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The European Academy of Andrology (EAA) ultrasound study on healthy, fertile men: clinical, seminal and biochemical characteristics

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Abstract

Background: Infertility affects 7%-12% of men, and its etiology is unknown in half of cases. To fill this gap, use of the male genital tract color-Doppler ultrasound (MGT-CDUS) has progressively expanded. However, MGT-CDUS still suffers from lack of standardization. Hence, the European Academy of Andrology (EAA) has promoted a multicenter study ("EAA ultrasound study") to assess MGT-CDUS characteristics of healthy, fertile men to obtain normative parameters.

Objectives: To report (a) the development and methodology of the "EAA ultrasound study," (b) the clinical characteristics of the cohort of healthy, fertile men, and (c) the correlations of both fertility history and seminal features with clinical parameters.

Methods: A cohort of 248 healthy, fertile men (35.3 ± 5.9 years) was studied. All subjects were asked to undergo, within the same day, clinical, biochemical, and seminal evaluation and MGT-CDUS before and after ejaculation.

Results: The clinical, seminal, and biochemical characteristics of the cohort have been reported here. The seminal characteristics were consistent with those reported by the WHO (2010) for the 50th and 5th centiles for fertile men. Normozoospermia was observed in 79.6% of men, while normal sperm vitality was present in almost the entire sample. Time to pregnancy (TTP) was 3.0[1.0-6.0] months. TTP was negatively correlated with sperm vitality (Adj.r =−.310, P = .011), but not with other seminal, clinical, or biochemical parameters. Sperm vitality and normal morphology were positively associated with fT3 and fT4 levels, respectively (Adj.r = .244, P < .05 and Adj.r = .232, P = .002). Sperm concentration and total count were negatively associated with FSH

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levels and positively, along with progressive motility, with mean testis volume (TV). Mean TV was 20.4 ± 4.0 mL, and the lower reference values for right and left testes were 15.0 and 14.0 mL. Mean TV was negatively associated with gonadotropin levels and pulse pressure. Varicocoele was found in 33% of men.

Conclusions: The cohort studied confirms the WHO data for all semen parameters and represents a reference with which to assess MGT-CDUS normative parameters.

KEYWORDS
clinical, hormonal, and metabolic parameters, healthy, fertile men, male genital tract ultrasound, seminal parameters, sperm vitality, time to pregnancy

1 | INTRODUCTION

Infertility affects 7%-12% of men of reproductive age.[1-3] Its etiology can be related to congenital or acquired conditions acting at a pre-testicular, testicular, or post-testicular level.[1,3,4] Depending on the etiology, infertile men may present different seminal and hormonal characteristics. Subjects with pre-testicular and testicular causes of infertility often show oligo- or azoospermia,[2,5] various degrees of other seminal abnormalities,[4] low testis volume (TV) and testosterone (T) levels and low (pre-testicular etiology) or high (testicular etiology) gonadotropin levels.[5] Subjects with post-testicular causes of infertility often show oligo- or azoospermia when a partial or a complete obstruction is present, respectively,[5,6] low semen volume and pH when ejaculatory duct obstruction or seminal vesicle/s agenesis/abnormalities occur,[5] and TV, gonadotropin and T levels in the normal range.[5]

Large previous studies performed on infertile men reported that, according to the World Health Organization (WHO),[7] oligozoospermia was detected in 10%[8] to 30%[9-11] of cases, asthenozoospermia in 30%,[9,11] teratozoospermia in 21%[8] to 49%,[9] and low semen volume in 11%[11] to 15%.[8,10] Severe male factor infertility has been reported in 20.4% of infertile men,[2] azoospermia in ~ 5%[2,9] to 11%,[12] and T deficiency in 10.1[12] to 25.6%[13] of cases. On the other hand, large previous studies performed on fertile men reported lower rates of semen abnormalities compared with those of infertile men, showing oligozoospermia in ~ 3%[14,15] to 6%[16,17] of cases, asthenozoospermia in 10.4%[14] to 17%,[16] teratozoospermia in 2.5%[14] to ~ 10%,[15,16] and low semen volume in 4%[16] to 5.8%.[14] Accordingly, fertile men showed better semen parameters,[6,18-21] larger TV[6,20,21] and lower FSH[6,19-22] and LH[6,19,21] levels than infertile men, while, regarding T levels, some authors[18,19] reported higher T values in fertile than infertile men, while others[6,20,21] found no difference between groups.

Despite the knowledge derived from the aforementioned studies, and many scientific efforts, mainly in genetics and sperm biology, the etiology of male infertility is still unknown in half of the cases.[1,3,5] In order to increase the understanding of the causes of infertility, the use of the imaging of the male genital tract (MGT) has been progressively expanded.[5] In particular, color-Doppler ultrasound (CDUS) provides useful information on MGT abnormalities.[5] However, although CDUS is widely used to explore the MGT, MGT-CDUS standardization is still lacking, often leading to subjective and vague diagnoses. For this reason, the European Academy of Andrology (EAA) has promoted the creation of a network of EAA Centers collaborating on an international multicenter study entitled "Standardization of the MGT-CDUS parameters in healthy, fertile men" (shortened to "EAA ultrasound study"; see http://www.andrologyacademy.net/studies)[23] aimed at establishing a cohort of healthy, fertile men of reference in order to define MGT-CDUS normative parameters. The definition of MGT-CDUS reference ranges will help outline the confines of the pathological characteristics of infertile men and improve our understanding of male infertility and its possible therapeutic solutions.

In the present manuscript (a) the development and methodology of the "EAA ultrasound study," (b) the clinical, seminal, and biochemical parameters of the cohort of healthy, fertile men investigated by the EAA multicentric consortium, and (c) the correlations of both fertility history and seminal features of healthy, fertile men with the aforementioned parameters have been reported and discussed. Scrotal and transrectal MGT-CDUS reference ranges and characteristics in healthy, fertile men will be reported separately.

2 | METHODS

The "EAA ultrasound study" was carried out in two steps.

- Step 1—Study onset, including a. Call for Expressions of Interest and investigator meetings; b. definition of the sample size, study design, and aim; and c. definition of the standard operating procedures (SOPs) and assessment of the MGT-CDUS intra- and inter-operator comparability.
- Step 2—Study progress, including a. subject enrollment, clinical evaluation, and semen analysis; b. biochemical evaluation; and c. creation of a dedicated database and data analysis.

2.1 | Step 1—Study onset

2.1.1 | Call for Expressions of Interest and investigator meetings

In September 2012, the Florence EAA Center (coordinator Center) launched a Call for Expressions of Interest to all the EAA Centers in order to create a multicentric network collaborating on an international study aimed at defining the MGT-CDUS normative parameters in a cohort of healthy, fertile men. Overall, eleven EAA Centers (Ancona, Italy; Barcelona, Spain; Cairo, Egypt; Catania, Italy; Florence, Italy; Giessen, Germany; Halle, Germany; L'Aquila, Italy; Muenster, Germany; Rome, Italy; and Tartu, Estonia) joined the project.

Afterward, three investigator meetings were organized. The first one took place in Florence (October 20, 2012) to define the standard operating procedures (SOPs) for the assessment of MGT-CDUS qualitative and quantitative parameters in healthy, fertile men. The second one took place in Berlin (November 29, 2012) to define the study design and aim and the SOPs related to data collection and blood sample management. The third one took place in Florence (April 20, 2013) to assess the intra- and inter-operator comparability of the MGT-CDUS parameters among different operators.

2.1.2 | Sample size, study design, and aim

The "EAA ultrasound study" was designed as a multicenter, international, observational study.

The sample size was defined according to the Clinical and Laboratory Standard Institute (CLSI) Guidelines,[24] which, in order to derive a non-parametric 95% reference interval, support, as a standard for general practice, the recommended minimum of 120 reference subjects. Although CLSI Guidelines suggest a minimum sample size of 120 subjects to calculate the reference range of clinical parameters, in order to increase the precision of the study estimates, a sample size of 180 subjects was decided upon. The recruitment period was established to be 36 months. In order to ensure the achievement of 180 cases for all the parameters investigated, the EAA consortium agreed to enroll the largest possible sample until the end of the recruitment period.

The aims of the "EAA ultrasound study" were defined as follows: 1. primary: to evaluate the quantitative and qualitative CDUS features of the scrotal and prostate-vesicular regions in a cohort of healthy, fertile men of reference to define MGT-CDUS normative parameters, and 2. secondary: to correlate the CDUS findings with clinical, seminal, and biochemical parameters evaluated within the same day.

"Fertile men" were defined by the EAA multicentric consortium as (a) partners of a pregnant woman in the second or third trimester of pregnancy occurred through natural conception or (b) men with a child less than one year old conceived through natural conception. The partners of a pregnant woman in the first trimester of pregnancy were excluded from the study in order to avoid the enrollment of males of couples at high risk of a miscarriage,[25,26] so as to enlist fertile men with a likely high rate of live births. "Healthy men" were defined as subjects with no personal history of previous or current systemic diseases, including tumors, cardiovascular, renal, liver, respiratory, neurological, gastrointestinal, hematological, endocrine, autoimmune, rheumatic, infectious, and dermatological diseases.

The inclusion criteria of the "EAA ultrasound study" were as follows: (a) healthy, fertile men (see above); (b) age ≥ 18 years; and (c) capacity to give consent for study participation. The exclusion criteria of the "EAA ultrasound study" were as follows: (a) previous or current treatments with a recognized long-lasting negative effect on semen parameters (ie, chemo- or radio-therapy)[3]; (b) use, in the six months prior to enrollment, of medications with a recognized transient negative effect on semen parameters[3]; and (c) latex allergy (transrectal probe cover). The health of the female partner was not an exclusion criterion.

A standardized protocol was designed to collect data within the same day from each enrolled subject, including blood sampling for the determination of biochemical parameters; personal and medical history and physical examination; scrotal and transrectal CDUS before and after ejaculation; and semen analysis (see below and http://www.andrologyacademy.net/studies).[23]

2.1.3 | Standard operating procedures (SOPs)

SOPs for primary aim

The CDUS parameters to be analyzed and the methods used to evaluate them were standardized and reported at http://www.andrologyacademy.net/studies.[23] Intra-operator comparability and inter-operator comparability of the main MGT-CDUS characteristics were also assessed. In addition, standardized schedules to report parameters detected before and after ejaculation in each EAA Center were uploaded and made available at http://www.andrologyacademy.net/studies.[23]A broader discussion of the SOPs evaluating the MGT-CDUS characteristics of healthy, fertile men will be reported in separate manuscripts focused on scrotal and prostate-vesicular CDUS.

2.1.4 | SOPs for secondary aims

Fertility history, medical history, and physical examination

An extensive fertility history, male personal and medical history and physical examination, as well as female partner's medical history (as derived from the men's declarations), were performed according to a standardized interview available at http://www.andrologyacademy.net/studies23. In particular, time to pregnancy (TTP), an epidemiological indicator used to assess fecundity,[27] was defined as the number of months taken to conceive,[28] as derived from the men's declarations. Anamnesis and general and andrological physical examination were performed according to previous reports,[1,29-31] including assessment of blood pressure (BP) (mean of three measurements 5 minutes apart, in sitting position, with a standard sphygmomanometer), waistline, height, weight, and TV (assessed using Prader orchidometer). Height and weight were used to calculate body mass index (BMI), and overweight and obesity were defined according to the WHO classification.[32] Pulse pressure was calculated as the difference between systolic and diastolic BP.[31] Mean BP was calculated as (diastolic BP + [1/3 × pulse pressure]).[31] Hypertension was defined according to the American College of Cardiology/American Heart Association (ACC/AHA) Guidelines.[33] Current smoking, alcohol and cannabis consumption, and physical activity levels were assessed using standard questions, and the answers were codified as dummy variables 0-1 (no/yes), according to a previous study.[34]

2.2 | Step 2–Study progress

2.2.1 | Subjects' enrollment, clinical evaluation, and semen analysis

Institutional Review Board (IRB) approval was required for all Centers before starting the enrollment of fertile men. As an example, the Florence Center IRB (June 6, 2013; Prot.2013/0024124) and Azienda Ospedaliero-Universitaria Careggi (November 11, 2013; Prot.37896/2013, Rubrica n.60/13) approvals are available at http://www.andrologyacademy.net/studies.[23]

Subjects were recruited by the EAA Centers and evaluated by a physician to establish the subjects' eligibility. Recruitment was mainly performed among male subjects of fertile couples consulting obstetrics and gynecology units during pregnancy or after delivery. All the relevant study information, summarized in a brochure, and an informed consent form (see http://www.andrologyacademy.net/studies)[23] were provided to each subject before enrollment. After voluntarily agreeing to sign the informed consent form, each subject could enter the study.

All subjects enrolled were asked to undergo a standardized protocol performed within the same day, including scrotal and transrectal CDUS before and after ejaculation (see above, "SOPs for primary aim"); personal and medical history and physical examination (see above, "SOPs for secondary aim"); blood sampling for the determination of biochemical parameters (see below, "biochemical evaluation") and semen analysis.

Semen analysis was performed according to the WHO criteria[7] in each EAA Center's laboratory. In accordance with the WHO Manual,[7] a sexual abstinence period of 2-7 days was allowed and had to be reported and expressed in "days". The presence of antibodies coating spermatozoa was evaluated by a direct test using the commercial SpermMAR (mixed antiglobulin reaction) test IgG (FertiPro), according to a previous study.[20] All laboratories reported following an external quality control (EQC).

2.2.2 | Biochemical evaluation

Blood samples had to be drawn in the morning, after an overnight fast, immediately centrifuged at 3.000 rpm for 20 minutes, and collected plasma had to be stored at − 80°C in each EAA Center. After completing the enrollment of fertile men, plasma samples were transferred to the Florence central laboratory to ensure uniform results of the measurement of biochemical parameters. Total T was measured using the high performance liquid chromatography tandem mass spectrometry (HPLC-MS) method, using the AB-Sciex 6500 QTRAP with Agilent HPLC System. Determination of follicle-stimulating hormone (FSH; assay sensitivity: 0.2 IU/L), luteinizing hormone (LH; assay sensitivity: 0.2 IU/L), prolactin (PRL; assay sensitivity: 4.24 mU/L), thyroid-stimulating hormone (TSH; assay sensitivity: 0.005 mU/L), free triiodothyronine (fT3; assay sensitivity: 0.77 pmol/L), free thyroxine (fT4; assay sensitivity: 1.3 pmol/L), and prostate specific antigen (PSA; assay sensitivity: 0.03 ng/mL) were performed using the electrochemiluminescent method (Modular Roche), while that of sex hormone-binding globulin (SHBG; assay sensitivity: 0.35 nmol/L) was obtained using modular E170 platform electrochemiluminescence immunoassay (Roche Diagnostics). Determination of blood glucose was done by glucose oxidase method (Aeroset Abbott; assay sensitivity: 0.06 mmol/L), while that of total cholesterol (assay sensitivity: 1.29 mmol/L), high-density lipoprotein cholesterol (assay sensitivity: 0.08 mmol/L) and triglycerides by automated enzymatic colorimetric method (Aeroset Abbott; assay sensitivity: 0.02 mmol/L). Calculated free T (cFT) was derived according to Vermeulen's formula[35] (available at http://www.issam.ch/freetesto.htm). Normal FSH levels (≤8 U/L),[6,36,37] hypogonadotropic hypogonadism (contemporary presence of total T < 10.5 nmol/L and LH < 9.4 U/L),[38] normal PRL values as well as mild and severe hyperprolactinemia,[39,40] euthyroidism and thyroid function abnormalities[41,42] and metabolic syndrome (classified according to both the International Diabetes Federation and the American Heart Association/National Heart, Lung, and Blood Institute [IDF&AHA/NHLBI][43] and the National Cholesterol Education Program Third Adult Treatment Panel [NCEP-ATPIII][44] definitions) were defined according to previous studies. In particular, subclinical hypothyroidism was defined as TSH between 4.5 and 10.0 mU/L and normal thyroid hormone levels.[41] In addition, a lower limit of

TABLE 1 Fertility history and socio-demographic parameters of the sample. Data were expressed as mean ± SD when normally distributed, as medians (quartiles) for parameters with non-normal distribution, and as percentages when categorical. The minimum and maximum range values for male and female partner age and TTP have been reported in brackets

	n = 248
Socio-demographic parameters	
Age (years)	
Males	35.3 ± 5.9 (23-53)
Female partners	31.2 ± 5.2 (19-43)
Female partners of age ≤ 35 years old[a]	80.6%
Education (%)	
University degree	48.4
High school diploma	42.4
Middle school	9.2
Physical activity (%)	55.2
1-3 h/day	41.2
4-6 h/day	9.2
>6 h/day	4.8
Current smokers (%)	25.2
<10 cigarettes/day	16.4
10-20 cigarettes/day	6.0
>20 cigarettes/day	2.8
Current alcohol consumption (%)	35.6
≤2 drinks/day	32.8
3-4 drinks/day	2.8
Current cannabis consumption (%)	5.6
1-2/week	3.6
3-4/week	0.8
>4/week	1.2
Occupational exposure to harmful substances (%)	8.4
Pesticides	1.6
Solvents	4.8
Petroleum derivates	3.6
High temperature	0.4
Fertility history	
Time to pregnancy (TTP; months)	3.0 [1.0-6.0] (1.0-24.0)
Sexual intercourses during the 3 months before pregnancy (%)	
1-2/month	6.8
3-6/month	31.2
>6/month	62
Previous natural conception with the same partner (%)	43.5
Previous miscarriage with the same partner (%)	10.5
Female partner's diseases (derived from the men's declarations)(%)	3.6
Autoimmune thyroiditis under levothyroxine treatment	2.8
Non-autoimmune hypothyroidism under levothyroxine treatment	0.4
Polycystic ovarian syndrome	2.8
Endometriosis	0.4
Ulcerative rectocolitis	0.4

[a]A decline in female fecundity after the age of 35 years old has been reported.[47,48]

the normal total T of 9.2 nmol/L according to Bhasin et al,[45] or a cFT < 225 pmol/L according to Wang et al,[46] was also considered to define hypogonadism.

2.2.3 | Creation of a dedicated database and data analysis

At the end of the study, all data collected by the EAA Centers were made anonymous and posted to the coordinator EAA Center (Florence), a dedicated dataset was created and statistical analysis was performed.

In the present study, data were expressed as mean ± SD when normally distributed, as medians (quartiles) for parameters with non-normal distribution, and as percentages when categorical. Correlations were assessed using Spearman's or Pearson's method whenever appropriate. Stepwise multiple linear or logistic binary regressions were applied for multivariate analyses whenever appropriate. When distribution could be normalized through logarithmic transformation, the same test was applied to logarithmically transformed data. For continuous parameters, comparison between two groups in an univariate setting was performed with unpaired two-sided Student's *t* tests for variables with normal distribution or Mann-Whitney U test for variables with non-normal distribution, and analysis of covariance (ANCOVA) was used for comparisons between two groups in a multivariate setting. Relative risk and 95% confidence interval were calculated for the association of categorical parameters, and chi-squared test was used for comparisons, using Fisher's exact test whenever appropriate. Multivariate analyses of categorical parameters were performed using a binary logistic regression model. Multivariate analyses were performed adjusting for confounders including male age, waistline, smoking habit, alcohol consumption, physical activity, cFT levels, and number (#) of EAA Centers, unless otherwise specified. In particular, current smoking, alcohol consumption, and physical activity were codified as dummy variables 0-1 (no/yes) (see above). Finally, the reference range for TV was estimated according to the CLSI Guidelines,[24] as the 5th and the 95th percentiles of its distribution. All statistical analysis was performed on SPSS (Statistical Package for the Social Sciences) for Windows 20.0. A $P < .05$ was considered as significant.

3 | RESULTS

Overall, 248 healthy, fertile men were enrolled from February 2016 to February 2019. Information on the socio-demographic characteristics and fertility history (Table 1), seminal (Table 2), and clinical (Table 3) parameters were available for all the subjects studied. Biochemical parameters were available for most, but not all, of the subjects studied (Table 4).

3.1 | Socio-demographic characteristics and fertility history (see Table 1)

The mean age of the males studied was 35.3 ± 5.9 years. Most female partners were ≤ 35 years old (threshold for the onset of the decline in female fecundity)[47,48] and had no history of systemic or gynecologic diseases. The median TTP was 3.0 [1.0-6.0] months, ranging from 1.0 to 24.0 months. Most of the subjects studied (94.4%) achieved pregnancy within the first 12 months of sexual attempts, while only a small portion (n = 14, 5.6%) conceived between 12 and 24 months.

3.2 | Seminal parameters (see Table 2)

The mean or median values of the seminal parameters found in the cohort studied were consistent with those reported by the WHO[7] for the 50th centile for fertile men. In addition, a high prevalence of subjects with seminal values above the 5th centile reported by the WHO[7] for fertile men was observed for each seminal parameter. According to the WHO,[7] normozoospermia was observed in 79.6% of men, while normal vitality was detected in almost the entire sample. Similar results were found when subjects with a TTP > 12 months were excluded from the analysis (not shown).

3.3 | Andrological and medical history and physical examination (see Table 3)

According to WHO classification,[12] 7.6% of the subjects studied were obese, while a mild (stage 1)[33] hypertension (≥130/85[33,43,44]) was observed in 16% of men.

At Prader orchidometer, the mean TV was 20.4 ± 4.0 mL. The lower reference range (5th percentile of the TV distribution) for the right and the left testis was 15.0 and 14.0 mL, respectively. Any clinical varicocoele was found in 33.0% of men, and 16.6% of the sample showed a grade II or III varicocele, respectively, according to WHO classification.[49]

3.4 | Biochemical parameters (see Table 4)

Most of the enrolled subjects showed hormonal levels in the normal range. Only a few subjects showed FSH > 8 U/L,[36] hypogonadotropic hypogonadism,[38,45,46] mild hyperprolactinemia,[39] or subclinical hypothyroidism.[41] According to IDF&AHA/NHLBI[43] or

TABLE 2 Seminal parameters of the sample. Data were expressed as mean ± SD when normally distributed, as medians (quartiles) for parameters with non-normal distribution, and as percentages when categorical. The minimum and maximum range values for each seminal parameter have been reported in brackets. The values reported by the WHO Manual[7] on the 50th centile for fertile men have been reported in the right column for comparison

	n = 248	WHO Manual,[7] 50th centile
Sexual abstinence (days)	3.8 ± 1.4 (2.0-7.0)	
Seminal parameters		
pH	7.7 ± 0.3 (7.0-8.3)	
≥7.2 (%)	98.8	
Semen volume (mL)	3.3 ± 1.6 (0.3-11.5)	3.7
≥1.5 mL (%)[a]	94.0	
Sperm concentration, $\times 10^6$/mL	67.6 [45.5-112.0] (4.0-288.0)	73
≥15 × 10^6/mL (%)[a]	95.6	
Sperm total count, $\times 10^6$/ejaculate	210.0 [115.0-333.0] (6.4-1932.0)	255
≥9 × 10^6/ejaculate (%)[a]	95.6	
Sperm progressive motility, %	49.0 ± 16.0 (6.0-78.0)	55
≥32% (%)[a]	84.4	
Sperm morphology, % normal forms	9.0 [6.0-13.0] (6.0-13.0)	15
≥4% (%)[a]	90.8	
Sperm vitality	75.0 ± 11.0 (44.0-95.0)	79
≥58 (%)[a]	94.0	
IgG-MAR test ≥ 1% (%)	4.8	
IgG-MAR test ≥ 50% (%)	0.4	
Leukocytospermia (%)	7.2	
Bacteriospermia (%)	7.6	
Abnormal viscosity (%)	25.2	
Prevalence of normozoospermia and isolated or combined sperm abnormalities		
Normozoospermia (%)	79.6	
Presence of at least one sperm abnormality	20.4	
Oligozoospermia (%)	4.4	
Asthenozoospermia (%)	15.6	
Teratozoospermia (%)	9.2	
Oligo-asthenozoospermia (%)	2.8	
Oligo-teratozoospermia (%)	2.0	
Astheno-teratozoospermia (%)	2.4	
Oligo-astheno-teratozoospermia (%)	1.2	
Azoospermia (%)	0.0	

[a]Percentage of subjects above the 5th centile reported for seminal parameters of fertile men by the WHO Manual.[7] The prevalence of normozoospermia and isolated or combined sperm abnormalities has also been reported.

TABLE 3 Clinical parameters of the sample. Data were expressed as mean ± SD when normally distributed, as medians (quartiles) for parameters with non-normal distribution, and as percentages when categorical. The minimum and maximum range values for each physical parameter have been reported in round brackets

	n = 248
Andrological and medical history	
History of cryptorchidism (%)[a]	0.4
History of mumps (%)	32.2
Before puberty	29.8
After or during puberty (without orchitis)	2.4
History of genito-urinary infections (%)	15.2
Prostatitis	5.6
Epididymitis	0.8
Orchitis	0.4
Urethritis	4.8
Sexually transmitted diseases	3.6
Current medications (%)	1.6
Proton pump inhibitors	0.4
Antihistamine drugs	1.2
Physical examination	
Systolic BP (mm Hg)	121.5 ± 11.2 (100-139)
Diastolic BP (mm Hg)	78.6 ± 7.5 (70-89)
Pulse pressure (mm Hg)[31]	42.9 ± 9.4 (15-70)
Mean BP (mm Hg)[31]	92.7 ± 7.3 (70-107)
Hypertension (≥ 130/85)[33,43,44]	16.0%
Body mass index (BMI, kg/m^2)[32]	25.0 ± 3.2 (17-40)
Normal weight (BMI = 18.5-24.9 kg/m^2)[32]	58.8%
Overweight (BMI = 25.0-29.9 kg/m^2)[32]	33.6%
Obesity (BMI ≥ 30.0 kg/m^2)[32]	7.6%
Waistline (cm)	93.0 ± 9.1 (72-133)
Central obesity (waistline > 102 cm)[44]	15.6%
Mean testis volume (Prader) (mL)	20.4 ± 4.0 [15.0-28.0][b]
Right testis volume (mL)	21.2 ± 4.2 [15.0-28.0][b]
Left testis volume (mL)	19.7 ± 4.1 [14.0-25.0][b]
Epididymis and vas deferens (%)	100
Gynecomastia (%)	1.6
Enlarged prostate at digito-rectal examination (%)	5.2
Penis length (mm)	11.6 ± 2.0
Penis circumference (mm)	9.5 ± 2.0
Penile plaques (La Peyronie's disease) (%)	0.0
Varicocoele, left side (%)	33.0
Grade I	16.4
Grade II	14.2
Grade III	2.4
Varicocoele, right side (%)	3.2
Grade I	2.8
Grade II	0.4
Grade III	0.0
Bilateral varicocoele (%)	3.2

Abbreviation: BP, blood pressure.

[a]This subject reported a spontaneous descent of the right testis at the age of five.

[b]For mean testis volume, the reference range, estimated according to the CLSI Guidelines[24] as the 5th and the 95th percentiles of its distribution, has been reported in square brackets. The prevalence of subjects with hypertension[33,43,44] and central obesity[44] or belonging to different BMI classes,[32] according to previous studies, has been also reported.

to NCEP-ATPII[44] criteria, one out of five men had abnormal lipid levels, 19.5% or none of the sample showed abnormal glycemia and 12.9% or 6.9% of the subjects studied had metabolic syndrome, respectively.

3.5 | Fertility history and seminal correlations

The possible correlations between (a) fertility history or (b) seminal features of the cohort studied and other parameters reported in Tables 1-4 were investigated and reported below. Figures 1-3 show the associations that were significant both at univariate and at multivariate analysis, and their level of significance. The multivariate analysis was performed adjusting for the confounders reported in the Methods section, including male age, waistline, current smoking (yes/no), alcohol consumption (yes/no), physical activity (yes/no), cFT levels, and # EAA Centers (unless otherwise specified).

3.6 | Correlations of fertility history-related parameters

After adjusting for confounders, TTP was negatively associated with sperm vitality (Figure 1, panel A), but not with other parameters reported in Tables 1-4 (not shown). The aforementioned association was confirmed even when female partner age was included in the multivariate model as a further covariate (Adj.r = −.249, P = .037). When subjects with TTP ≤ 12 months were compared to the rest of the sample (n = 14), no difference in the parameters in Tables 1-4 was observed (not shown).

The frequency of sexual intercourse was not related to TTP or semen parameters (not shown). Subjects reporting a previous pregnancy or a previous miscarriage showed no differences in TTP or semen parameters compared with the rest of the sample, respectively (not shown).

TABLE 4 Biochemical parameters of the sample. Data are expressed as mean ± SD or as medians (quartiles) when appropriate, and as percentages when categorical

Biochemical parameters	# of subjects	Results
FSH (U/L)	233	3.3 [2.3-4.4] (1.0-12.1)
FSH > 8 U/L[6,36,37]	10 (4.3%)	
LH (U/L)	232	3.2 [2.4-4.5] (0.8-8.7)
LH > 9.4 U/L[38]	0 (0.0%)	
Total testosterone (TT; nmol/L) (HPLC-MS method)	207	19.8 ± 7.5 (10.1-41.5)
Sex hormone-binding globulin (nmol/L)	236	37.8 ± 14.5 (8.6-91.9)
Calculated free testosterone (cFT; pmol/L)[35]	207	403 ± 144 (199-970)
Hypogonadism (TT < 10.5 nmol/L and LH < 9.4 U/L)[38]	2 (1.0%)	
Hypogonadism (TT < 9.2 nmol/L)[45]	0 (0.0%)	
Hypogonadism (cFT < 225 nmol/L)[46]	6 (2.9%)	
PRL (mU/L)	233	153.0 [118.8-226.0] (56-683)
Mild hyperprolactinemia (PRL = 420-735 mU/L)[39,40]	10 (4.3%)	
Severe hyperprolactinemia (PRL > 735 mU/L)[39,40]	0 (0.0%)	
TSH (mU/L)	234	1.48 [1.04-2.20] (0.47-7.76)
fT3 (pmol/L)	233	4.8 ± 0.6 (3.5-6.4)
fT4 (pmol/L)	232	13.6 ± 1.7 (11.5-20.65)
Euthyroidism[41,42]	230 (99.1%)	
Subclinical hypothyroidism[41,42]	2 (0.9%)	
Glycemia (mmol/L)	205	5.0 ± 0.5 (3.5-6.06)
≥5.6 mmol/L[43]	40 (19.5%)	
≥6.1 mmol/L[44]	0 (0.0%)	
Total cholesterol (mmol/L)	205	4.91 ± 0.83 (3.0-7.0)
HDL cholesterol (mmol/L)	205	1.24 ± 0.3 (0.49-2.17)
<1.03 mmol/L[43,44]	45 (22.0%)	
Triglycerides (mmol/L)	203	1.14 [0.86-1.70] (0.45-4.56)
≥1.7 mmol/L[43,44]	54 (26.6%)	
LDL cholesterol (mmol/L)	203	3.02 ± 0.75 (1.16-5.04)
Insulin levels (mU/L)	203	7.5 [5.2-11.9] (1.1-59.5)
PSA (ng/mL)	231	0.74 [0.51-1.06] (0.09-10.27)

Note: The minimum and maximum range values for each biochemical parameter have been reported in round brackets. The number (#) of subjects for whom biochemical parameters were available has been reported. Hormonal and glyco-metabolic abnormalities, according to previous studies,[6,36-46] have been also reported (see the Methods section).

Abbreviations: FSH, follicle-stimulating hormone; fT3, free triiodothyronine; fT4, free thyroxine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LH, luteinizing hormone; PRL, prolactin; PSA, prostate-related antigen; TSH, thyroid-stimulating hormone.

3.7 | Correlations between seminal and socio-demographic or medical history-related parameters

After adjusting for confounders, alcohol consumers showed lower sperm vitality (Figure 1, panel B) and sedentary subjects more often showed abnormal semen viscosity (Figure 1, panel C) than the rest of the sample. No other association between seminal and socio-demographic or medical history-related parameters was observed (not shown).

3.8 | Correlations between seminal and physical examination-related parameters

After adjusting for confounders, sperm concentration, total count, and progressive motility were positively associated with mean TV at Prader evaluation (Figure 2, panels A-C). No other association between seminal and physical parameters/abnormalities (including varicocoele of any grade, hypertension, obesity, or metabolic syndrome—both aforementioned classifications) was observed (not

shown). As a corollary, mean TV was negatively associated with FSH and LH levels, as well as with pulse pressure (Figure 2, panels D-F), but not with other biochemical or physical parameters (not shown).

3.9 | Correlations between seminal and biochemical parameters

After adjusting for confounders, sperm concentration and total count were negatively associated with FSH levels (Figure 3, panels A and B), while sperm vitality and normal sperm morphology were positively associated with fT3 and fT4 levels, respectively (Figure 3, panels C and D). No other association between seminal and biochemical parameters was observed (not shown).

4 | DISCUSSION

The present manuscript represents an introductory report on the development and methodology of the "EAA ultrasound study" (http://www.andrologyacademy.net/studies)[23] and on the clinical results derived from a cohort of healthy, fertile men used as a reference to define MGT-CDUS normative parameters. The "EAA ultrasound study" is the first study aimed at identifying the reference range of the CDUS parameters of the entire human MGT, as derived from a cohort of healthy, fertile men. The development and methodology of the "EAA ultrasound study" have been reported in the Methods section of the present manuscript. The clinical, seminal, and biochemical characteristics of the enrolled healthy, fertile men, as well as correlations of both fertility history and seminal features with the other parameters investigated, are reported herein, whereas the MGT-CDUS characteristics will be described and discussed in separate manuscripts.

The main characteristics of the semen parameters found in the cohort were consistent with those reported by the WHO[7] for both the 50th and the 5th centiles for fertile men. This is relevant, because in other studies[50-52] comparing their data with those of the WHO[7] the mean or median values of the semen parameters of the investigated cohort were consistent with those of the WHO,[7] but there was a higher prevalence of subjects with seminal values below the 5th centile than in the present study.

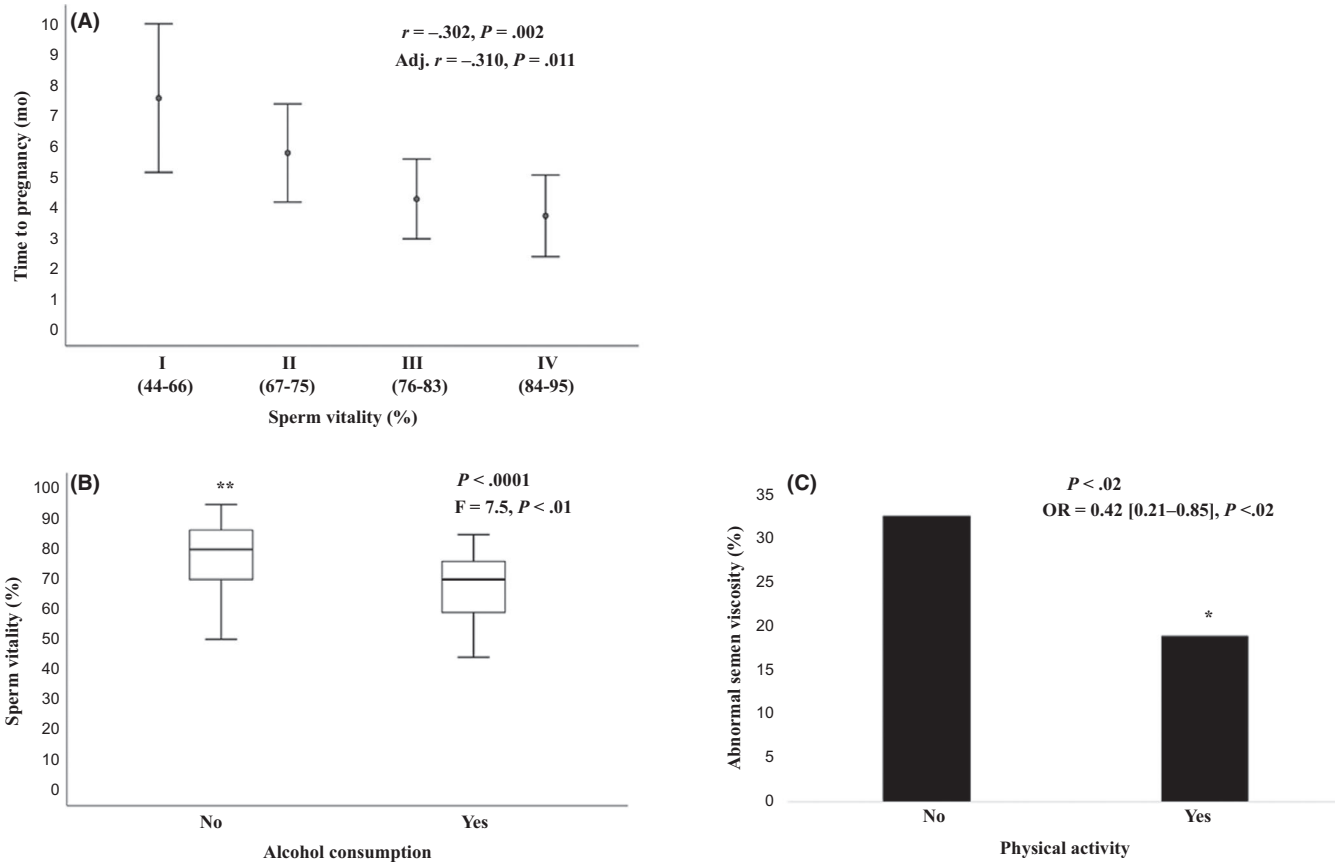

FIGURE 1 Association between sperm vitality and time to pregnancy (panel A) and comparison of sperm vitality between alcohol consumers and no consumers (panel B) and of the prevalence of abnormal semen viscosity between physically active and sedentary men (panel C). Panel (A) Sperm vitality has been reported as quartiles for graphical purposes. Unadjusted and adjusted (for male age, waistline, smoking habit, alcohol consumption, physical activity, cFT levels, and # EAA Centers) associations have been reported. Panel (B) Unadjusted and adjusted (for male age, waistline, smoking habit, physical activity, cFT levels, and # EAA Centers) comparisons have been reported. Panel (C) Unadjusted and adjusted (for male age, waistline, smoking habit, alcohol consumption, cFT levels, and # EAA Centers) comparisons have been reported. OR = odds ratio. *P < .02; **P < .01

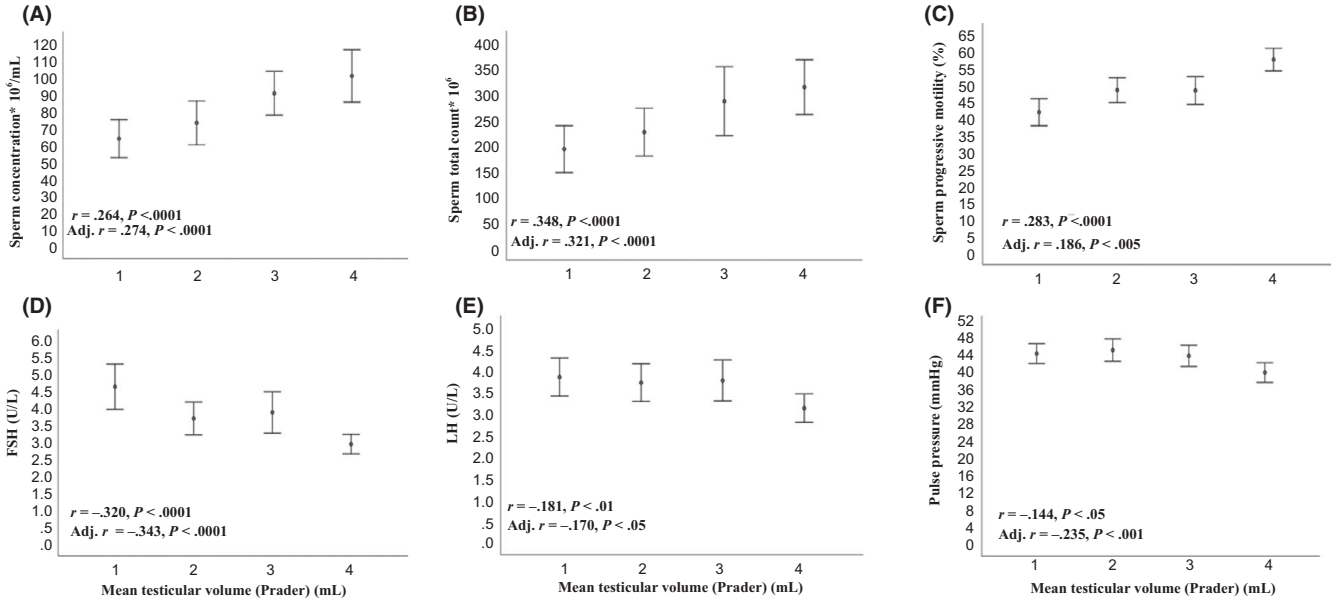

FIGURE 2 Main associations between seminal parameters and mean testicular volume (TV) and between mean TV and hormonal or clinical parameters. Panels (A-C) associations between sperm concentration (panel A), total count (panel B) and progressive motility (panel C) with mean TV (Prader orchidometer evaluation). Panels (D-F) associations between mean TV and FSH levels (panel D), LH levels (panel E), and pulse pressure[31] (panel F). Unadjusted and adjusted (for male age, waistline, smoking habit, alcohol consumption, physical activity, cFT levels, and # EAA Centers) associations have been reported

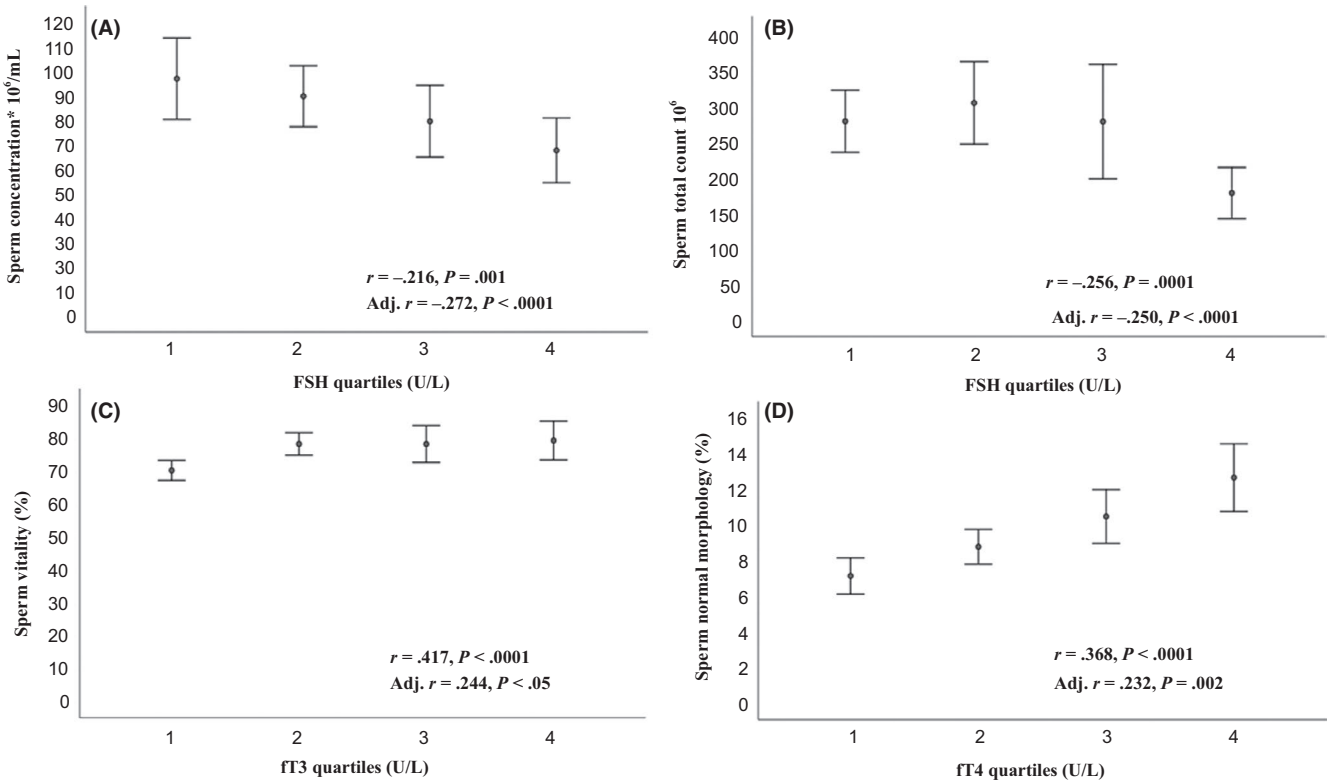

FIGURE 3 Associations between sperm parameters and FSH or thyroid hormones levels. Panels (A, B) associations between sperm concentration (panel A) or total count (panel B) and FSH levels. Panels (C, D) associations between sperm vitality and fT3 levels (panel C) and between normal sperm morphology and fT4 levels (panel D). Unadjusted and adjusted (for male age, waistline, smoking habit, alcohol consumption, physical activity, cFT levels, and # EAA Centers) associations have been reported

Interestingly, the large majority of the enrolled cohort showed a TTP ≤ 12 months, hence supporting the previous definition of infertility (failure to conceive threshold of 12 months) released by the WHO[53] and by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) in partnership with several international societies.[54] However, a few subjects fathered between 12 and 24 months. These subjects (n = 14) can still be classified as fertile, according to the National Institute for Health and Clinical Excellence's (NICE) definition of infertility (failure to conceive threshold of 24 months).[55] In fact, it is well-known that half of the couples not achieving pregnancy within 12 months will naturally achieve it within 24 months.[56]

According to WHO criteria,[7] normozoospermia was found in four out of five fertile subjects; however, about 20% of the sample showed at least one semen abnormality. Previous studies performed on fertile men[14-17,28,57] reported various degrees of seminal abnormalities. Overall, these results confirm that the presence of normal sperm parameters exert a positive impact on pregnancy outcome; however, they do not represent a necessary condition for fertility. In fact, fertility is defined as the capacity to establish a clinical pregnancy,[54,58] while normozoospermia refers to the contemporary presence of three sperm parameters (concentration, motility and morphology) above the 5th centile reported by the WHO.[7]

Sperm vitality represents the proportion of live, membrane-intact spermatozoa.[7,59] In previous studies, poor sperm vitality has been associated with a male infertility status[60] and with a higher rate of sperm DNA fragmentation.[60-63] In this cohort, a normal sperm vitality was observed in almost the entire sample. In addition, among the semen parameters studied, only sperm vitality showed a negative correlation with TTP. This association is even more relevant considering that the female partners were relatively young (mean age 31.2 ± 5.2 years) and had no relevant history of comorbidities. Although, according to WHO,[7] sperm vitality represents an important parameter to be assessed in a routinary semen analysis, no previous study has analyzed its possible association with TTP in fertile men. A previous European multicentric study,[28] evaluating 942 fertile couples, reported a positive association between TTP, sperm concentration, and normal morphology. Conversely some authors,[57] evaluating 315 proven fertile men, found no associations between TTP and sperm parameters. In addition, studies evaluating couples with unknown risk factors for infertility, and who had discontinued contraception to get pregnant,[64-66] reported associations between TTP and sperm concentration,[64,65] motility,[66] and normal morphology.[64-66] However, the relationships found by some authors[66] were not confirmed in multivariate models. In the present study, the relationship between TTP and sperm vitality was confirmed even after adjusting for several confounders. The results suggest that, besides the presence of normal values of the conventional semen parameters, a normal sperm vitality can positively contribute to male fertility. However, further studies are recommended to confirm these results.

Any positive IgG-MAR test was present in about 5% of the sample, according to previous reports in fertile men,[20] while an IgG-MAR test ≥ 50%—considered as a pathological threshold according to the WHO[7]—was virtually absent. Abnormal semen viscosity—considered as a possible cofactor of male infertility through impairment of normal sperm motility[67]—was present in one out of four fertile men. Since semen viscosity increases as a function of ejaculatory abstinence,[68] it could be speculated that, at the time of investigation, the subjects studied had a lower ejaculatory frequency, as their wives were in the second or third trimester of pregnancy or shortly after delivery.

Considering the fertility history of the sample studied, the median TTP reported by fertile men was three months, in line with previous studies.[28,57] The subjects studied often reported frequent sexual activity, confirming its positive role in couple fecundity.[69,70] In fact, two to three sexual intercourses per week ensure that ejaculation falls within the ovulatory period and semen quality is optimal.[69,70] In addition, half of the fertile men reported fathering a previous pregnancy. Accordingly, a history of previous pregnancy is associated with higher chances for a successful second pregnancy.[71] Finally, one out of ten men reported a previous miscarriage, suggesting that fertile couples experience a relatively lower rate of spontaneous pregnancy loss as compared to its worldwide estimation of one out of four/five couples.[25,26,72]

Evaluating hormonal parameters, the majority of the sample showed gonadotropin and T levels within the normal range. Only a few subjects reported FSH levels > 8 U/L, while hypogonadism was virtually absent. These results confirm that a normal hormonal milieu plays a key role in male reproductive health.[73] As expected, FSH showed a negative association with sperm concentration and total count. Conversely, no other sex hormones (including LH and T) or glyco-metabolic parameters showed associations with semen characteristics. However, thyroid hormone levels were positively associated with sperm vitality and normal morphology, even in adjusted models. The relationship between thyroid function and spermatogenesis in adult men is still controversial.[74] Thyroid hormone nuclear receptors are located in several portions of the male genital tract,[40,74-76] including the testis, where they are expressed in Sertoli, Leydig, and germ cells.[77] Hypothyroidism negatively affects ejaculate volume,[78] sperm motility[78,79] and morphology,[79] and its treatment leads to the normalization of semen abnormalities.[78,79] In addition, a positive association between fT3 levels and semen volume has been reported in infertile men.[40] Thyroid hormones in vitro show a beneficial role on sperm mitochondrial function, oxidative stress, and DNA integrity.[80] These mechanisms could be involved in promoting sperm vitality and morphology; however, further studies are needed to clarify this point.

Considering physical examination, 33.6% of subjects were overweight and 7.6% obese, while according to IDF&AHA/NHLBI[43] or NCEP-ATPII[44] criteria, 12.9% or 6.9% of subjects had metabolic syndrome. Overweight or obese men, as well as those with metabolic syndrome, showed no differences in semen parameters compared with the rest of the sample. To date, the relationship between obesity or metabolic syndrome and male fertility is debated.[81] The relatively high reported prevalence of overweight, obesity, or metabolic

syndrome in fertile men and the lack of their impact on semen parameters support their marginal role in male infertility. On the other hand, a mild hypertension was found in 16% of fertile men. Although systolic BP and diastolic BP, as well as being hypertensive, were not associated with semen parameters, pulse pressure showed a negative association with mean TV. Hypertension is frequent among European men with primary infertility[82] and has been associated with a high rate of sperm DNA fragmentation[83] and with abnormal sperm morphology.[83,84] On the other hand, it has been reported that anti-hypertensive treatment improved sperm parameters in men with idiopathic oligozoospermia.[85] The aforementioned data could support the negative relationship between pulse pressure and mean TV found in this study. In contrast, previous studies showed a positive association between mean[31] or systolic[86] BP and mean TV. However, the aforementioned studies were performed in infertile men[31] and adolescents,[86] which may have different hormonal patterns than fertile men. Hence, the relationship between BP and TV needs further investigation.

Considering andrological physical examination, the results of this study confirm that TV represents a reliable surrogate of testicular function.[5] In fact, in the sample studied, TV was positively associated with sperm concentration, total count, and progressive motility, and negatively with FSH and LH levels. Previous studies performed in the general population,[87] infertile[31,88,89] and fertile[21] men, reported that a reduced TV was related to poor sperm parameters and to hormonal abnormalities, including increased gonadotropin levels. So far, the clinical mean TV reported in the general European population was 20.0 ± 5.0 mL.[87,90] In addition, normal TV at Prader evaluation has been previously suggested as > 14-15 mL, although this threshold was derived from a few studies.[91,92] In this cohort of fertile men, the clinical mean TV was 20.4 ± 4.0 mL. The lower reference value for right and left testes were 15 and 14 mL, respectively, hence defining thresholds for clinical testis hypotrophy in an evidence-based way.

Any clinical varicocoele was found in about one out of three fertile men, with a prevalence similar to that of men with primary infertility.[93] In addition, 16.6% of fertile men showed a grade II or III varicocoele. This is relevant, since grade II or III varicocoele is considered as possible candidate for surgical correction in subjects with abnormal sperm parameters both by the American Urology Association/American Society for Reproductive Medicine[94] and the EAU Guidelines on Male Infertility.[95] The relatively high prevalence of varicocoele in fertile men and the lack of association between any grade of varicocoele and sperm parameters suggest that varicocoele may exert a scanty effect on male fertility and that its surgical correction should be limited to highly selected populations. Accordingly, current EAU Guidelines on Male Infertility[95] support very specific indications for varicocoele treatment both in adults and adolescents.

Cryptorchidism is usually associated with an increased risk of infertility and testicular cancer,[5,96] while a history of orchitis or epididymitis is frequently associated with abnormal sperm parameters.[5,97] In the present cohort, those conditions were virtually absent. Conversely, a history of prostatitis or of urinary infection was reported by a relatively high rate of fertile men (15.2%), suggesting a low impact on male fertility. So far, the impact of urogenital infections/inflammation on semen quality and male infertility are still under debate.[98]

Evaluating socio-demographic characteristics, in this cohort current smokers were about one out of four men, mainly reporting a mild consumption. Previous studies on fertile men[14,21,99-101] found relatively higher rates of current smokers than the present study. To date, considering previous studies[14,21,99-101] and the present study, the prevalence of smoking habit in fertile populations is estimated to be 25%-41%. Such a high frequency, similar to that reported by the WHO in the general European male population (38.7%),[102] suggests that a possible negative effect of smoking on male fertility may be marginal. Accordingly, in this study, current smokers showed no differences in semen parameters as compared with non-smokers. As a corollary, some authors reported a similar frequency of tobacco consumption comparing fertile and infertile men[21] although others[100] reported a higher frequency in the latter group. Although a recent meta-analysis showed that cigarette smoking exerts a negative effect on semen parameters,[103] the American Society for Reproductive Medicine (ASRM) Practice Committee[104] reported that smoking has not yet been conclusively shown to reduce male fertility. Hence, further studies are needed to elucidate the effect of smoking on male fertility.

In the present study, alcohol consumption was reported by one out of three men, mainly as a mild intake, and was associated with a lower sperm vitality compared to the rest of the sample. Previous meta-analyses showed that alcohol intake has a detrimental effect on semen volume[105,106] and normal sperm morphology.[106] The results herein suggest a possible further detrimental effect of alcohol on male fertility by reducing sperm vitality, which could affect TTP (see above). Although these data suggest some negative effects of alcohol consumption on male fertility, a large cross-sectional study on healthy men,[107] including 1872 fertile European males, found no association between low/moderate alcohol consumption and semen parameters. However, sperm vitality was not considered in the latter study. Therefore, the relationship between alcohol consumption and male fertility has not been clarified.

About 6% of the present sample reported cannabis consumption, in line with the prevalence reported by the European Monitoring Center for Drug and Drug Addiction[108] in the general population. In this study, no differences in semen parameters were found comparing cannabis users with the rest of the sample. Recent systematic reviews support a negative effect of cannabis consumption on sperm parameters[109,110] and fertilizing capacity.[110] However, further studies are needed to validate these findings in relation to couple fertility.

A modest, occasional, occupational exposure to harmful substances was reported by about 8% of the sample. Although occupational hazards are documented risk factors for male reproductive function,[111] the exposure reported by this study's fertile men was too marginal to draw any conclusion.

Finally, the sample reported regular physical activity in about half of the cases, with a prevalence relatively higher than that reported for the adult European population by the Eurostat.[112] Active subjects showed lower abnormal viscosity compared with sedentary ones, but no difference in other semen parameters. A recent meta-analysis[113] reported that recreational, but not elite, physical activity seems to have a positive effect on sperm concentration and progressive motility. Further studies are needed to better understand the possible beneficial effect of physical activity on male fertility.

5 | CONCLUSIONS

The present study investigated a multinational cohort of 248 healthy, fertile men with semen parameters consistent with those reported by the WHO.[14] Hence, this cohort represents a valid reference point for assessing MGT-CDUS normative parameters. Normozoospermia was observed in four out of five men, while normal sperm vitality was seen in almost the entire sample. The median TTP was three months. TTP was negatively correlated with sperm vitality but not with other seminal features, nor with clinical or biochemical parameters. Sperm vitality was lower in alcohol consumers compared with the rest of the sample and positively correlated with fT3 levels, while normal sperm morphology was positively associated with fT4 levels. Sperm concentration and total count were negatively associated with FSH levels and positively, along with progressive motility, with mean testis volume (TV). Mean TV was 20.4 ± 4.0 mL, and the lower reference values for right and left testes were 15 and 14 mL. Mean TV was negatively associated with gonadotropin levels and pulse pressure. Varicocoele was found in one out of three men. The present results, along with those derived from the MGT-CDUS evaluation of healthy, fertile men (which will be reported in separate studies), will help in better understanding infertility etiology and in modifying its therapeutic management (eg, avoiding uncritical varicocelectomy).

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