in this study, confirming our [1] and other authors' [29,42] previous results. With the exception of a recent study showing a prevalence of hypogonadism (4.4% with a TT cut-off of 348 ng/ml; 12.4% with a cFT cut-off of 70 pg/ml) similar to this study in PLWH <50 years [5] the prevalence of male hypogonadism in PLWH in the ART era is highly heterogeneous, ranging from 13% up to 40% [3,4], being of 33% on average, as settled by our recent meta-analysis [4]. This heterogeneity is due to differences among studies in terms of serum TT assays, use of cFT or TT, use of different cut-offs, and mean age of patients [3,4]. Overall, it seems that the prevalence of male hypogonadism among PLWH has been decreasing in recent years thanks to improvement of their management and general health status [4], although the finding of compensated hypogonadism remains quite common in these patients. The low prevalence found in this study is close to that described among HIV-uninfected men of similar age groups [46–48] and is in line with the above-mentioned decrease in last years. This low prevalence could be due to the younger age of our patients (inclusion criterion <50 years), to underestimation caused by exclusion of all patients of our cohort with previous or ongoing TRT(ruling out patients already diagnosed with overt hypogonadism), to the fact that almost all of them belong to HIV-cohorts that have beneficiated of last generation ART regimens (they have been enrolled since 2013) and infection contracted in recent years (i.e. 7.7% of patients with length of disease <54 months) that avoided the exposure to older, more toxic drugs. Thus, the infection has had little time to determine its effects and complications including those on the hypothalamus-pituitary-gonadal axis [3].

There is growing evidence suggesting that male hypogonadotropic hypogonadism could be induced by an unhealthy status among PLWH as an adaptive mechanism to spare energy [8,12,49], similarly to aging men [3,26,50]. We have recently described the possible functional nature of hypogonadism in HIV, where low T could be considered more an epiphenomenon and a biomarker of poor health rather than a form of true clinical hypogonadism [8,49]. Patients with a recent diagnosis of HIV, in fact, experience a better general health status and a lower incidence of comorbidities [51–54]. To the best of our knowledge, no previous study has compared serum E2 measured with CI and LC-MS/MS in a cohort of male PLWH. Serum E2 measured with LC-MS/MS resulted significantly lower than with CI, thus confirming that immunoassays are not reliable in PLWH as well as in male population for the measurement of serum E2 in the male range [18,19,21], LC-MS/MS being the only reliable assay in men including PLWH [15–18,20–22]. Serum E2 measured by LC-MS/MS did not differ between hypogonadal and eugonadal PLWH, as in a recent study [32], but relative estrogen deficiency was almost frequent in PLWH with hypogonadism suggesting that when testosterone decreases serum E2 drops too even in PLWH [12,19], notwithstanding increased visceral adiposity and HIV lipodystrophy and related aromatase over-expression in adipose tissue [8,19,55]. Relative estrogen deficiency may contribute worsening some health conditions already common in male PLWH, such as osteoporosis, fat redistribution, dyslipidaemia, and glucose metabolism alterations [19,56].

This study has several strengths and limitations. Considering strengths, this is a properly-designed, prospective cohort study performed by using the reference method LC-MS/MS for serum TT measurement in association with the assessment of SHBG and gonadotropins, thus it provides a comprehensive view of the hypothalamus-pituitary-gonadal axis of male PLWH. In addition, the large recruitment of only young to middle-aged patients allowed us to avoid the effects of physiological aging on gonadal status and the possible forms of LOH. As regards to limitations, morning serum TT has been determined on a single blood sample rather than in two separate mornings as guidelines describe [27], however it was assessed by using two different methodologies, including the recommended LC-MS/MS. Moreover, this study lacks a specific control group of age-matched PLWH, but the prevalence of biochemical hypogonadism in HIV-uninfected men has been widely explored in literature allowing indirect comparison [45–48]. Again, these data refer only to biochemical hypogonadism, not to a clinical diagnosis of the disease.In clinical practice well-validated immunoassays methods are adequate and sufficient for a baseline evaluation of serum TT and cFT, as supported by the good correlation between CI and LC-MS/MS in this study and overall acceptable sensitivity of commercially available CI [17,57]; this is not the case, however, for serum E2 [14]. Mass spectrometric methods are superior and are becoming more available in clinical laboratories; thus, male gonadal function assessment will improve in the near future [11,13,17,57]. Our results suggest that SHBG should be considered mandatory in the clinical work-up for the diagnosis of male hypogonadism in PLWH in order to calculate cFT, independently from the methodology used for serum TT determination [3,9,12,17,27], thus reinforcing available advice from Endocrine Society guidelines and other studies [3,5,27–29]. Also serum gonadotropins are mandatory since they allow correctly classifying the type of hypogonadism and refining the diagnosis. At present, gonadotropins remain poorly considered in HIV research, especially in studies not designed by endocrinologists or experts in the field of hypogonadism [3], thus preventing a wide and comprehensive use of gonadotropins in infectious diseases specialists' clinical practice for the diagnosis of male hypogonadism. This study suggests that clinicians should maintain close monitoring of gonadal function overtime in patients with compensated hypogonadism, a condition that is known to precede primary overt gonadal failure as also advised by other authors [29]. Finally, it should be remarked that the clinical diagnosis of hypogonadism cannot be based only on the finding of biochemical hypogonadism but needs to be coupled with signs and symptoms of hypogonadism [1,3,29] as in HIV-uninfected men [50,58]. However, coupling biochemical hypogonadism with clinical signs and symptoms remains challenging in male PLWH [3] due to the high prevalence of some symptoms and signs such as bone loss and erectile dysfunction both in PLWH with and without hypogonadism [1,29,44,55].

In conclusion, LC-MS/MS methodology is the best-performance methodology compared to CI in detecting the presence of serum T deficiency in young to middle-aged PLWH. SHBG levels should be assessed in PLWH allowing the calculation of cFT to not underdiagnose biochemical hypogonadism.

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Disclosure statement

The authors report no conflict of interest.

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