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Cortisol in seminal plasma: a potential addition to the multidimensional approach to male infertility

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SUMMARY

ABSTRACT

Background: several experimental evidence on animal models suggests a potential effect of glucocorticoids (GCs) on male reproductive function. Indeed, stress-related increase in endogenous levels of serum GCs can induce apoptosis in Leydig cells and male germ cells, and the administration of exogenous GCs can lower number and functionality of mature sperm cells. On the other hand, the complete absence of GCs is associated with atrophy of seminiferous tubules and spermatogenesis arrest, suggesting the existence of a physiological range of GC levels for optimal testicular function.

Objective: the aim of the present study was to measure cortisol levels in human semen plasma and to evaluate the possible relation between sperm cortisol levels and semen, hormone, and ultrasound (US) parameters.

Materials and methods: semen samples were provided from 149 men referring to Endocrinology Clinic, University Hospital delle Marche, Ancona, for fertility evaluation, between July 2020 and June 2021. After liquefaction, all sperm samples were evaluated for sperm parameters according with the 5th edition of WHO Manual. Concentrations of cortisol in seminal plasma were measured by electrochemiluminescence (ECLIA).

Results: Mean age and body mass index (BMI) of patients was 33.3 ± 8.4 years and 24.6 ± 3.1 kg/m^2, respectively. According with the WHO definitions of normal values, 55 patients (36.9%) were considered as normozoospermic, whereas 94 (63.1%) presented various forms of pathospermia, including oligo-astheno-teratozoospermia (OAT) (32, 21.5%), isolated asthenozoospermia (34, 22.8%), and azoospermia (9, 6.0%). High variability in sperm cortisol levels emerged (median 2.64, IQR 1.76-3.62 mcg/dl). We found significantly higher levels of sperm cortisol in men with varicocele (+18.8%, p=0.039) and in those with positive semen culture (+14.3%, p=0.045). In men without varicocele, sperm cortisol levels showed negative weak-moderate correlation with all semen parameters, and diagnostic power for abnormal total motile sperm count (TMSC) was slightly higher than that of serum follicle-stimulating hormone

(FSH). In the whole group, we also found a negative correlation between sperm cortisol levels and serum estradiol (E2) levels (ρ=-0.330, p=0.02). Interestingly, the latter showed a significant positive correlation with total motile sperm count (TMSC) that remained significant after multivariate analysis (except when BMI was also considered).

Conclusion: to the best of our knowledge, our study was the first to evaluate the presence of cortisol in seminal plasma and its relationship with semen, hormone, and US parameters. Our results showed that subjects with risk factors for male infertility such as varicocele and positive semen culture have higher seminal cortisol levels, and sperm quality is inversely correlated with sperm cortisol levels in men without varicocele. We also found a negative correlation between sperm cortisol and serum E2, which in turn was positively correlated with sperm quality, suggesting that cortisol might influence male reproductive function.

KEYWORDS

Sperm cortisol; idiopathic male infertility; adrenal; testicular; steroidogenesis; spermatogenesis.

INTRODUCTION

Couple infertility, defined by the World Health Organization (WHO) as the absence of conception after 12 months of regular, unprotected intercourse, affects 48.5 million couples around the world, and a male factor, alone or in combination with a female factor, can be identified in up to 50% of cases [1, 2]. Unfortunately, in 30–50% of male infertility cases, a specific cause is not identified, and these are therefore defined as "idiopathic" (idiopathic male infertility, IMI), even if risk factors such as smoking, alcohol, obesity, and aging may contribute to the impairment of semen quality [3].

Several experimental studies on animal models have suggested a possible effect of glucocorticoids (GCs) on male reproductive function. Research in rodents, indeed, has shown that a stress-related endogenous increase in corticosterone levels (the main GC in many animal species) is capable of triggering apoptotic phenomena in Leydig cells, spermatogonia, and first-order spermatocytes and reducing circulating levels of testosterone; similarly, administration of exogenous hydrocortisone is capable of reducing sperm number and function [4]. In addition, intratesticular cortisol concentration has showed an inverse correlation with the number of Sertoli cells in boar testis [5]. On the other hand, in rats, the complete lack of GCs caused by adrenalectomy leads to seminiferous tubule damage and ineffective spermatogenesis that can be partially corrected by dexamethasone replacement therapy [6], while cortisol appears to be a critically important mediator in driving spermatogonial differentiation, meiosis and spermatogenesis in the *zebrafish* gonad [7]. Thus, it could be hypothesized that a physiological range of gonadal GCs necessary to ensure a well-functioning testicular microenvironment may exist.

Although evidence in humans is much scarcer, it has recently been shown that hydrocortisone can act as a partial agonist at the level of the *CatSper* (Cation channel of Sperm) receptor [8], which activation, primarily mediated by progesterone, is implicated in the maintenance of sperm motility and the completion of the capacitation process by the acrosomal reaction [9]. It is also possible that the action of GCs at the gonadal level may be modulated at the receptor

level. The receptor for GCs NR3C1 (nuclear receptor subfamily 3 group C member 1), indeed, has several polymorphisms, among which *Bcll* has been found to be directly related to the presence of alterations in seminal parameters, first and foremost asthenozoospermia [10]. Considering these data, it is evident that the study of the testicular effects of GCs could be a key element in understanding the pathophysiology of male infertility, especially for IMI. Therefore, we conducted a study to investigate whether the addition of sperm cortisol measurement to a complete multidimensional evaluation could provide an additional tool in the diagnostic workflow of the infertile man.

METHODS

Patients

Between July 2020 and June 2021, 189 male patients from couples referring to the Andrology unit of our Endocrinology Division for couple infertility were evaluated for inclusion in the present study. Patients with adrenal and/or testicular diseases (including congenital adrenal hyperplasia -CAH- and primary hypogonadism due to Klinefelter syndrome or post-orchiectomy), those with body mass index (BMI) < 18.5 or > 30 kg/m^2, and those taking drugs known to alter the hypothalamus-pituitary-adrenal axis (e.g., glucocorticoids) or testicular function (e.g., androgens or antiandrogens) were excluded. All the included patients underwent complete andrological evaluation that included collection of medical history (including information about previous pregnancies and health issues), height and weight, and examination of the external genitalia. The study was conducted in accordance with the principles of the Declaration of Helsinki, and written informed consent was obtained from all the participants before the start of the study.

Biochemical data

Blood samples were taken between 9:00 and 11:00 a.m. after at least 8 hours fasting, and the levels of total testosterone (TT), follicle-stimulating hormone (FSH), luteinizing hormone (LH), sex hormone binding globulin (SHBG), prostate-specific antigen (PSA), prolactin (PRL), thyroid-stimulating hormone (TSH), and 17-β-estradiol (E2) were measured. All the hormones were measured by chemiluminescence immunoassay. Reference range for TT was 2.70-11.0 ng/dl, for FSH 1.4-16.0 mIU/ml, for LH 1.3-9.0 mIU/ml, for SHBG 14.5-48.4 nmol/l, for PSA < 4.00 ng/ml, for PRL 2.0-18.0 ng/ml, for TSH 0.350-3.500 mcUI/ml, and for E2 11.0-47.0 pg/ml. Free testosterone (FT) levels were calculated using Vermeulen's formula [11], with 6.4 ng/dl considered as cut-off for diagnosis of hypogonadism [12].

Semen collection

Sperm quality was evaluated according to the 2010 guidelines of the WHO [13] using semen samples obtained by masturbation after 3-5 day of sexual abstinence. Normal values were defined as follows: sperm concentration ≥ 15 x 10^6/ml, total motility ≥ 40%, progressive motility ≥ 32%, abnormal morphology < 96%, sperm vitality ≥ 58%. Total motile sperm count (TMSC) was calculated as (semen volume X sperm concentration X total sperm motility) /100, and normal value was considered ≥ 20 x 10^6 [14].

Ultrasound evaluation

All patients underwent ultrasound (US) examination (HD 7XE Philips, Bothell, WA, USA) with a 5-12 MHz linear transducer in both the B-mode and the color-flow mode. Scrotal US was performed with the patient lying supine, after ejaculation. The three diameters (antero-posterior, transverse, and longitudinal) of each testis were measured, and testicular volume was calculated according to the ellipsoid formula (length x height x width x 0.52). Epididymal antero-posterior diameter was evaluated in three segments (head, body, and tail) in longitudinal scans, and the antero-posterior diameter of vas deferens was measured in similar way. The pampiniform plexus was studied performing grayscale and echo color Doppler (ECD) examination, in transverse and longitudinal scans, with the patient standing, at resting and during a Valsalva maneuver. Venous vessel dilatation > 3 mm at rest was defined as varicocele, the latter being described according to the five-point scale in grade I-III (venous reflux only during a Valsalva maneuver limited to the upper, or extended to the middle and the lower portion of the testicle, respectively) and IV-V (continuous venous reflux increasing or not during a Valsalva maneuver, respectively), as previously described [15].

Sperm cortisol measurement

After semen analysis, the seminal fluids were stored at -20°C until use. Cortisol concentration in seminal plasma was measured by electrochemiluminescence (ECLIA, Eclecsys Cortisol II test - Roche Diagnostics GmbH, Mannheim, Germany – designed for measurement of cortisol in human serum, plasma, and saliva and adapted for seminal plasma) using an automatic immunodiagnostic analyzer (Cobas E 601). Dilution 1:2 was prepared if high viscosity was reported. According with our preliminary investigations, intra-assay variation coefficient (CV) for measurement of cortisol in semen plasma was considered acceptable (< 10%).

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 26.0 (SPSS Inc., Chicago, IL) for Microsoft Windows. Normal distribution for continuous variables was assessed using the Kolmogorov-Smirnov test for normality if not evident from the histogram and/or normality graph. Consequently, data are shown as mean ± standard deviation (SD), or median and interquartile range (IQR) according with their distribution. Comparison between two groups was performed by T-test for independent samples in case of normality, and by Mann-Whitney U-test in case of non-normal distributions. Similarly, comparison between three or more groups was performed by ANOVA one-way test or Kruskal-Wallis test. Chi-squared (Chi^2) test was used to compare categorical data. Bivariate correlations were investigated by Pearson's test or Spearman's test (whether the distribution was normal or not). In this purpose, 0-0.19 is regarded as very weak, 0.2-0.39 as weak, 0.40-0.59 as moderate, 0.6-0.79 as strong and 0.8-1 as very strong correlation. Multivariable analysis was conducted using multiple linear regression and multiple logistic regression if the dependent variables were continuous or dichotomous, respectively. Finally, receiver operating characteristics (ROC) analysis was conducted to evaluate the diagnostic power and optimal cut-off for the identification of relevant clinical conditions based on different biochemical-

instrumental parameters. The Youndens's J statistic (or Younden's index), defined by sensitivity (SE) + specificity (SP) – 1, was calculated to assess the optimum cut-off point for each diagnostic test. Values of $p<0.05$ were considered statistically significant.

RESULTS

Descriptive statistics

From the 189 couples initially evaluated, 149 male patients met the inclusion/exclusion criteria and were involved in the study. Semen data and sperm cortisol levels were available for all the 149 patients, whereas complete hormone, semen, and US data were available for 73 patients. Mean age and BMI of patients was 33.3 ± 8.4 years and 24.6 ± 3.1 kg/m^2, respectively. The results of semen analysis are reported in *Table 1*. As shown, median sperm concentration was 45.0 x 10^6/ml, with median total and progressive motility 40.0% and 30.0%, respectively. According with the above-mentioned definitions of normal values, 55 patients (36.9%) were considered as normozoospermic, whereas 94 (63.1%) presented various forms of pathospermia, including oligo-astheno-teratozoospermia (OAT) (32, 21.5%), isolated asthenozoospermia (34, 22.8%), and azoospermia (9, 6.0%). In addition, 103 (69.1%) had normal TMSC (≥ 20 x 10^6), and 46 (30.9%) abnormal TMSC (< 20 x 10^6).

Table 1: semen characteristics of the population (n=149)

Sperm parameter	Mean ± SD; Median (IQR)	Reference range
Abstinence (days)	4.0 (3.8-5.0)	2-7
Semen volume (ml)	3.3 ± 1.6	> 1.5
pH	7.7 ± 0.2	> 7.2
Sperm concentration (10^6/ml)	45.0 (13.3-90.0)	≥ 15
Total motility (%)	40.0 (15.0-55.0)	≥ 40
Progressive motility (%)	30.0 (7.0-40.0)	≥ 32
Atypical morphology (%)	93.2 ± 4.4	> 4
Leucocytes (10^6/ml)	0.6 (0.4-1.0)	< 1
Sperm vitality (%)	56.0 (29.0-74.3)	≥ 58
TMSC (x 10^6)	46.4 (8.2-143.2)	≥ 20

TMSC: total motile sperm count

Serum hormone data was available for 75 patients. Data are reported in *Table 2*. As shown, TT levels showed a normal distribution and a low variability among patients (4.89 ± 2.02 ng/ml), whereas FT and gonadotropin levels showed higher variability. No frank alterations emerged in PRL, TSH or PSA levels.

Table 2: serum hormones of the population (n=75)

	Mean ± SD; Median (IQR)	**Reference range**
FSH (mIU/ml)	4.5 (3.3-8.6)	1.4-16.0
LH (mIU/ml)	3.6 (2.4-5.7)	1.3-9.0
TT (ng/ml)	4.89 ± 2.02	2.70-11.0
SHBG (nmol/l)	42.07 ± 19.1	14.5-48.4
FT (ng/dl)	9.1 (7.2-10.4)	> 6.5
E2 (pg/ml)	25.4 ± 9.4	11.0-47.0
PRL (ng/ml)	8.0 ± 3.6	2.0–18.0
TSH (mcIU/ml)	1.637 ± 1.041	0.350-3.500
PSA (ng/ml)	0.63 ± 0.27	<4,00

E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TSH: thyroid-stimulating hormone; TT: total testosterone

Grayscale and ECD US data was available for 73 patients. Data regarding testicular volume, epididymal dimension (head, body, and tail), diameter of proximal tract of vas deferens, and characteristics of varicoceles are shown in *Table 3*. Notably, 29 patients (39.7%) suffered from varicocele, which was monolateral in 24 (21 left side, 3 right side) and bilateral in 5 cases.

Table 3: ultrasound data of the population (n=73)

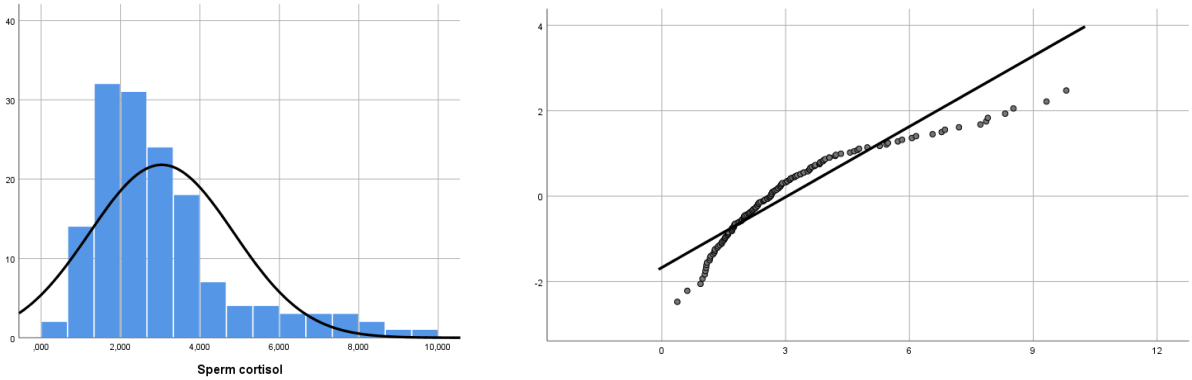
Grayscale	**Right side**	**Left side**	**Mean**
Testicular volume (ml)	15.6 ± 5.5	14.4 ± 5.4	15.1 ± 5.2
Epididymal head (mm)	9.7 ± 2.1	9.6 ± 1.8	9.6 ± 1.4
Epididymal body (mm)	3.2 ± 0.9	3.2 ± 0.9	3.2 ± 0.8
Epididymal tail (mm)	3.3 ± 1.0	3.3 ± 0.9	3.3 ± 0.8
Vas deferens (mm)	3.4 ± 0.9	3.5 ± 1.1	3.4 ± 0.9
Echo-colordoppler	**Right side**	**Left side**	**Total**
Varicocele	9 (12.3%)	27 (37.0%)	29 (39.7%)
I	1	3	
II	3	2	
III	2	10	
IV	3	9	
V	0	3	

Interestingly, no significant differences were found in hormone and US data between men with pathospermia (*data not shown*) and in those with abnormal TMSC (*data not shown*).

Sperm cortisol measurement

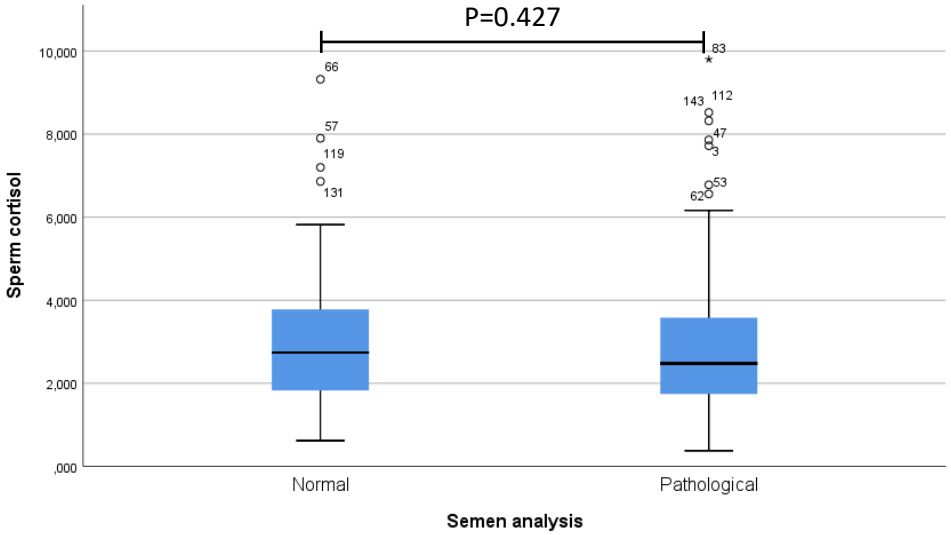
Sperm cortisol levels were evaluated in all the 149 samples. Mean value was 3.04 ± 1.82 mcg/dl. Median value was 2.64 (1.76-3.62) mcg/dl. Minimum and maximum values were 0.376 and 9.800 mcg/dl, respectively. The variable showed a skewed non-normal distribution (*Figure 1*), as confirmed by the Kolmogorov-Smirnov test (p<0.0001).

Figure 1: histogram of sperm cortisol and normal probability Q-Q plot



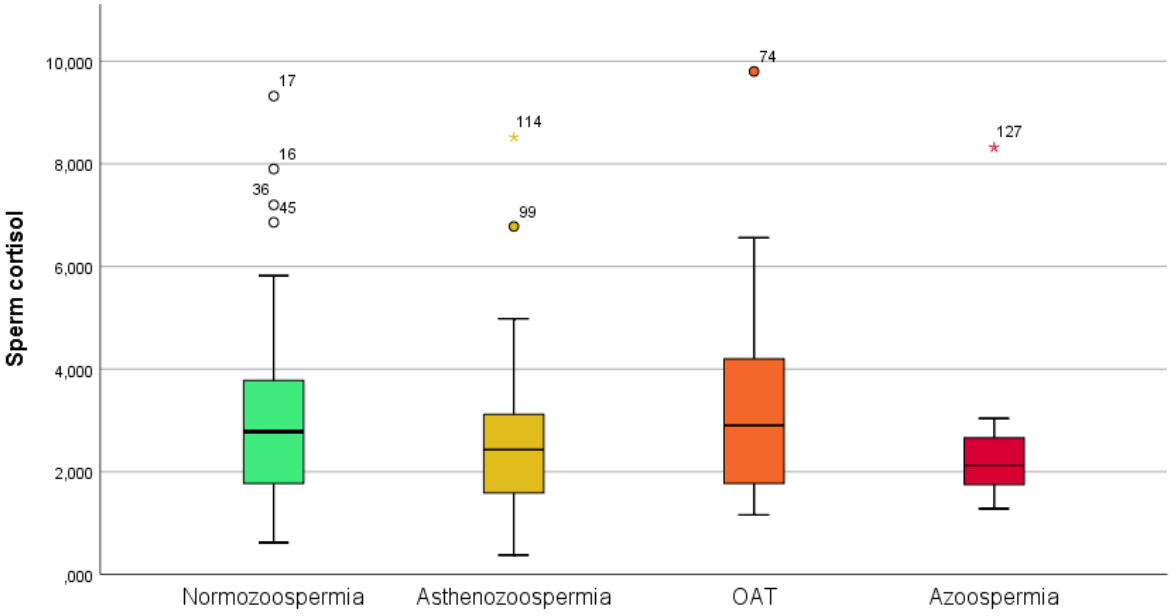
A comparison between sperm cortisol levels between men with normal and pathological semen parameters was performed, but no significant difference emerged: 2.74 (1.78-3.84) versus 2.48 (1.73-3.58) mcg/dl (p=0.427) (*Figure 2*).

Figure 2: sperm cortisol levels in men with normal and pathological semen analysis



Similarly, no significant differences in sperm cortisol levels emerged during comparison of different groups of pathospermia (*Figure 3*).

Figure 3: comparison of sperm cortisol levels in normozoospermic, asthenozoospermic, oligoasthenoteratozoospermic, and azoospermic men.



Median (IQR):

2.78 (1.77-3.81)	2.43 (1.58-3.13)	2.90 (1.74-4.27)	2.12 (1.55-2.85)

Sperm cortisol and varicocele

According with the presence of varicocele, we divided the patients in two groups, comparing semen and hormone data. All the classic semen parameters were significantly worse in men with varicocele, whereas no significant differences emerged in serum hormone levels between the two groups (*Table 4*). In addition, a significant relationship between varicocele and abnormal TMSC was found (Chi^2=6.828, p=0.013).

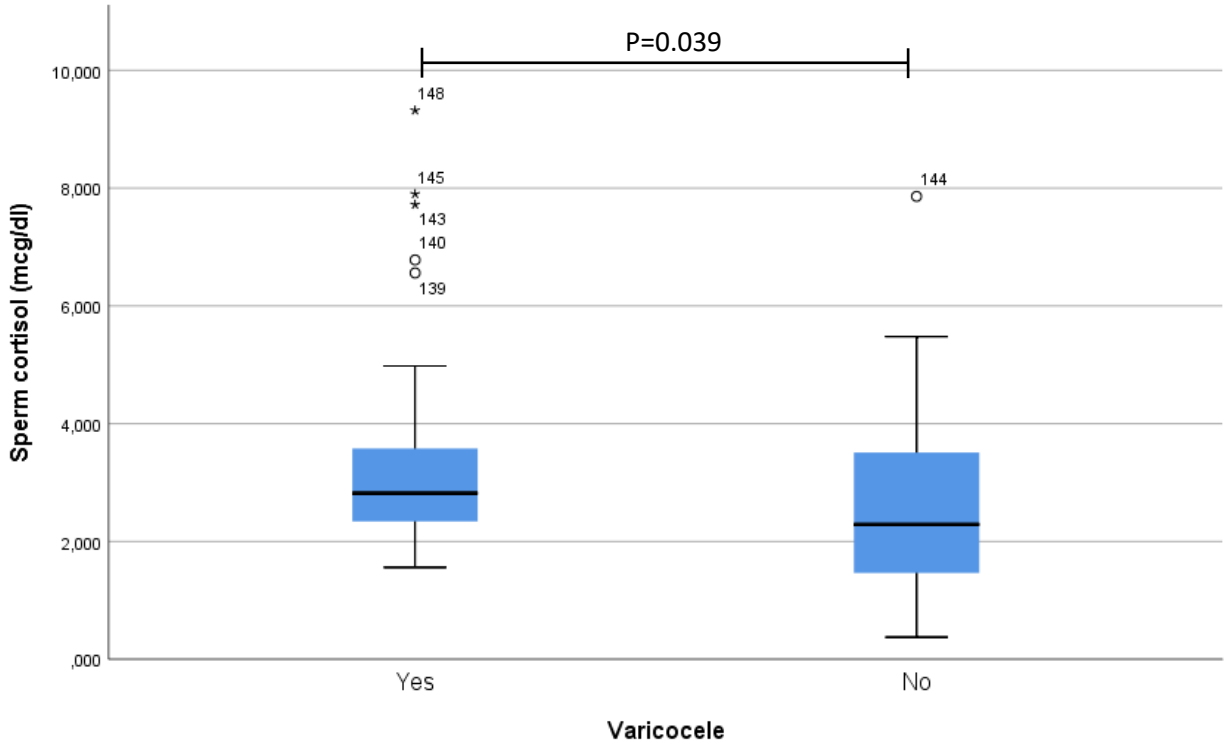
Table 4: semen and hormone levels in men with and without varicocele

n=71	**Varicocele (29)**	**No varicocele (44)**	**p-value**
Age (years)	33.9 ± 9.9 (36.0)	35.0 ± 7.5 (35.6)	P=0.870
Abstinence (days)	4.2 ± 1.4 (4.0)	4.0 ± 1.5 (4.0)	P=0.706
Semen volume (ml)	3.3 ± 1.6 (2.9)	3.2 ± 1.3 (3.2)	P=0.933
pH	7.7 ± 0.2 (7.6)	7.7 ± 0.2 (7.6)	P=0.607
Sperm concentration (10^6/ml)	**31.0 ± 35.7 (20.0)**	**57.9 ± 50.6 (45.0)**	**P=0.010***
Total motility (%)	**20.2 ± 19.1 (15.0)**	**35.3 ± 18.9 (40.0)**	**P=0.003***
Progressive motility (%)	**13.4 ± 17.7 (5.0)**	**25.2 ± 16.7 (30.0)**	**P=0.003***
Atypical morphology (%)	**96.1 ± 2.8 (97.0)**	**92.9 ± 4.5 (93.0)**	**P=0.003***
Leucocytes (10^6/ml)	0.9 ± 0.7 (0.6)	0.8 ± 0.5 (0.6)	P=0.997
Sperm vitality (%)	**36.9 ± 24.8 (29.0)**	**50.4 ± 24.0 (56.0)**	**P=0.032***
TMSC (x 10^6)	**30.3 ± 45.8 (7.2)**	**78.7 ± 80.5 (43.7)**	**P=0.002***
FSH (mIU/ml)	8.1 ± 7.4 (5.6)	7.7 ± 7.9 (4.3)	P=0.424
LH (mIU/ml)	4.8 ± 3.0 (4.7)	4.1 ± 2.7 (3.2)	P=0.194
TT (ng/ml)	5.1 ± 2.3 (4.9)	4.9 ± 1.9 (4.4)	P=0.896
SHBG (nmol/l)	40.4 ± 22.6 (32.8)	43.4 ± 17.5 (40.6)	P=0.199
FT (ng/dl)	9.6 ± 2.9 (9.6)	12.6 ± 23.0 (9.1)	P=0.311
E2 (pg/ml)	26.2 ± 7.4 (25.6)	25.4 ± 10.4 (22.6)	P=0.502
PRL (ng/ml)	8.4 ± 2.6 (8.3)	7.7 ± 3.9 (6.7)	P=0.134
TSH (mcIU/ml)	1.543 ± 0.750 (1.350)	1.691 ± 1.198 (1.440)	P=0.975
PSA (ng/ml)	0.6 ± 0.3 (0.6)	0.7 ± 0.3 (0.7)	P=0.759

E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TMSC: total motile sperm count; TSH: thyroid-stimulating hormone; TT: total testosterone. Values are presented as mean ± SD (median)

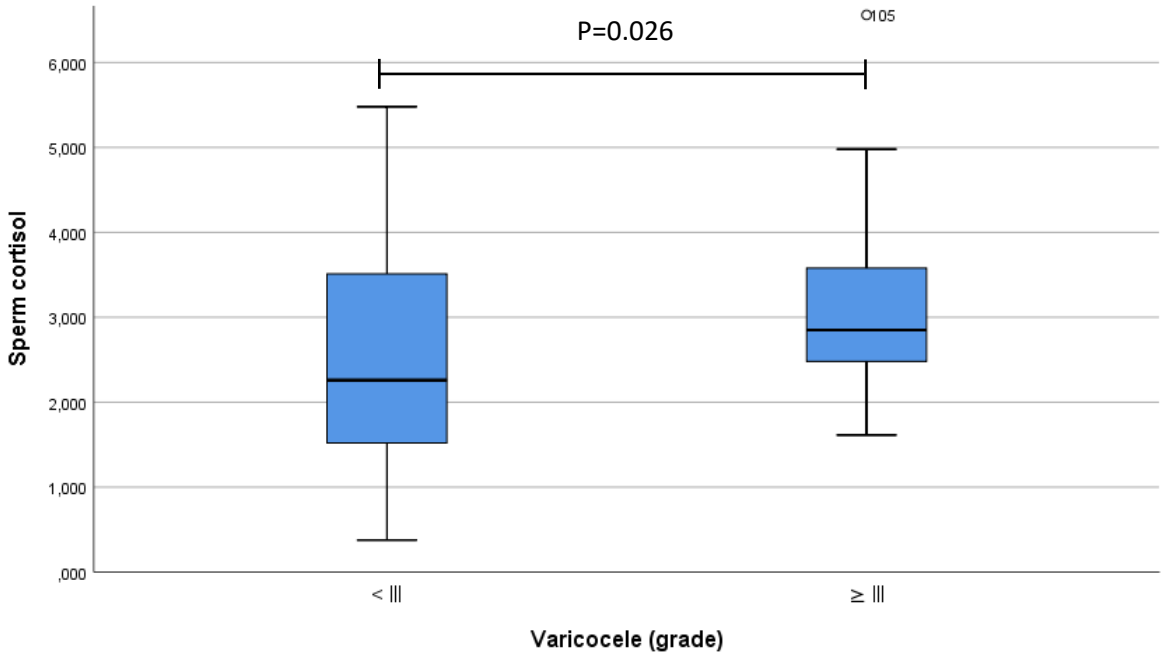
Cortisol levels in semen were significantly higher in men with varicocele compared with men without varicocele (3.56 ± 2.06; 2.82 (2.33-3.70) mcg/dl *versus* 2.63 ± 1.49; 2.29 (1.46-3.55) mcg/dl, P=0.039) (*Figure 4*).

Figure 4: sperm cortisol levels in men with and without varicocele



Interestingly, we also found that grade of varicocele may influence sperm cortisol levels. Indeed, men with varicocele grade III or above had higher cortisol sperm levels than men with lower grades of varicocele (3.49 ± 1.78; 2.85 (2.45-3.64) mcg/dl *versus* 2.77 ± 1.76; 2.26 (1.48-3.58) mcg/dl, P=0.026) (*Figure 5*).

Figure 5: sperm cortisol levels in men with different grades of varicocele



Regarding testicular volume measured by US, right testicular volume was slightly higher than left testicular volume (15.6 ± 5.5 ml versus 14.5 ± 5.6 ml, P<0.0001). This difference was also present when evaluated in men with varicocele (15.6 ± 4.4 ml versus 14.1 ± 5.0 ml, P<0.0001) and in men without varicocele (15.6 ± 6.2 ml versus 14.8 ± 5.5 ml, P<0.0001) in an overlapping manner (mean difference +1.5 ml versus +0.8 ml, p=0.696).

Sperm cortisol and semen culture

We further divided semen samples according with positive (n=27, 29.7%) or negative semen culture (n=64, 70.3%) and then we compared all the available data. Semen characteristics and hormone levels were not different between groups (Table 5). In particular, TMSC was lower in patients with positive sperm culture, but the difference was not significant. Similarly, no significant differences in US data emerged (*data not shown*).

Table 5: semen and hormone levels in men with positive or negative semen culture

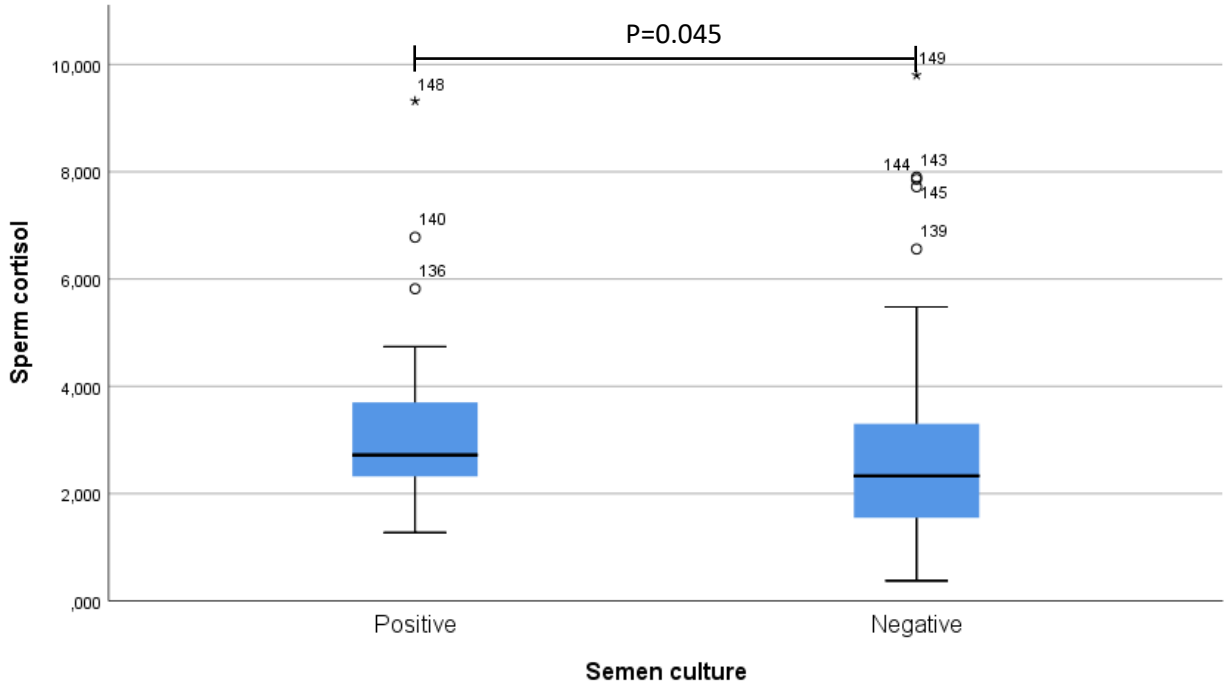
n=91	**Positive (27)**	**Negative (64)**	**p-value**
Age (years)	35.9 ± 7.5 (37.0)	34.7 ± 6.9 (36.0)	P=0.481
Abstinence (days)	4.4 ± 1.6 (4.0)	4.4 ± 1.7 (4.0)	P=0.817
Semen volume (ml)	3.3 ± 1.6 (3.3)	3.3 ± 1.5 (3.1)	P=0.728
pH	7.6 ± 0.2 (7.6)	7.7 ± 0.2 (7.6)	P=0.539
Sperm concentration (10^6/ml)	51.1 ± 35.0 (53.1)	59.3 ± 61.9 (33.0)	P=0.352
Total motility (%)	28.8 ± 19.8 (21.0)	32.8 ± 20.1 (35.0)	P=0.469
Progressive motility (%)	19.5 ± 18.3 (11.0)	23.5 ± 18.3 (25.0)	P=0.390
Atypical morphology (%)	94.7 ± 4.1 (95.5)	93.9 ± 4.3 (95.0)	P=0.368
Leucocytes (10^6/ml)	0.7 ± 0.4 (0.6)	0.9 ± 0.7 (0.7)	P=0.559
Sperm vitality (%)	45.6 ± 25.4 (42.0)	49.9 ± 25.2 (52.5)	P=0.521
TMSC (x 10^6)	66.2 ± 108.4 (21.3)	81.8 ± 105.2 (42.6)	P=0.217
FSH (mIU/ml)	6.3 ± 5.7 (4.1)	8.3 ± 7.3 (5.1)	P=0.263
LH (mIU/ml)	3.3 ± 1.6 (3.0)	4.7 ± 3.1 (3.8)	P=0.077
TT (ng/ml)	4.4 ± 1.3 (4.8)	5.2 ± 2.3 (4.7)	P=0.441
SHBG (nmol/l)	39.5 ± 9.2 (39.8)	44.9 ± 21.7 (37.8)	P=0.938
FT (ng/dl)	8.4 ± 2.4 (8.3)	9.1 ± 2.9 (9.1)	P=0.738
E2 (pg/ml)	21.3 ± 8.3 (19.7)	26.7 ± 10.0 (26.0)	P=0.140
PRL (ng/ml)	7.6 ± 2.3 (8.3)	7.6 ± 2.8 (7.1)	P=0.877
TSH (mcIU/ml)	1.482 ± 0.820 (1.340)	1.441 ± 0.688 (1.400)	P=0.894
PSA (ng/ml)	0.5 ± 0.4 (0.2)	0.7 ± 0.3 (0.7)	P=0.072

E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TMSC: total motile sperm count; TSH: thyroid-stimulating hormone; TT: total testosterone. Values are presented as mean ± SD (median)

Conversely, men with leucocytospermia (sperm leucocytes > 1 x 10^6/mmc), compared with men without leucocytospermia showed a significantly lower semen volume (2.8 ± 1.3 ml versus 3.5 ± 1.6 ml, p=0.012) and higher pH (7.8 ± 0.2 *versus* 7.6 ± 0.2, p<0.0001), whereas other sperm characteristics and hormone levels (including sperm cortisol) showed no significant differences (*data not shown*). Leucocytospermia, in addition, was not significantly associated with positive semen culture (Chi^2= 1.021, p=0.312).

Interestingly, a significant difference in sperm cortisol was noted, with higher levels in men with positive semen culture (3.31 ± 1.74; 2.72 (2.32-3.82) mcg/dl *versus* 2.80 ± 1.89; 2.33 (1.54-3.33) mcg/dl, P=0.045) (*Figure 6*).

Figure 6: sperm cortisol levels in men with positive and negative semen culture



We also evaluated compared US data between patients with positive and negative semen culture, but no differences emerged (*data not shown*).

Bivariate analysis

Then we conducted an exploratory bivariate analysis to investigate any relationship between serum hormone levels and semen characteristics (*Table 6*). We found a weak negative correlation between sperm concentration and FSH (ρ=-0.375, p=0.002) and LH levels (ρ=-0.313, p=0.002), and a moderate negative correlation with FT levels (ρ=-0.540, p=0.031). Conversely, atypical morphology showed a weak positive correlation with FSH (ρ=0.264, p=0.034) and LH levels (ρ=0.362, p=0.03), and a moderate correlation with FT levels (ρ=0.309, p=0.033). A weak negative correlation between LH levels and progressive motility (ρ=-0.263, p=0.034) was also noted. Interestingly, a positive weak correlation between serum E2 levels and sperm vitality and TMSC (ρ=0.314, p=0.043 and ρ=0.380, p=0.014, respectively) was observed. In addition, we found a weak negative correlation between TMSC and FSH (ρ=-0.320, p=0.011) and LH levels (ρ=-0.271, p=0.033), and between age and PRL levels (ρ=-0.316, p=0.009). Finally, a weak negative correlation between serum PSA levels and days of abstinence was observed (ρ=-0.338, p=0.041). We did not find significant correlations between BMI and any semen or US parameter (*data not shown*).

Table 6: bivariate analysis between hormone levels and semen parameters

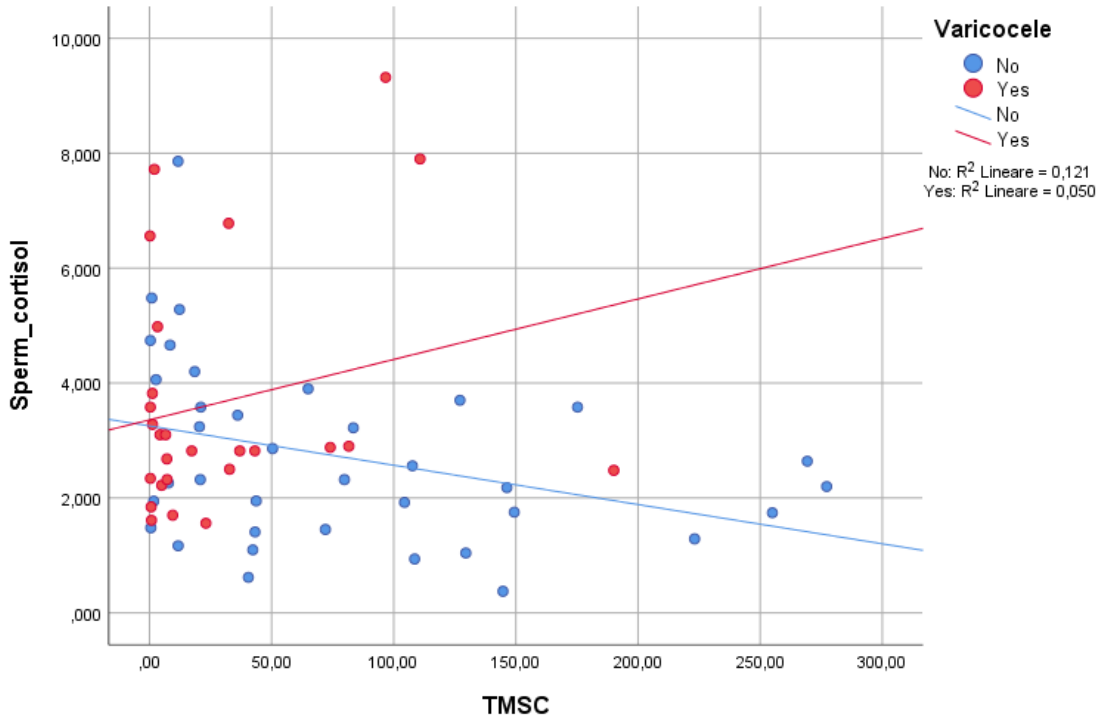
	FSH	LH	TT	SHBG	FT	E2	PRL	TSH	PSA	Sperm cortisol
Age	ρ=-0.070	ρ=-0.226	ρ=-0.071	ρ=0.025	ρ=-0.109	ρ=-0.141	**ρ=-0.316****	ρ=-0.222	ρ=0.058	ρ=-0.048
BMI	ρ=-0.079	ρ=-0.179	ρ=-0.176	ρ=-0.127	ρ=-0.031	ρ=0.238	ρ=-0.242	ρ=-0.031	ρ=-0.013	ρ=-0.151
Abstincence	ρ=-0.089	ρ=-0.076	ρ=0.040	ρ=-0.162	ρ=0.271	ρ=0.096	ρ=0.005	ρ=0.028	**ρ=-0.338***	ρ=-0.085
Volume	ρ=0.062	ρ=0.005	ρ=0.053	ρ=0.819	ρ=0.420	ρ=0.263	ρ=-0.164	ρ=-0.055	ρ=-0.124	ρ=-0.044
pH	ρ=-0.037	ρ=-0.094	ρ=-0.079	ρ=-0.187	ρ=-0.155	ρ=0.018	ρ=0.040	ρ=-0.158	ρ=0.111	ρ=-0.051
Sperm concentration	**ρ=-0.375****	**ρ=-0.313***	ρ=-0.144	ρ=-0.036	**ρ=-0.540***	ρ=0.135	ρ=-0.108	P=-0.223	ρ=0.036	ρ=-0.045
Total motility	ρ=-0.124	ρ=-0.297	ρ=0.011	ρ=0.236	ρ=-0.273	ρ=0.107	ρ=-0.208	ρ=0.031	ρ=0.105	ρ=-0.051
Progressive motility	ρ=-0.071	**ρ=-0.263***	ρ=-0.025	ρ=0.214	ρ=-0.231	ρ=0.070	ρ=0.073	ρ=0.041	ρ=0.112	ρ=-0.042
Atypical morphology	**ρ=0.264***	**ρ=0.362***	ρ=0.149	ρ=-0.124	**ρ=0.309 ***	ρ=-0.097	ρ=0.178	ρ=0.090	ρ=-0.052	ρ=0.024
Leucocytes	ρ=-0.107	ρ=-0.183	ρ=0.016	ρ=0.064	ρ=-0.457	ρ=0.060	ρ=0.054	ρ=0.083	ρ=0.085	ρ=-0.035
Sperm vitality	ρ=-0.211	ρ=-0.229	ρ=0.055	ρ=0.172	ρ=-0.147	**ρ=0.314***	ρ=-0.179	ρ=-0.047	ρ=0.086	ρ=-0.049
TMSC	**ρ=-0.320***	**ρ=-0.271***	ρ=0.026	ρ=0.047	ρ=0.076	**ρ=0.380***	ρ=-0.146	ρ=-0.096	ρ=0.038	ρ=-0.067

*p<0.05, **p<0.01

BMI: body mass index; E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TMSC: total motile sperm count; TSH: thyroid-stimulating hormone; TT: total testosterone.

Although no significant correlation between sperm cortisol and basic semen parameters emerged when the whole group was analyzed, when only patients without varicocele were considered, sperm cortisol showed weak negative correlation with sperm concentration (ρ=-0.323, p=0.048) and moderate negative correlation with sperm motility (ρ=-0.412, p=0.01 and ρ=-0.404, p=0.012 for total and progressive motility, respectively), sperm morphology (ρ=-0.434, p=0.007) and sperm vitality (ρ=-0.428, p=0.008), and a weak-moderate negative correlation between sperm cortisol and TMSC emerged (ρ=-0.395, p=0.016) (Figure 7).

Figure 7: sperm cortisol levels and TMSC (dispersion graph)



Then, we evaluated the relationship between sperm cortisol and serum hormone levels in the general group, finding a weak negative correlation with E2 (ρ=-0.330, p=0.02) (*Table 7, Figure*

8). The negative correlation between sperm cortisol and serum E2 was even stronger and more significant when only patients without varicocele were considered (ρ=-0.559, p=0.002).

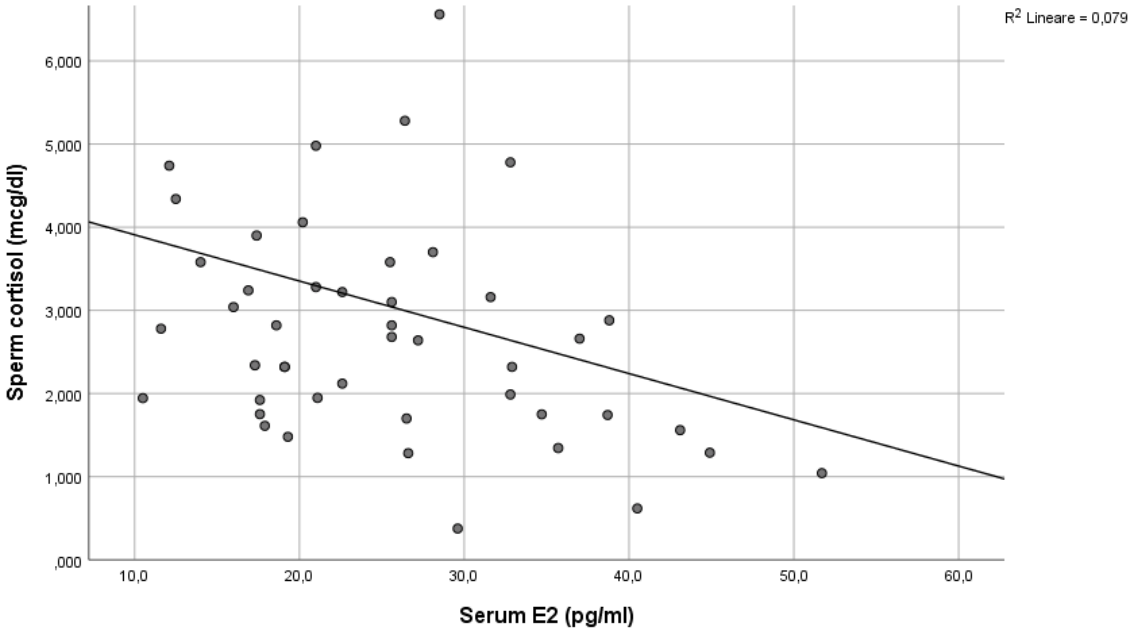
Table 7: bivariate analysis between serum hormone levels and sperm cortisol levels

	FSH	LH	TT	SHBG	FT	E2	PRL	TSH	PSA
Sperm cortisol	ρ=-0.148	ρ=-0.071	ρ=-0.105	ρ=-0.034	ρ=-0.138	**ρ=-0.330***	ρ=-0.104	ρ=-0.028	ρ=-0.127

*p<0.05

E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TSH: thyroid-stimulating hormone; TT: total testosterone.

Figure 8: sperm cortisol levels and serum E2 levels (dispersion graph)



Subsequently, we explored the gonadal status evaluating the relationship between the different serum hormones (*Table 8*). As expected, FSH and LH showed a strong positive correlation between each other (ρ=-0.656, p<0.0001), whereas LH levels were also weakly correlated with TT (ρ=0.270, p=0.023) and PRL levels (ρ=-0.296, p=0.017). A strong and obvious positive correlation was also observed between TT and SHBG (ρ=0.675, p<0.0001) and FT levels

(ρ=0.848, p<0.0001), and between SHBG and FT levels (ρ=0.373, p=0.019). We also noted a weak-moderate positive correlation between TT and E2 levels (ρ=0.388, p=0.006). In addition, we noted a significant correlation with PSA both for FT (ρ=0.486, p=0.016) and E2 (ρ=0.483, p=0.008), but not for TT. Finally, a moderate positive correlation between TSH and PRL levels (ρ=0.418, p=0.002) emerged.

Table 8: bivariate analysis between serum hormone levels

	FSH	LH	TT	SHBG	FT	E2	PRL	TSH	PSA
FSH	-	**ρ=-0.656*** **	ρ=0.020	ρ=-0.034	ρ=0.124	ρ=-0.081	ρ=0.078	ρ=0.054	ρ=0.020
LH	-	-	**ρ=0.270***	ρ=-0.003	ρ=0.273	ρ=0.159	**ρ=-0.296***	ρ=-0.085	ρ=0.035
TT	-	-	-	**ρ=0.675*** **	**ρ=0.848*** **	**ρ=0.388** **	ρ=0.023	ρ=-0.098	ρ=0.240
SHBG	-	-	-	-	**ρ=0.373***	ρ=0.199	ρ=-0.070	P=-0.081	ρ=0.060
FT	-	-	-	-	-	ρ=0.266	ρ=0.234	ρ=0.124	**ρ=0.486***
E2	-	-	-	-	-	-	ρ=-0.229	ρ=-0.085	**ρ=0.483** **
PRL	-	-	-	-	-	-	-	**ρ=0.418** **	ρ=-0.163
TSH	-	-	-	-	-	-	-	-	ρ=-0.111

E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TSH: thyroid-stimulating hormone; TT: total testosterone.

*p<0.05, **p<0.01, ***p<0.0001

Finally, grayscale US data were explored to investigate any uni- or bidirectional correlation with seminal data or hormone levels (*Table 9*). In this regard, we found a weak-moderate positive correlation between mean testicular volume and semen parameters, including sperm concentration (ρ=0.364, p=0.003), total sperm motility (ρ=0.258, p=0.036), sperm vitality (ρ=-0.297, p=0.015), and TMSC (ρ=0.385, p=0.002), whereas we observed a strong negative correlation with LH (ρ=-0.594, p<0.0001) and FSH levels (ρ=-0.696, p<0.0001). Interestingly, we observed a weak positive correlation between sperm leucocytes and diameters of the distal epididymal tract (body: ρ=0.343, p=0.005; tail: ρ=0.386, p=0.001) and the vas deferens

(ρ=0.249, p=0.04). This was confirmed comparing diameters of epididymal body and tail and vas deferens in men with and without leucocytospermia (p=0.008, p=0.002, and p=0.004).

Table 9: bivariate analysis between grayscale US data, hormone levels, and semen parameters.

	Testicular volume	**Epididymal head**	**Epididymal body**	**Epididymal tail**	**Vas deferens**
Age	ρ=0.134	P=-0.0.84	ρ=0.015	ρ=-0.013	ρ=-0.031
Abstincence	ρ=0.025	ρ=-0.066	ρ=0.076	ρ=0.121	ρ=0.091
Volume	ρ=-0.059	ρ=0.022	ρ=-0.190	ρ=-0.187	ρ=-0.036
pH	ρ=0.019	ρ=0.155	ρ=0.113	**ρ=0.248***	ρ=0.145
Sperm concentration	**ρ=0.364****	ρ=-0.139	ρ=0.115	ρ=0.019	ρ=-0.209
Total motility	**ρ=0.258***	ρ=-0.178	ρ=0.064	ρ=-0.129	ρ=-0.204
Progressive motility	ρ=0.226	ρ=-0.175	ρ=0.014	ρ=-0.174	ρ=-0.222
Atypical morphology	ρ=-0.297	ρ=0.200	ρ=-0.110	ρ=0.088	ρ=0.195
Leucocytes	ρ=0.146	ρ=0.181	**ρ=0.343****	**ρ=0.386****	**ρ=0.249***
Sperm vitality	**ρ=-0.297***	ρ=-0.218	ρ=0.066	ρ=0.299	ρ=-0.101
TMSC	**ρ=0.385****	ρ=-0.128	ρ=0.089	ρ=0.297	ρ=-0.153
FSH	**ρ=-0.696*****	ρ=-0.005	ρ=-0.248	ρ=-0.161	ρ=-0.027
LH	**ρ=-0.594*****	ρ=0.093	**ρ=-0.265***	ρ=-0.104	ρ=0.095
TT	ρ=-0.002	ρ=0.229	ρ=-0.172	ρ=0.005	ρ=-0.101
SHBG	ρ=0.086	ρ=0.111	ρ=-0.176	ρ=-0.223	**ρ=-0.289***
FT	ρ=-0.101	ρ=0.154	ρ=-0.080	ρ=0.070	ρ=0.010
E2	ρ=0.252	ρ=0.138	ρ=-0.078	ρ=0.087	ρ=0.128
PRL	ρ=-0.081	ρ=0.161	ρ=0.113	ρ=-0.064	ρ=0.145
TSH	ρ=-0.079	ρ=0.229	ρ=0.219	ρ=-0.116	ρ=0.274
PSA	ρ=0.049	ρ=0.068	ρ=-0.062	ρ=0.671	ρ=0.191
Sperm cortisol	ρ=0.072	ρ=0.101	ρ=0.091	ρ=0.160	ρ=0.033

E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TMSC: total motile sperm count; TSH: thyroid-stimulating hormone; TT: total testosterone.

*p<0.05, **p<0.01, ***p<0.0001

Multivariate analysis

We further conducted a multivariate analysis to investigate which hormone mostly contributed to semen quality. E2 levels confirmed an independent association with TMSC even after adjustment for age, FSH, LH, TT, SHBG, FT, and sperm cortisol, but not when BMI was also included (*Table 10*).

Table 10: multiple linear regression (dependent variable = TMSC).

	Model 1	Model 2	Model 3	Model 4
Age	-	0.067 (-2.894-4.427)	0.018 (-3.596-4.008)	-0.658 (-52.164-28.123)
BMI	-	-	-	-0.207 (-44.214-27.420)
FSH	-0.060 (-53.766-88.918)	0.041 (-4.683-5.658)	-0.018 (-5.531-5.115)	-1.369 (-97.307-51.561)
LH	-0.205 (-23.560-8.401)	-0.242 (-26.774-9.112)	-0.257 (-27.341-8.547)	-0.043 (-59.596-54.157)
TT	-0.121 (-16.492-8.312)	-0.428 (-37.007-8.831)	-0.437 (-37.253-8.519)	8.382 (-572.6-1349.1)
SHBG	-	0.261 (-1.099-2.918)	0.311 (-0.948-3.114)	-5.943 (-84.821-32.599)
FT	-	-	-	-3.570 (-605.27-230.33)
E2	**0.501** (1.389-6.725)**	**0.558** (1.701-7.106)**	**0.497** (1.082-6.768)**	0.502 (-6.288-16.117)
Sperm cortisol	-	-	-0.181 (-19.685-5.963)	-0.453 (-162.78-79.252)

E2: 17-β-estradiol; FSH: follicle-stimulating hormone; LH: luteinising hormone; Free T: free testosterone; PRL: prolactin; PSA: prostate-specific antigen; SHBG: sex hormone binding globulin; TSH: thyroid-stimulating hormone; TMSC: total motile sperm count; TT: total testosterone.

Data are presented as β (CI 95%).

$p<0.05$, $^{**}p<0.01$

A multiple linear regression model was created to further explore the relationship between ultrasound data and TMSC, confirming the significant association between testicular volume and sperm quality (β=0.321, p=0.024). In addition, multiple logistic regression analysis confirmed that higher values of testicular volume are associated to a lower risk of oligozoospermia (OR=0.797, p=0.008) and necrozoospermia (OR=0.808, p=0.008) (*Table 11*).

Table 11: multiple linear regression and multiple logistic regression.

Dependent variable	TMSC	Oligozoospermia	Asthenozoospermia	Teratozoospermia	Necrozoospermia
Testicular volume	**0.321* (0.620-8.350)**	**0.797** (0.674-0.942)**	0.958 (0.835-1.098)	0.885 (0.774-1.013)	**0.808** (0.691-0.946)**
Epididymal head	0.027 (-13.463-16.211)	1.238 (0.891-1.719)	0.962 (0.690-1.340)	1.045 (0.787-1.388)	1.173 (0.850-1.619)
Epididymal body	-0.245 (-15.585-63.543)	0.739 (0.177-3.092)	0.589 (0.162-2.140)	0.374 (0.107-1.314)	0.773 (0.237-2.525)
Epididymal tail	-0.126 (-41.408-20.155)	0.440 (0.126-1.541)	0.971 (0.358-2.633)	1.343 (0.503-3.582)	1.824 (0.651-5.114)
Vas deferens	-0.283 (-52.657-9.900)	3.250 (0.90-10.886)	2.977 (0.951-9.319)	2.670 (0.980-7.275)	1.492 (0.540-4.124)

TMSC: total motile sperm count.

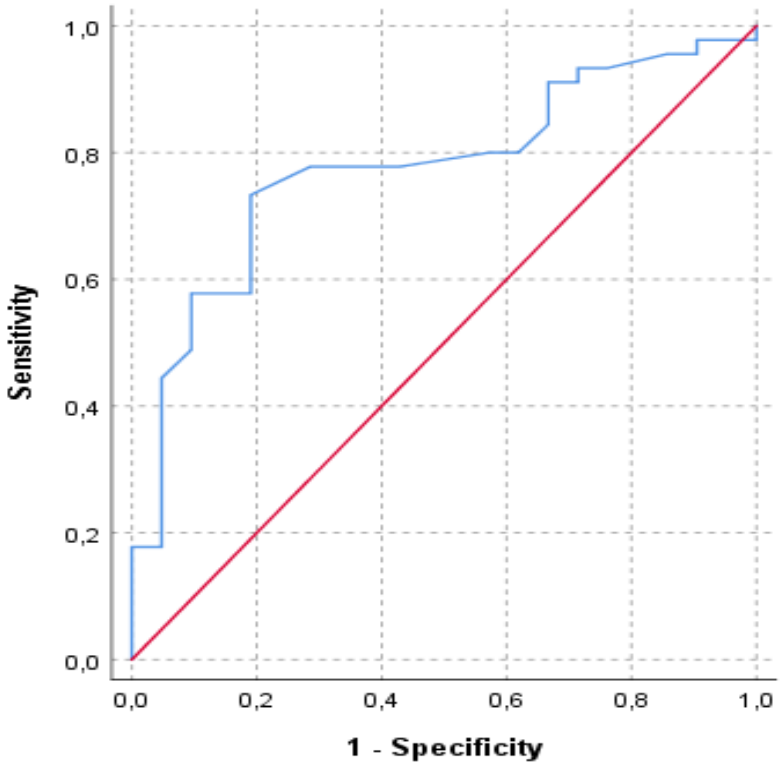
Data are presented as β (CI 95%) and OR (CI 95%).

*p<0.05, **p<0.01

Receiver-operating characteristic (ROC) analysis

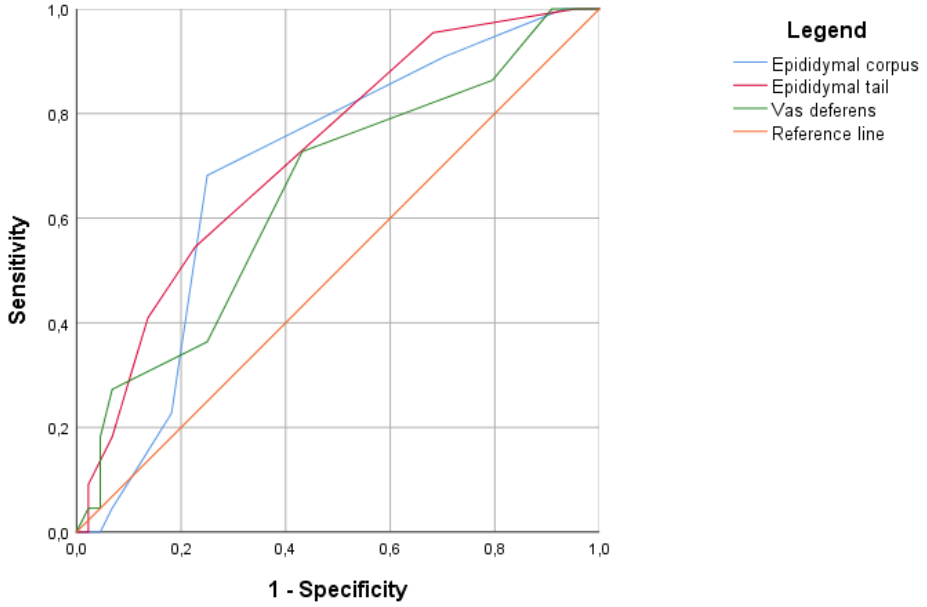
To estimate the predictive value of testicular volume toward oligozoospermia, we performed a ROC analysis that showed that a cut-off of 14.8 ml for testicular volume alone could exclude the probability of oligozoospermia with 0.7 SE and 0.8 SP with a 0.775 area under the curve (AUC) (*Figure 9*).

Figure 9: ROC curve plot for oligozoospermia and testicular volume.



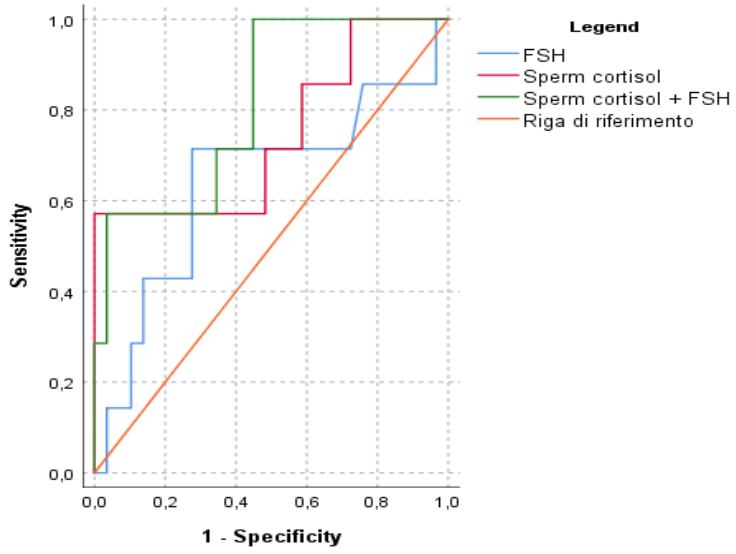
Similarly, ROC analysis for epididymal body, epididymal tail, vas deferens, and leucocytospermia was performed, showing that a cut-off of 3.3 mm for each parameter was associated with SE 0.7 SP 0.8 for the epididymal body, SE 0.6 and SP 0.8 for the epididymal tail, and SE 0.7 and SP 0.6 for the vas deferens. AUC was 0.694, 0.723, and 0.650, respectively (*Figure 10*).

Figure 10: ROC curve plot for leucocytospermia and epididymal body, epididymal tail, and vas deferens antero-posterior diameters measured by grayscale US.



Interestingly, when we compared diagnostic value of sperm cortisol and serum FSH level toward abnormal TMSC in men without varicocele, we found that sperm cortisol showed higher AUC (0.744 versus 0.638), with cut-offs of 3.64 mcg/dl being highly specific (SE 0.6, SP 0.9) and 1.93 mcg/dl highly sensitive but unspecific (SE 0.9, SP 0.4). In addition, the AUC was even higher (0.813) if FSH and sperm cortisol were combined (Figure 11).

Figure 11: ROC curve plot for TMSC and sperm cortisol, FSH, and sperm cortisol + FSH



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DISCUSSION

In recent years, cortisol measurement has been performed in a wide variety of matrices, including blood, saliva, hair, and earwax [16]. This is not surprising, given the fundamental role that GCs play in modulating a plenty of metabolic and immune processes [17, 18]. The widespread use of steroid drugs, moreover, has not been accompanied by an adequate number of studies aimed at investigating their possible impact on fertility. In fact, the effects of exposure to high doses of exogenous GCs, as well as endogenous GCs related to hypothalamic-pituitary-adrenal (HPA) axis disorders (e.g., Cushing's disease) are poorly known. Shift workers, who often exhibit circadian rhythm disturbances that may result in altered adrenal and testicular steroidogenesis, with higher cortisol and lower testosterone levels [19], may also suffer from infertility and sperm abnormalities [20]. Accordingly, Wdowiak et al. reported a significant negative effect of emotional disorders (anxiety and depression) on sperm density and semen volume, together with increased serum PRL and cortisol secretion [21]. Anxiety and depression are conditions known to alter HPA axis function creating a paraphysiological situation of hypercortisolism known as pseudo-Cushing's (or, according to the most recent definition, non-neoplastic hypercortisolemia [22]), but no data is currently available regarding impairment of sperm quality in men with pathologically altered levels of serum cortisol.

For the first time in the past 20 years, we measured cortisol levels in semen fluid. Even if our initial analysis showed no correlation between sperm cortisol and semen parameters, when patients with varicocele were excluded an overall significant negative correlation between sperm cortisol and semen quality emerged. This very interesting finding suggest that high cortisol levels, in the male genital tract, may be associated with an unfavorable condition for spermatogenesis. However, the cross-sectional nature of our study does not allow us to understand whether there is a causal relationship between this "sperm hypercortisolism" and impaired spermatogenesis, but we can make some hypotheses. It is possible that intratesticular cortisol impair Sertoli cells proliferation. This was recently reported by Berger et al., who

artificially reduced testicular corticosteroid concentration in pigs at one week of age using letrozole, finding an inverse correlation between testicular GC concentration and Sertoli cells number [5]. In addition, a previous report by Ren et al. demonstrated that the corticosterone treatment induced Sertoli cells dysfunction and consequent impaired spermatogenesis also in adult animals [23]. In humans, the functionality and Sertoli cells can be indirectly evaluated by serum FSH measurement, which is produced by the pituitary under the stimulation of hypothalamic gonadotropin releasing hormone (GnRH) and regulated through negative feedback by testicular inhibin B [24]. Higher FSH levels are a distinctive marker of defective spermatogenesis and correlates with lower testicular volume and poorer sperm quality [15]. In our study, not only FSH levels correlated with both semen and US parameters, but sperm cortisol showed even better diagnostic power for detection of abnormal TMSC in men without varicocele, suggesting that sperm cortisol could become an additional marker of impaired spermatogenesis.

Notably, in our sample, the relationship between sperm cortisol and seminal parameters loses significance in subjects with varicocele. We hypothesize that varicocele, which is known to accompany male infertility, may result in alterations in seminal quality by nonhormonal mechanisms (e.g., testicular hyperthermia) [25] that may mask the effects of testicular hypercortisolism. Despite this, subjects with varicoceles have both marked seminal changes and higher seminal cortisol levels, so a potential effect of cortisol in the pathophysiology of varicocele-related seminal damage deserves further investigation.

Thus, we found that cortisol in semen is detectable and can be detected in conditions associated with infertility. But where this cortisol comes from is unclear. In this purpose, the very first measurement of seminal cortisol occurred more than 40 years ago. In this study, the authors reported lower seminal cortisol levels than plasma levels, hypothesizing that cortisol may pass from systemic circulation to the testicular environment through a sort of a filter [26]. From there, about 10 years passed before Brotherton published another study in 1990 in which cortisol was

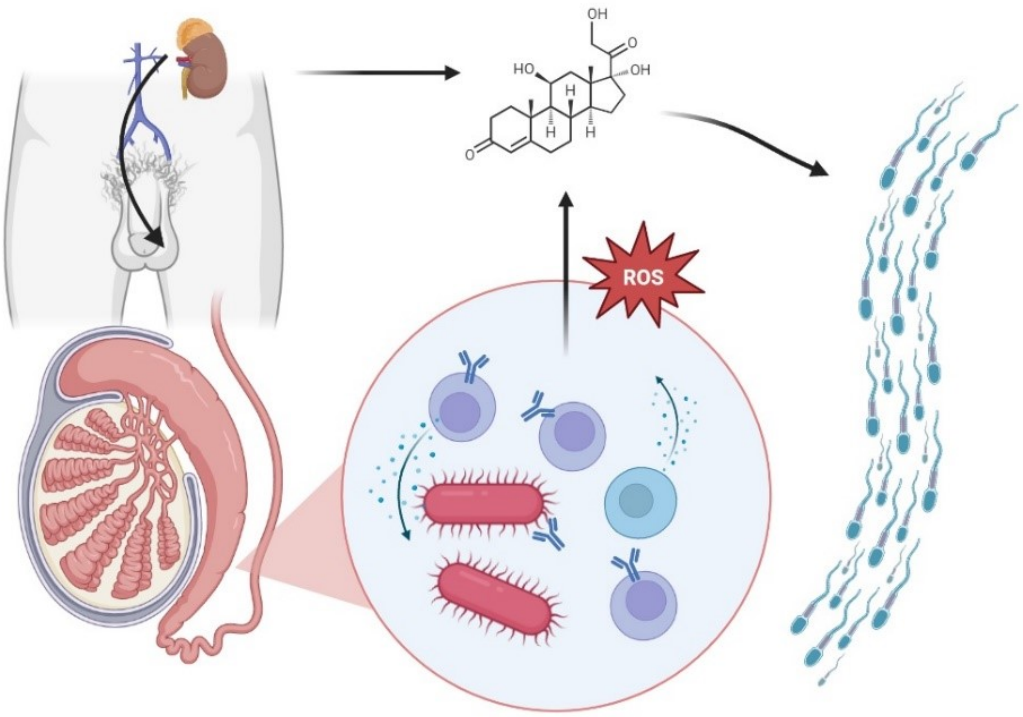
measured in 21 seminal fluid samples, but without providing any information on the relationship with seminal parameters or the fertility status of the subjects tested [27]. The most recent evidence comes from the 2003 study by Hampl et al. in which 40 subjects (26 from infertile couples, 14 healthy volunteers) provided semen and blood samples for evaluation of four different hormones, including cortisol. Interestingly, the authors reported a significant correlation between plasma and seminal cortisol levels ($r=0.3344$, $p=0.003$), but the relationship with seminal parameters had not been explored [28]. Taken together, previous studies reported seminal cortisol levels ranging from 2.14 to 6.38 mcg/dl [28], that is in line with our observation (median value: 2.64 mcg/dl). Interestingly, we also found significantly higher levels of sperm cortisol in men with varicocele. Varicocele is a well-known cause of male infertility which can adversely affect semen quality [29]. In our study group, varicocele was associated with lower sperm count, lower total and progressive motility, higher percentage of abnormal sperms, and lower sperm vitality and TMSC, confirming previous reports [30]. If we accept the hypothesis that seminal cortisol derives from systemic circulation, the higher levels of seminal cortisol that we observed may be due to adrenal metabolite reflux from through the internal spermatic veins. This is in line with our observation that sperm cortisol levels were directly correlated with the severity of varicocele. This, in turn, may lead to testicular damage and impaired testosterone production, as observed in animal studies [31]. Moreover, we found a higher concentration of seminal cortisol in patients with bacteriospermia. This was very interesting due to a wide variety of reasons. First, there is no current consensus on significance of positive semen culture in men with infertility. Indeed, the prevalence of bacteriospermia in infertile men ranges from 6 to 68%, with several bacterial species being involved, including *Escherichia spp*, *Staphylococcus spp*, *Streptococcus spp*, *Enterococcus*, and *Ureaplasma spp*, and consequent heterogeneous effects (negative, neutral, or even positive) on semen quality [32]. Second, we did not observe a significant relationship between leucocytospermia and bacteriospermia, consistently with previous reports [33]. In addition, microscopic parameters of semen were not impaired neither in men with leucocytospermia nor in men with positive semen culture. This suggests that

bacteriospermia is not always associated with inflammation of the male genital tract. According with the most accepted definition of male accessory gland inflammation (MAGI) [34], we observed higher pH and lower semen volume in men with leucocytospermia, who also showed US abnormalities consistent with dilatation of the epididymo-deferential tract, that may persist also after resolution of an acute infection, leading to persistent alteration in the function of male accessory glands [35]. On the other hand, the association between positive semen culture and higher sperm cortisol levels leads to the hypothesis that cortisol may be produced locally to counteract inflammation generated by bacterial contamination of male genital tract. In this regard, bacterial load of sperm has been found to be associated with elevated levels of pro-inflammatory cytokines, including interleukin (IL) -1, IL-2, IL-6, and tumor necrosis factor alpha (TNF-α) [36]. This could lead to the local production autonomous production of immunomodulatory steroids in the male reproductive tracts, as previously suggested by Sterzl et al. [37].

Together, this data suggests that cortisol in semen could partly derive from systemic circulation and partly from local production (*Figure 12*), and we found higher sperm cortisol in two different conditions associated (or which association in under debate) with male infertility.

In addition, both these conditions are associated to increased oxidative stress (OS) in semen [38, 39]. OS occurs when the production of reactive oxygen species (ROS) overcomes the antioxidant capacity of seminal fluid. Indeed, ROS are natural byproduct of adenosine triphosphate production, and small quantities of ROS are required to support spermatogenesis, sperm capacitation and acrosome reaction. When ROS levels increase to a pathological level, OS may cause sperm membrane and DNA damage, impairing the functionality of spermatozoa [40]. If the association between OS and increased sperm cortisol levels is confirmed, the latter could be a surrogate marker of OS, allowing better characterization of male infertility subjects. However, further studies are needed to confirm these hypotheses.

Figure 12: origin of sperm cortisol.



The main source of cortisol in the human body is the adrenal gland. Through the bloodstream, circulating cortisol reaches the male genital tract and pass in seminal fluid. In men with varicocele, sperm cortisol levels could be increased due to the retrograde flow of adrenal metabolites through the left renal vein and the internal spermatic vein. In subjects with bacteriospermia, the presence of bacteria and the activation of white cells lead to a release of pro-inflammatory cytokines and immunomodulatory steroids, including cortisol. Both conditions increase levels of reactive oxygen species (ROS), which in turn cause oxidative stress (OS), impairing semen quality.

Notably, sperm quality does not always reflect the real reproductive capacity of the couple, so that that about one third of cases of male infertility remain “idiopathic” [41]. In recent years, new tests became available to better define the fertility status of male patients, and some of them were included in the last edition of the “WHO laboratory manual for the examination and processing of human semen” under the “extended examinations” and “advanced examinations” chapters [42]. Consequently, the relationship between sperm cortisol and non-classic seminal parameters such as sperm DNA fragmentation (SDF) and semen cytokines, deserve further investigation.

Regarding serum steroids, we found a negative correlation between sperm cortisol and serum E2. Interestingly, we also found a positive correlation between serum E2 and TMSC, that maintained statistical significance even after correction for multiple factors. In line with our findings, recent studies demonstrated that physiological levels of E2 are needed to sustain spermatogenesis, being produced by Leydig and Sertoli cells, and estrogen receptors (ERs) have been found in brain, penis, and testis [43]. Of note, the relationship between serum E2 levels and TMSC become non-significant when BMI was included in the multiple regression analysis, suggesting that additional factors (such as aromatization of testosterone to E2 by adipose tissue) could have influenced our observations. Indeed, increased conversion from testosterone to E2 by aromatase activity of adipose tissue and consequent inhibition of gonadotropin release from the pituitary is considered one of the major cause of impaired sperm quality in extreme obesity [44]. In contrast, obese patients were previously excluded in our study, so we hypothesize that our observation may reflect the modulation of E2 at testicular level that occur under physiological condition, when the normal testosterone/E2 ratio is maintained [45], whereas the relationship between E2 and seminal quality changes only under pathological conditions. The negative correlation that we observed with sperm cortisol, on the other hand, could indirectly suggest an association between impairment of spermatogenesis for higher values of cortisol, but it needs further confirmation.

From a clinical practice perspective, we observed interesting concordance between seminal, hormonal and imaging data. In particular, scrotal US correctly allowed to identify subjects with varicocele and consequent impaired sperm quality, and testicular volume was positively correlated with semen quality and negatively with gonadotropin levels. Testicular volume directly reflect the health status of the seminiferous tubules, and our data are perfectly in line with previous findings [46]. In addition, the diameters of the distal epididymal and the proximal deferential tract were positively correlated with seminal leucocytes and pH, confirming the role of scrotal US in the identification of MAGI [34]. We also conducted a ROC analysis to identify

the best US predictor for impairment of semen quality, but any single parameters provided sufficient predictive power to be used alone in the diagnostic work-up of the infertile male, underlining the importance of a multidimensional approach to the male infertility.

To sum up, we observed indirect evidence that suggest that pathophysiology of male infertility may involve sperm cortisol, which may derive both from systemic circulation and from autonomous production in the male genital tract. Further investigations may help to better define the relationship between cortisol and spermatogenesis. A particularly interesting pathological condition, from this point of view, is CAH. CAH is a group of autosomal recessive disorders in which impaired cortisol biosynthesis leads to various alterations in GC, mineralocorticoid and sex steroid secretion, and the most common type of CAH is 21-hydroxylase deficiency [47]. Men with CAH due to 21-hydroxylase deficiency often suffer from testicular adrenal rest tumors (TARTs), which are cause of pain and male infertility due to impaired blood flow and functional impairment of seminiferous tubules. Since proper GC therapy my completely restore fertility in men with complete azoospermia due to TARTs [48], any additional diagnostic tools to assess testicular health in response to GC therapy would be desirable. In addition, steroid 11β-hydroxylase activity, and consequent capability of cortisol production of TARTs have been demonstrated more than 40 years ago [49], intriguingly suggesting a potential role of the of intratesticular GCs in the development of infertility in these patients. Moreover, given the above-mentioned relationship between shift work and impaired sperm quality, the evaluation of sperm cortisol in male shift workers would deserve more interest.

Our study has some limitations. First, we used a detection kit that was originally projected for other types of matrices, and seminal plasma presents a characteristic viscosity that differs from serum and saliva, but our results are in line with previous, albeit limited, data. Second, we cannot exclude that the process of freezing the samples may have altered seminal cortisol levels, but no technical issues emerged in the de-freezing process and the same method of measurement

was systematically applied for all the samples. In addition, we do not have data regarding serum cortisol levels to draw firm conclusions about the provenience of cortisol measured in semen, but previous reports suggest that a correlation between cortisol in plasma and semen exist [28]. Finally, our data derives from male partners of infertile couples, that is an umbrella term which may include healthy men with infertile partners, true infertile men, and sub-fertile subjects [1]. Further studies are therefore needed to compare sperm cortisol levels in fertile and infertile men to better define the role of seminal GCs in male infertility.

Despite this, our study is the first to have explored, in a sufficiently large population, the possible correlation between sperm cortisol levels and seminal, hormonal and US parameters. Our results are encouraging but need further confirmation by introducing more in-depth seminal assessments (e.g., SDF, seminal cytokines, assessment with computer-assisted-sperm-analysis - CASA - systems), on healthy volunteers, also comparing cortisol levels on other matrices, such as blood and saliva. If our hypotheses were confirmed, sperm cortisol could become an additional tool in the diagnostic pathway of the infertile male that is low cost, relatively easy to perform, and does not require the purchase of additional instrumentation.

CONCLUSION

To the best of our knowledge, our study was the first to evaluate the presence of cortisol in seminal plasma and its relationship with semen, hormone, and US parameters. Our results showed that subjects with risk factors for male infertility such as varicocele and positive semen culture have higher seminal cortisol levels, and we found a negative correlation between sperm cortisol and all classic semen parameters in men without varicocele. Serum E2, which was positively correlated with sperm quality, in turn was negatively correlated with sperm cortisol, indirectly suggesting that cortisol might influence male reproductive function as well. Given the limitations that exist in the infertile male pathway, despite the possibility of a multimodal diagnostic approach, further studies to explore the potential of sperm cortisol are desirable.

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