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ORIGINAL ARTICLE ***Reproductive endocrinology***

Vitamin D deficiency and low ionized calcium are linked with semen quality and sex steroid levels in infertile men

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STUDY QUESTION: Are low vitamin D levels linked with semen quality and sex steroids in infertile men?

SUMMARY ANSWER: Infertile men with vitamin D deficiency had lower sperm motility, total numbers of motile sperm, Inhibin B, sex-hormone-binding-globulin (SHBG) and testosterone/estradiol ratio, but higher levels of free sex steroids, than infertile men with normal vitamin D levels.

WHAT IS KNOWN ALREADY: Low vitamin D levels have been associated with decreased sperm motility in healthy men, but a relationship between vitamin D and calcium with semen quality and especially sex steroids has not been sufficiently described in infertile men.

STUDY DESIGN, SIZE, DURATION: This study comprises baseline characteristics of 1427 infertile men screened from 2011 to 2014 for inclusion in a randomized clinical trial, the Copenhagen-Bone-Gonadal Study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In total 1427 infertile men, consecutively referred to our tertiary andrological centre for fertility workup, underwent a physical examination and had semen quality assessed based on two samples and blood analysed for serum testosterone, SHBG, estradiol, inhibin B, luteinizing hormone, follicle-stimulating hormone (FSH), 25-hydroxyvitamin D (25-OHD), ionized calcium (Ca^{2+}) and karyotype. There were 179 men excluded due to serious comorbidities or anabolic steroid usage, leaving 1248 patients for analyses.

MAIN RESULTS AND THE ROLE OF CHANCE: Men with 25-OHD >75 nmol/l had higher sperm motility and 66 and 111% higher total numbers of motile spermatozoa after 45 and 262 min, respectively, than men with 25-OHD <25 nmol/l (all $P < 0.05$). SHBG levels and testosterone/estradiol ratios were 15 and 14% lower, respectively, while free testosterone and estradiol ratios were 6 and 13% higher, respectively, in men with 25-OHD <25 nmol/l (all $P < 0.05$). Men with lower Ca^{2+} levels had higher progressive sperm motility and inhibin B/FSH ratio but lower testosterone/estradiol ratio (all $P < 0.05$).

LIMITATIONS, REASONS FOR CAUTION: All outcomes presented are predefined end-points but inferral of causality is compromised by the descriptive study design. It remains to be shown whether the links between vitamin D, calcium, semen quality and sex steroids in infertile men are causal.

WIDER IMPLICATIONS OF THE FINDINGS: The associations between vitamin D deficiency and low calcium with semen quality and sex steroids support the existence of a cross-link between regulators of calcium homeostasis and gonadal function in infertile men.

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Introduction

The actions of vitamin D extend beyond the regulatory role in calcium and bone homeostasis. The vitamin D receptor (VDR) is expressed in several organs including the testes, and vitamin D may be a modifiable regulator of fertility and reproductive function (Blomberg Jensen, 2014).

Synthesis of vitamin D primarily takes place in the skin. Skin exposure to UV-radiation initiates conversion of 7-dehydrocholesterol to biological inactive vitamin D_3 (cholecalciferol). Cholecalciferol has to undergo hepatic 25-hydroxylation, forming 25-hydroxyvitamin D and 1α-hydroxylation in the kidney before the active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) is generated (Prosser and Jones, 2004). Serum 25-hydroxyvitamin D (25-OHD) levels are associated with parathyroid hormone and calcium levels and used clinically to determine vitamin D status (Ross *et al.*, 2011; Rosen *et al.*, 2012; Blomberg Jensen, 2014). Vitamin D deficiency is defined as serum 25-OHD <25 nmol/l (10 ng/ml), while the threshold for insufficiency has been debated but can be considered as 25-OHD <50 nmol/l (Ross *et al.*, 2011; Rosen *et al.*, 2012). Most physiological actions of vitamin D are mediated by binding of the active metabolite $1,25(OH)_2D_3$ to VDR in the target tissue. VDR and the vitamin D metabolizing enzymes are expressed in germ cells, spermatozoa, Leydig cells and the epithelial cells lining the male reproductive tract (Blomberg Jensen *et al.*, 2010, 2012b). This expression profile indicates a direct effect on spermatogenesis, sex hormone production and sperm maturation. Indeed, male rodents with vitamin D deficiency have impaired fertility (Kwiecinski *et al.*, 1989; Uhland *et al.*, 1992; Blomberg Jensen, 2012), while mice with global loss of VDR or 1α-hydroxylase have low sperm motility although the phenotype varies from complete infertility to near normality (Kinuta *et al.*, 2000; Panda *et al.*, 2001; Erben *et al.*, 2002; Blomberg *et al.*, 2013; Sun *et al.*, 2015). VDR regulates synthesis and signalling of estrogens in the reproductive organs of rodents, which has been shown to be important for semen quality (Kinuta, *et al.*, 2000; Krishnan *et al.*, 2010; Blomberg Jensen *et al.*, 2013). However, recent studies have questioned whether the observed reproductive and endocrine effects in VDR and 1α-hydroxylase knockout mice are elicited by direct VDR-mediated effects or by the concomitant hypocalcaemia (Kinuta *et al.*, 2000; Panda *et al.*, 2001; Erben *et al.*, 2002; Blomberg Jensen *et al.*, 2013; Sun *et al.*, 2015). Rescue of the impaired reproductive phenotype in VDR and 1α-hydroxylase knockout models with calcium ± estradiol supplementation indicates an impact of such hypocalcaemia-induced changes. However, time to pregnancy and litter size could not be normalized in vitamin D deficient rats supplemented with calcium (Uhland *et al.*, 1992; Blomberg Jensen, 2012). This observation, combined with the modest stimulatory effect of $1,25(OH)_2D_3$ on intracellular calcium concentration and motility of human spermatozoa, indicates that VDR also may have a direct regulatory role in the reproductive organs of higher primates (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011, 2012a).

The link between vitamin D and calcium complicates interpretation of causality from animal and human studies. So far contribution of calcium-mediated effects has been neglected in most human studies focusing on vitamin D and male reproduction. Positive associations between vitamin D and sperm motility have been consistently reported in most retrospective studies (Blomberg Jensen, 2014), while the presumed impact of vitamin D, calcium or phosphate on sex steroid production is more controversial (Blomberg Jensen, 2012; Jorde *et al.*, 2013). Recent mechanistic studies in humans and rodents have indicated a stimulatory effect of $1,25(OH)_2D_3$ on testosterone and estradiol production (Kinuta *et al.*, 2000; Parikh *et al.*, 2010; Hofer *et al.*, 2014). However, cross-sectional studies in young men found no significant associations between serum 25-OHD and sex steroid levels (Blomberg Jensen, 2012; Jorde *et al.*, 2013). Studies in older men with multiple comorbidities showed a positive association between 25-OHD and total testosterone. This indicates that the influence of vitamin D on sex steroid levels could be an age-dependent or disease-related effect. Serum estradiol in men originates predominantly from peripheral conversion of circulating testosterone (Longcope *et al.*, 1969). The converting enzyme (aromatase: CYP19A1) (Krishnan *et al.*, 2010) is regulated by multiple factors including $1,25(OH)_2D_3$ and calcium (Blomberg Jensen, 2014). Indeed, VDR is considered to be a tissue-specific regulator of aromatase due to the activity of different promotors in the gene for CYP19A1. This enables $1,25(OH)_2D_3$ to stimulate aromatase function in bone and gonad, while VDR represses conversion of androgens into estrogens in adipose tissue and breast (Krishnan *et al.*, 2010).

The stimulatory reproductive effects observed in animal and human studies, and the complex interplay between vitamin D, calcium, bone and gonadal function prompted us to initiate a single centre randomized clinical trial (RCT) called the 'Copenhagen Bone-Gonadal Study'. In this study, vitamin D insufficient infertile men were supplemented with high dose cholecalciferol + calcium or placebo for 5 months. Here, we present the baseline characteristics of 1427 infertile men screened for eligibility to participate in the 'Copenhagen Bone-Gonadal Study' and associations between serum 25-OHD and ionized calcium with testicular function.

Materials and Methods

Study population

A total of 1427 men, referred from January 2011 until August 2014 to the University Department of Growth and Reproduction, Rigshospitalet Denmark (tertiary centre) for andrological evaluation due to male infertility, were screened for inclusion in the randomized clinical trial Copenhagen Bone-Gonadal Study (CBG study NCT01304927). The men were included prospectively in the present study in accordance with the Helsinki Declaration and after approval from the local ethics committee (permit no: H-4-2010-138), irrespectively of whether they met the inclusion criteria for the intervention study. Informed consent was obtained at the clinical visit. Prior to the clinical visit, all men were scheduled to deliver two semen samples and a blood sample on the day they delivered the first semen sample. The blood sample was assessed for biochemical analyses, karyotype

and Y chromosome microdeletions. At the clinical visit, all men underwent a physical examination including ultrasound of the scrotum, and weight and height were measured. All clinical information available at the initial visit was used including information about age, previous diseases, puberty, cryptorchidism, any known history of fertility, when the couple started trying to conceive, medication, co-morbidities, smoking, abuse or use of anabolic steroids etc. Sixteen men were excluded due to current usage of anabolic steroids or drugs interfering with sex hormone levels (Fig. 1). Of the remaining 1411 men, 163 had a previous/current serious co-morbidity that influences vitamin D-calcium homeostasis or reproductive function. All analyses were performed on the whole group of 1411 men and the subgroup of 1248 men without serious co-morbidities. Reproductive hormones were measured in 1370 men (97%), while 25-OHD_{2+3} and ionized calcium were determined in 95 and 93%, respectively. There were 1367 men (97%) who delivered a semen sample on the day of blood sampling and 93% of all men delivered 2 semen samples, allowing semen and biochemical data on 1189 men without serious co-morbidities (Table I).

Biochemical analyses

Blood sampling was done exclusively between 8.00 and 9.30 a.m. Measurements of 25-OHD rely on determination of both 25-OH vitamin D_2 and D_3 using isotope dilution liquid chromatography tandem mass spectrometry (LC–MS/MS). The inter-assay coefficients of variation (CV) for 25-OHD_3 were <10% at 25 nmol/l and <8% at 75 and 250 nmol/l. For 25-OHD_2, CVs were <12% at 20 and <10% at 58 and 220 nmol/l. Serum FSH, LH and SHBG levels were determined using a time-resolved immunofluorometric assay (Delfia, Wallac, Turku, Finland) and inhibin-B was determined by a specific two-sided enzyme-linked immunoassay (InhibinB genII, Beckman Coulter, USA). The CV for measurements of FSH, LH, SHBG and Inhibin B were <4, <4, <6 and 11%, respectively. Testosterone and estradiol levels were measured by RIA (Coat-a-Count, Siemens and Pantex, Santa Monica, CA, respectively) with a detection limit of 0.23 nmol/l for testosterone and 18 pmol/l for estradiol, an intra-assay CV <8% and an inter-assay CV <13% within the normal range. Free testosterone (FT) and estradiol were calculated on the basis of the measured serum concentrations of total testosterone, estradiol and SHBG using the method of Vermeulen (Vermeulen *et al.*, 1999) and Mazer (Mazer, 2009) with a fixed albumin concentration of 43.8 g/l. Serum ionized calcium (Ca^{2+}) was measured using Konelab 30i with a CV <2.0%. More than 80% of the samples were analysed within 60 min. Karyotype and Y chromosome microdeletions were investigated as described previously (Andersson *et al.*, 2004a). Reproductive hormones are presented compared with a population of healthy males (Andersson *et al.*, 2004b) (Fig. 2). In addition, serum 25-OHD, total and ionized calcium, SHBG and albumin (Cobas 8000, CV <4%) were measured in the 300 included men on the first day of treatment.

Semen analysis

Semen samples were produced by masturbation. On average, the two samples were delivered with a 16-day interval. Self-reported information on the duration of ejaculation abstinence, fever and spillage was obtained and semen analysis was conducted as described previously (Jorgensen *et al.*, 2001). Briefly, semen volume was estimated by weighing and sperm concentration determined using Bürker–Türk hemocytometer. Only spermatozoa with tails were counted. Smears were prepared and Papanicolaou stained to determine sperm morphology according to strict criteria. Sperm motility was determined in duplicates and classified as progressive motile (WHO class A + B), non-progressive motile (Class C) or immotile (Class D). AB and ABC sperm motility was determined at two time points after ejaculation (mean T1: 45 min and T2: 4 h and 22 min) and total number of motile sperm was calculated by multiplying with total sperm number. Finally, the averages of the duration of abstinence, time from ejaculation to motility assessment, total sperm count, semen volume, sperm concentration, ABC and AB sperm motility and morphology of the two semen samples (available in 93%) from each individual were determined and used for the analysis. For the remaining 7%, data from one sample were presented.

Statistical analysis

Hormone data are presented in comparison with reference levels based on the general Danish male population (Fig. 2). Descriptive statistics are presented as medians in four strata defined by the level of 25-OHD : <25 nmol/l (deficiency), 25–49 nmol/l (insufficiency), 50–74 nmol/l (sufficiency) and ≥75 nmol/l (high vitamin D status) (Table I). The distributions in the strata were compared using the Kruskal–Wallis test. Significant results were investigated further using multiple regression analyses to ensure that the findings were not biased by confounding factors. In all the regression models, the following biologically relevant confounders were included: age, BMI and smoking. In addition, relevant additional confounders were identified that were significantly ($P < 0.05$) associated with the outcome variable. The following confounders were tested: season, duration of ejaculation abstinence and time from ejaculation to analysis. The following outcome variables were adjusted for additional confounders in the models: sperm concentration or total sperm count for the duration of abstinence, sperm motility variables for time from ejaculation to analysis, total motile sperm for the duration of abstinence and time from ejaculation to analysis, SHBG, total/free estradiol, FT and testosterone/estradiol ratio for season. In the regression analyses, outcome variables were transformed in order to obtain approximate normality of the model residuals as well as variance homogeneity. The best transformations were: sperm motility variables (logit or ln transformed), total sperm count, sperm concentration and the total number of motile and morphological normal spermatozoa (ln transformed). Total testosterone, SHBG, free androgen index (FAI), testosterone/estradiol ratio and testosterone/LH ratio (ln transformed), estradiol, FSH, LH, inhibin B and inhibin B/FSH were untransformed. The explanatory variable 25-OHD or ionized calcium entered the model untransformed. Outcome variables were stratified according to calcium tertiles or quartiles. Interactions between 25-OHD, ionized calcium and possible confounding factors were also investigated. All observations were visually inspected in the residual plots to judge whether or not they were outliers. No observations were excluded. Differences in total number of motile spermatozoa were also tested using the Mann–Whitney *U*-test in men with deficiency or high vitamin D status. All statistical analyses were undertaken using the SPSS software package (version 22, TX, USA).

Results

Basic characteristics of all infertile men without serious comorbidities are presented in Table I and Figs 2–5. All the data described below and presented in Figs 2–5 and Supplementary Figs S1–S3 are from these 1189 men only. Supplementary Table SI comprises similar information on all men with available serum 25-OHD for comparison. Average 25-OHD was 57.5 nmol/l and differed significantly with age ($P = 0.009$), BMI ($P = 0.0004$) and season ($P < 0.0005$) (Supplementary Fig. S1). BMI was negatively associated with 25-OHD, and the highest proportion of vitamin D deficiency was found in men below 25 years of age. Serum 25-OHD was lowest during winter and spring compared with summer and autumn ($P < 0.01$) (Supplementary Fig. S1). Average ionized calcium (Ca^{2+}) was 1.20 mmol/l and 22% of all men had hypocalcaemia defined as serum Ca^{2+} <1.18 mmol/l (Fig. 2). Ca^{2+} was positively

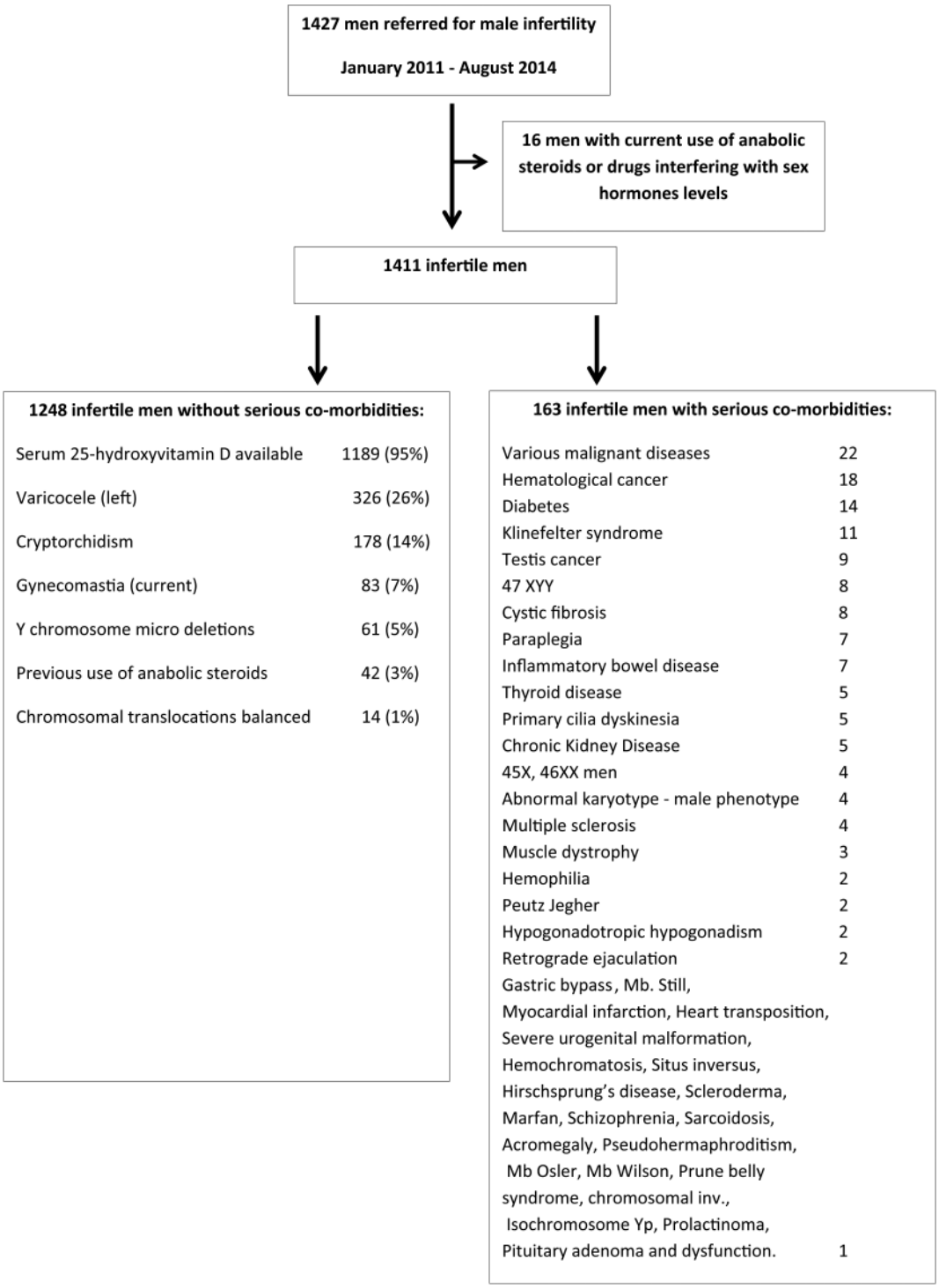

Figure 1 Flowchart baseline characteristics of Copenhagen Bone Gonadal study.

associated with 25-OHD ($P = 0.006$), negatively with age ($P = 0.005$) and differed according to season (Supplementary Fig. S1). To validate ionized calcium measurements albumin, total and ionized calcium were determined in a subgroup of 309 infertile men (who later started in the intervention). The exact same methodology and routine was used for ionized calcium measurements in the 1427 men. We found a positive association between total calcium and ionized calcium $R^2 = 0.15$ (Supplementary Fig. S1, $P < 0.0005$).

Semen quality, inhibin B and FSH

Vitamin D deficient men had significantly lower total (ABC) and progressive (AB) sperm motility and total number of motile spermatozoa. No

Table I Characteristics of 1189 men from infertile couples.

Variable	Total	Serum 25-hydroxyvitamin D (nmol/l)				*P*-Value
Median (25–75 quartiles) or frequency (%)		<25	25–50	50–75	>75	
Included men with serum (*n*)	1189 (100%)	99 (8.3%)	374 (31.5%)	433 (36.6%)	283 (23.9%)	–
Age (years)	34.1 (31–38)	32.3 (28–38)	34.0 (30–38)	34.2 (30–38)	34.4 (31–38)	0.032*
Height (cm)	181.0 (176–186)	178.5 (175–185)	180.7 (176–185)	180.9 (176–186)	182.1 (177–186)	0.024*
Weight (kg)	83.8 (77–93)	83.3 (75–91)	84.2 (77–94)	83.9 (77–93)	83.5 (76–91)	0.443
BMI (kg/m^2)	25.5 (23.5–28.0)	25.8 (22.8–29.2)	25.8 (23.7–28.2)	25.6 (23.5–27.9)	24.9 (23.3–27.1)	0.059
Daily smokers (%)	20	35	22	17	20	0.006**
Duration of infertility (months)	24 (16–30)	24 (18–36)	24 (16–29)	24 (18–30)	18 (12–24)	0.061
Cryptorchidism (%)	16	16	16	15	16	0.993
Testis size orchidometer (ml)	18.0 (15–22)	17 (15–22)	20 (15–22)	18 (15–22)	20 (15–25)	0.097
Testis size ultrasound (ml)	12 (9–16)	11 (8–15)	13 (10–16)	12 (9–16)	13 (10–17)	0.026*
Ultrasound testis score (1–5)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)	0.731
Duration of abstinence (days)	3.8 (3.0–4.0)	3.6 (3.0–4.4)	3.7 (3.0–4.5)	3.8 (3.3–4.3)	3.8 (3.0–4.3)	0.780
Time to motility analysis (h)	0.75 (0.58–0.96)	0.79 (0.63–0.92)	0.71 (0.54–0.92)	0.79 (0.58–0.96)	0.75 (0.58–0.92)	0.335
Semen volume (ml)	3.6 (2.6–4.7)	3.7 (2.8–5.1)	3.7 (2.7–4.8)	3.7 (2.9–4.9)	3.7 (3.0–4.7)	0.801
Total sperm number (10^6)	31.2 (2–96)	20.5 (1.8–83.0)	39.5 (4.0–119)	29.0 (2.9–99.5)	35.5 (4.3–105)	0.144
Sperm concentration (10^6/ml)	8.0 (0.8–27.0)	5.0 (0.4–23.8)	8.7 (1.0–32.5)	7.4 (0.7–25.0)	10.1 (1.5–28.5)	0.106
Sperm motility (ABC) (%)	43 (27–59)	34 (22–54)	45 (28–45)	41 (26–58)	45 (31–63)	0.036*
Progr. sperm motility (AB) (%)	30 (17–46)	23 (11–40)	31 (17–45)	30 (17–44)	31 (20–48)	0.075
Sperm morphology (%)	2.8 (1–5)	2.3 (1–5)	2.5 (1–6)	2.8 (1–5)	2.8 (1–5)	0.898
Ionized calcium (mmol/l)	1.20 (1.18–1.23)	1.20 (1.17–1.23)	1.20 (1.17–1.22)	1.21 (1.19–1.23)	1.21 (1.18–1.23)	0.009
FSH (U/l)	5.0 (3.1–8.2)	5.4 (3.5–7.5)	4.7 (3.0–7.8)	5.2 (3.1–10.2)	5.0 (3.0–8.4)	0.125
Inhibin B (pg/ml)	147 (89–201)	148 (81–183)	150 (95–206)	139 (83–200)	156 (94–205)	0.070
LH (IU/l)	3.9 (2.9–5.4)	4.0 (3.0–5.7)	4.0 (2.9–5.2)	4.0 (2.9–5.7)	4.0 (3.0–5.2)	0.502
Total testosterone (nmol/l)	15.4 (12.3–19.4)	14.3 (11.1–17.8)	15.6 (12.6–19.6)	15.1 (12.1–19.3)	15.9 (12.9–19.4)	0.070
SHBG (nmol/l)	34 (26–45)	28 (21–36)	35 (26–47)	34 (26–45)	37 (28–48)	<0.0005***
FAI	45 (37–56)	51 (41–65)	45 (38–55)	45 (36–56)	44 (36–54)	0.004**
Free testosterone (pmol/l)	300 (251–358)	320 (252–374)	306 (256–360)	297 (246–358)	298 (256–349)	0.369
Free testosterone (%)	1.99 (1.7–2.3)	2.21 (1.9–2.4)	1.98 (1.7–2.2)	1.99 (1.7–2.2)	1.93 (1.7–2.2)	<0.0005***
Estradiol (pmol/l)	65 (51–80)	70 (56–89)	66 (52–82)	62 (49–79)	63 (49–78)	0.013*
Free estradiol (pmol/l)	1.52 (1.1–2.0)	1.73 (1.3–2.3)	1.56 (1.2–2.0)	1.50 (1.1–1.9)	1.48 (1.1–1.9)	<0.0005***
Free estradiol (%)	2.42 (2.1–2.7)	2.61 (2.4–2.9)	2.39 (2.1–2.7)	2.42 (2.1–2.7)	2.33 (2.1–2.6)	<0.0005***
Testosterone/estradiol ratio	242 (187–321)	219 (164–287)	238 (189–309)	249 (190–337)	261 (199–331)	0.003**

The men were all without serious co-morbidities. The results for the entire group as well as stratified according to serum 25-hydroxyvitamin D levels are shown. Cryptorchidism includes both previous and current cases and testis size is based on right testis. Free testosterone and free estradiol were calculated using the Vermeulen and Mazer equation. FAI calculated by total testosterone divided by SHBG multiplied by 100. *P*-Value determined by the Kruskal–Wallis test.

Semen variables are presented as median of two samples except for the 7% of men who only delivered one sample. For conversion of 25-OHD from nmol/l to ng/ml, divide by 2.496.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.0005$

significant differences in total sperm counts, sperm concentration, semen volume or sperm morphology between men with low and high vitamin D status were found (Fig. 3). Positive linear associations between 25-OHD and AB and ABC sperm motility were found 45 min (T1) ($P = 0.02$ and 0.04) and 4 h (T2) ($P = 0.004$ and <0.0005) after ejaculation. Vitamin D deficient men had lower AB and ABC motility at both T1 and T2 ($P < 0.05$), also after adjustment for time from ejaculation to motility assessment, age and ionized calcium (Fig. 3). The link between 25-OHD and sperm motility was strongest on Day 1 but the same tendency was found in the second semen sample delivered on average 16 days after the blood sample (Supplementary Fig. S3). Total numbers of motile and progressive motile sperm were 66 and 111% higher at T1 and T2 in the high vitamin D status group compared with vitamin D deficient men ($P = 0.003$ and 0.02, respectively) (Fig. 3). Median numbers of total motile or progressive motile sperm were lower in vitamin D deficient men at both T1 and T2 (data not shown, $P < 0.05$). Surprisingly, ionized calcium was negatively associated with AB motility at both time points ($P = 0.02$ and 0.04). Men in the

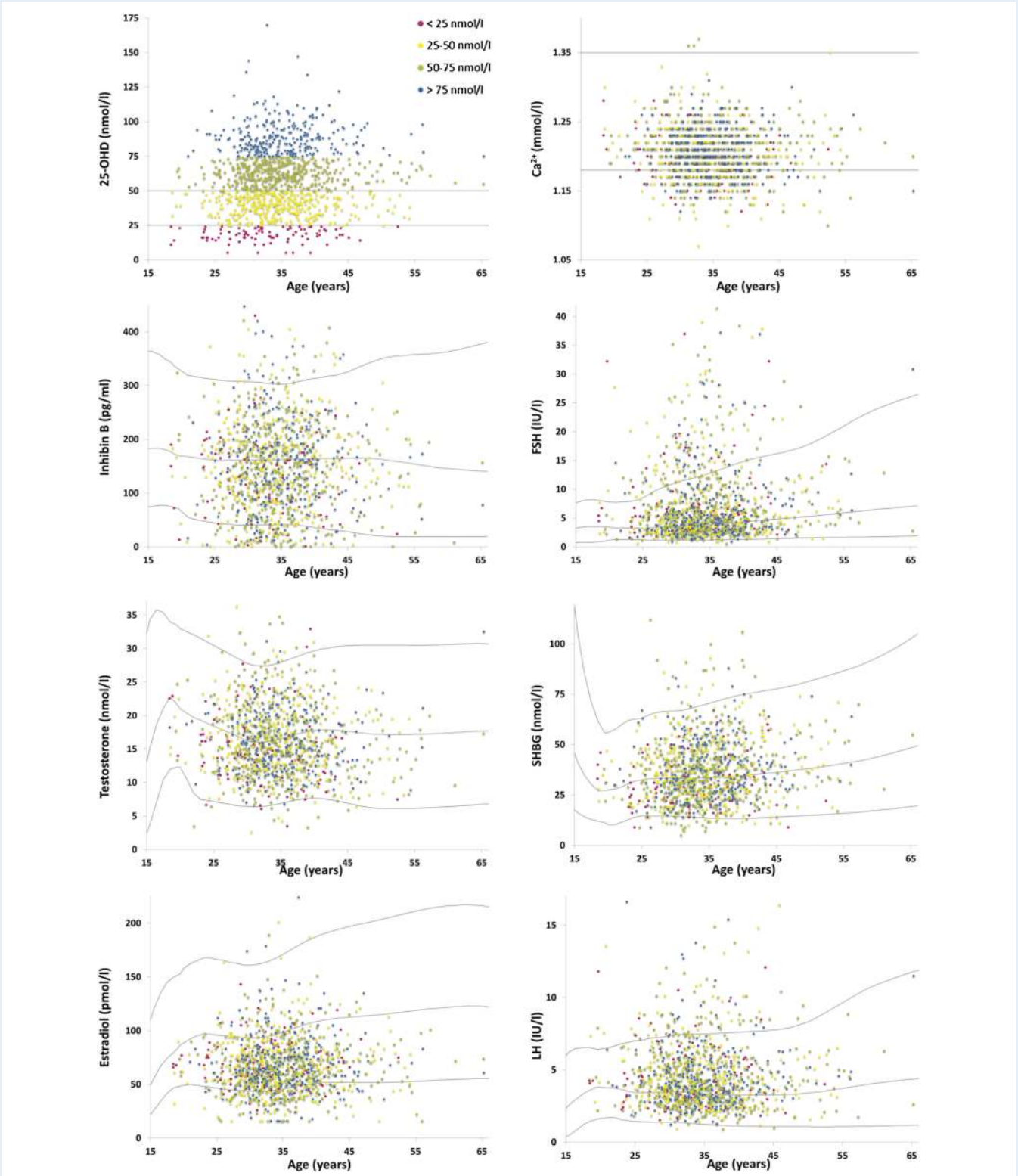

Figure 2 Serum 25 hydroxyvitamin D (25-OHD), ionized calcium (Ca^{2+}) and reproductive hormones according to age and 25-OHD status in 1189 infertile men. Each measurements are coloured according to 25-OHD status (red: <25 nmol/l; yellow: 25–50 nmol/l; green: 50–75 nmol/l; blue >75 nmol/l. Lines represent age-specific reference ranges for reproductive hormones (2.5, 50 and 97.5 percentiles). For ionized calcium, the upper line = 1.35 and lower line = 1.18 mmol/l mark reference limit used to determine hypo- and hypercalcaemia are based on data from healthy men. Tertiles of ionized calcium are <1.19, 1.19–1.22, >1.22 mmol/l and quartiles are <1.18, 1.19–1.20, 1.21–1.23, >1.23 mmol/l.

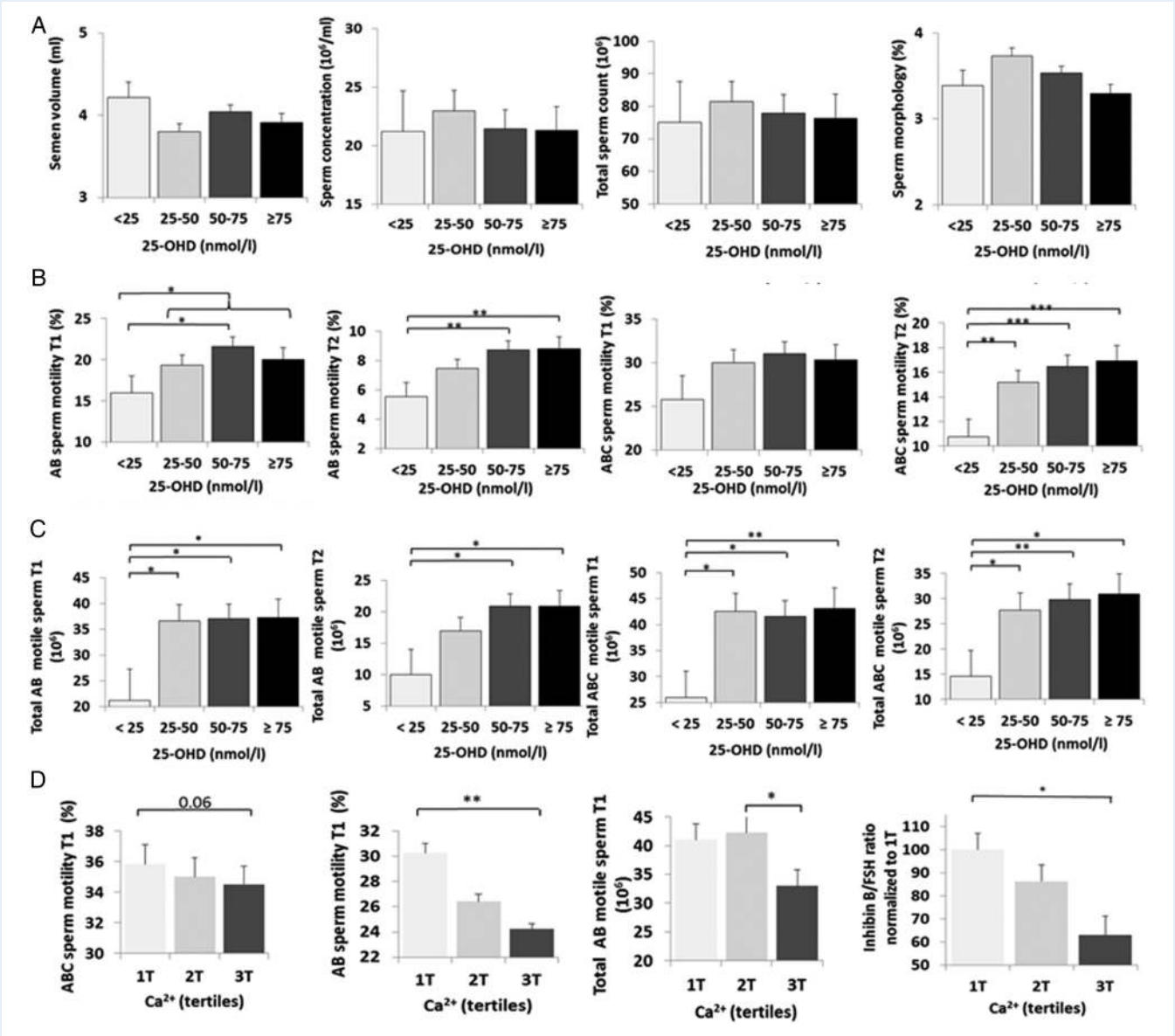

Figure 3 Semen quality and reproductive hormones according to vitamin D and calcium status in 1189 infertile men. Outcomes stratified according to serum 25-OHD: deficiency (<25 nmol/l), insufficiency (25–50 nmol/l), sufficiency (50–75 nmol/l) and high status (≥75 nmol/l). (**A**) Semen volume, sperm concentration, total sperm count and sperm morphology. (**B**) Percentage progressive (AB) sperm motility after 45 min (T1) and 4 h and 22 min (T2) and total (ABC) sperm motility at T1 and T2. (**C**) Total number of progressive (AB) and all (ABC) motile sperm (in millions) at T1 and T2. (**D**) Sperm motility, progressive sperm motility, total progressive motile sperm at T1 and inhibin B/FSH ratio stratified according to tertiles (1T: <1.19, 2T: 1.19–1.22, 3T: >1.22 mmol/l) of ionized calcium. Data presented as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

highest ionized calcium group had significantly lower AB motility after adjustment of relevant confounders including 25-OHD (Fig. 3). No vitamin D and calcium interaction was significantly associated with sperm motility variables. Serum inhibin B and inhibin B/FSH ratio (markers of spermatogenesis) were positively associated with 25-OHD ($P < 0.05$). Inhibin B levels were borderline ($P = 0.051$) significantly lower in vitamin D deficient men compared with high status men after adjustment of relevant confounders including calcium (Supplementary Fig. S2) but no difference in inhibin B/FSH ratio was found. Men in the highest calcium tertile had lower inhibin B levels (data not shown, $P = 0.03$) and a lower inhibin B/FSH ratio ($P = 0.03$) (Supplementary Fig. S2). Moreover, the duration of infertility was inversely associated with serum levels of 25-OHD and Ca^{2+} (both $P = 0.04$). Men with vitamin D deficiency or the lowest calcium tertile had the longest duration of infertility ($P = 0.06$ and 0.04, respectively) (Supplementary Fig. S2).

Testosterone, SHBG and LH

Total testosterone and testosterone/LH ratio were not significantly associated with 25-OHD or ionized calcium. Total testosterone was non-significantly lower ($P = 0.082$) in men with vitamin D deficiency (Table I and Fig. 4). Instead, 25-OHD was positively associated SHBG

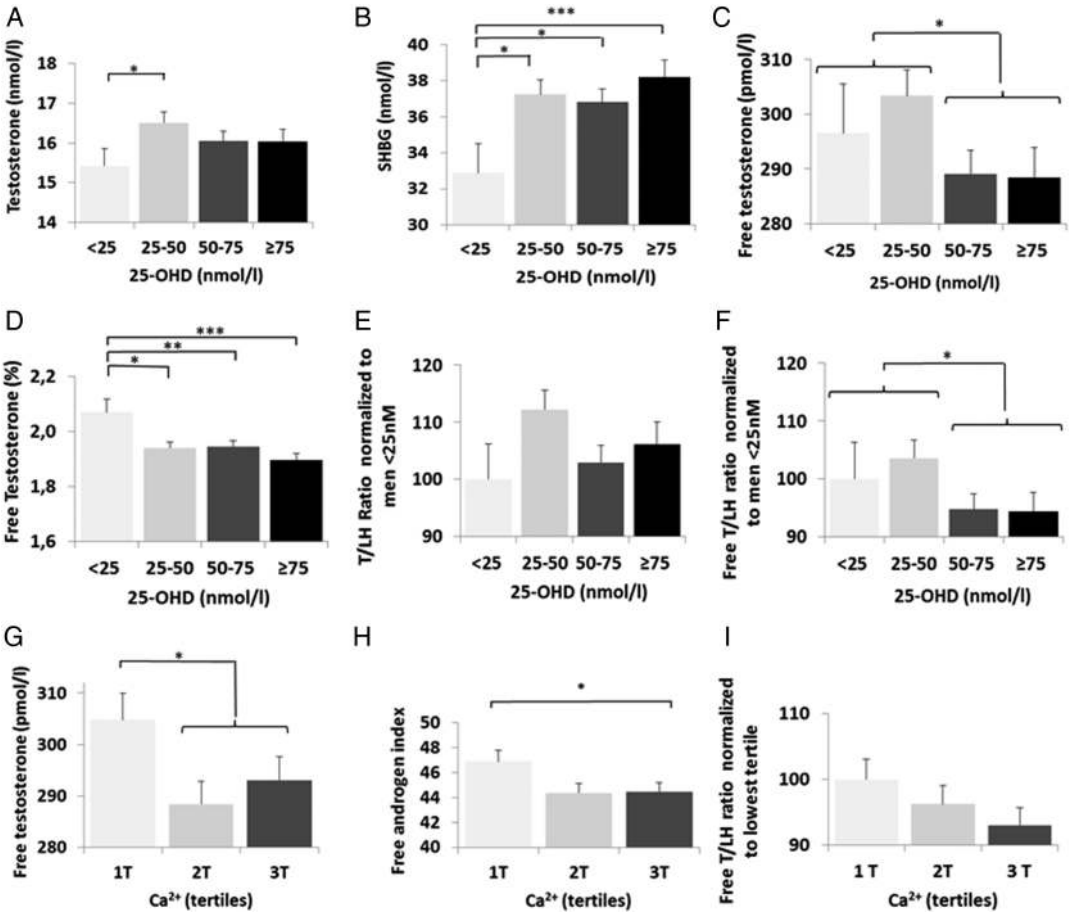

Figure 4 Testosterone and SHBG levels according to vitamin D and calcium status in 1189 infertile men. Outcomes stratified according to serum 25-OHD: deficiency (<25 nmol/l), insufficiency (25–50 nmol/l), sufficiency (50–75 nmol/l) and high status (≥75 nmol/l). (**A**) Total testosterone, (**B**) SHBG, (**C**) free testosterone, (**D**) free testosterone percentage of total testosterone, (**E**) testosterone/LH (T/LH) ratio. (**F**) free testosterone/LH ratio as relative to vitamin D deficient men. Outcomes stratified according to tertiles (1T: <1.19, 2T: 1.19–1.22, T3: >1.22 mmol/l) of ionized calcium level. (**G**) Free testosterone, (**H**) free androgen index, (**I**) free testosterone/LH ratio. T/LH ratio presented as relative to lowest calcium tertile (=100). Data presented as means ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$.

($P = 0.047$) and men with high vitamin D status had the highest SHBG level also after adjustment of calcium (Fig. 4). In accordance, 25-OHD was negatively associated with FT ($P = 0.03$), the percentage of FT (FT% $P = 0.02$) and FAI ($P = 0.04$) (Fig. 4). Moreover, vitamin D deficient men had the highest FT%. Men with 25-OHD below 50 nmol/l had higher FT compared with men ≥50 nmol/l ($P = 0.03$) (Fig. 4), but the significant difference disappeared ($P = 0.08$) after adjustment for Ca^{2+}. Testosterone/LH ratio was not significantly different between vitamin D groups, but FT/LH ratio was higher in men with 25-OHD below 50 nmol/l (Fig. 4). Also ionized calcium was negatively associated with FT ($P = 0.04$) (Fig. 4). Men with low Ca^{2+} had significantly higher serum FT, while FT%, total testosterone, SHBG and T/LH ratio did not differ between calcium groups (Fig. 4 and Supplementary Fig. S2).

Estradiol and testosterone/estradiol ratio

Serum 25-OHD was negatively associated with total and free estradiol levels ($P = 0.011$ and 0.018). A 13% higher free estradiol level was found in men with low vitamin D status ($P = 0.01$) (Fig. 5). The difference remained significant ($P = 0.02$) after adjustment of serum Ca^{2+}. Total estradiol was 3.4 pmol/l lower in men with 25-OHD levels above 50 nmol/l ($P = 0.032$) (Fig. 5) but the significance disappeared after adjustment for ionized calcium ($P = 0.13$). Interestingly, the interaction term (25-OHD*ionized calcium) was associated with both total and free estradiol ($P < 0.0005$). 25-OHD* Ca^{2+} was positively associated with total and free estradiol when calcium and vitamin D levels were low (in vitamin D deficient and hypercalcaemic men) and negatively associated when vitamin D and calcium levels were high. Total estradiol levels were inversely associated with serum Ca^{2+} ($P = 0.003$), and the lowest total and free estradiol were found in the highest Ca^{2+} quartile (Fig. 5). The percentage of free estradiol (FE%) was also negatively associated with 25-OHD. FE% was higher in vitamin D deficient men compared with both vitamin D sufficient and high status men ($P = 0.015$ and 0.022) also after adjustment for ionized calcium (Fig. 5). Serum 25-OHD and Ca^{2+} were both positively associated with the testosterone/estradiol (T/E) ratio ($P = 0.04$ and 0.02). Vitamin D deficient men had a 14% lower T/E ratio compared with men having a sufficient

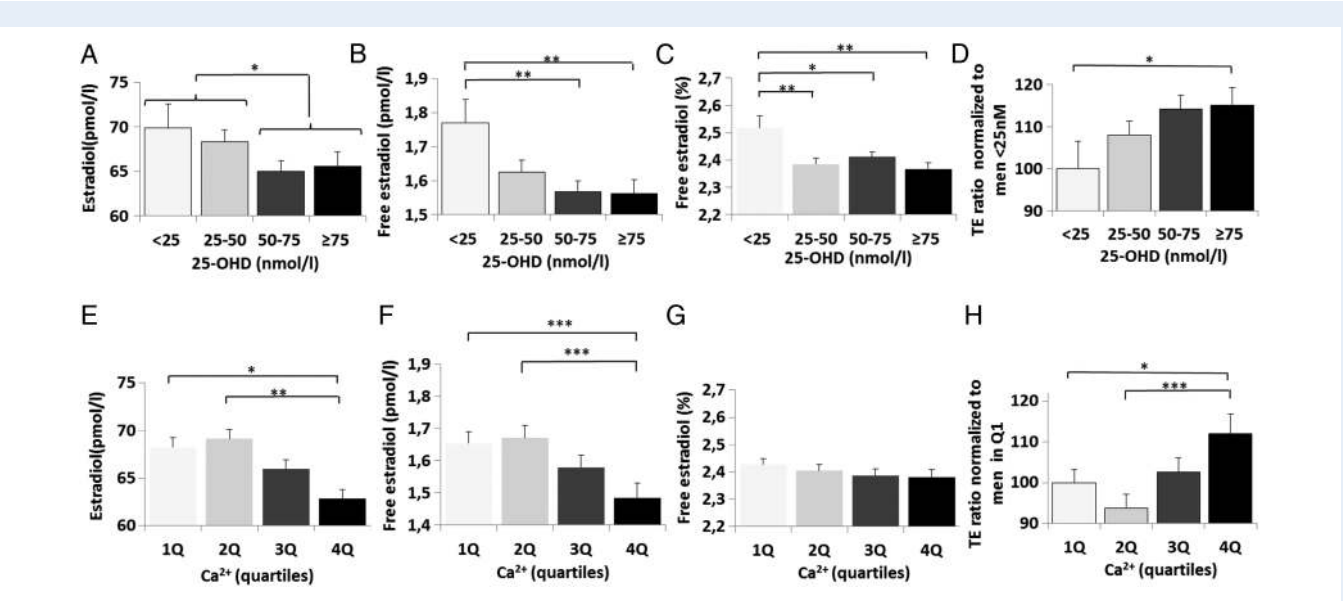

Figure 5 Total and free estradiol stratified according to vitamin D and calcium status in 1189 infertile men. Outcomes stratified according to serum 25-OHD: deficiency (<25 nmol/l), insufficiency (25–50 nmol/l), sufficiency (50–75 nmol/l) and high status (≥75 nmol/l). (**A**) Total estradiol, (**B**) free estradiol, (**C**) free estradiol percentage of total estradiol, (**D**) testosterone/estradiol (T/E) ratio. Outcomes stratified according to quartiles (<1.18, 1.19–1.20, 1.21–1.23, >1.23 mmol/l) of ionized calcium level. (**E**) Total estradiol, (**F**) free estradiol, (**G**) free estradiol percentage, (**H**) T/E ratio. T/E ratio presented as relative to vitamin D deficient or lowest calcium quartile (=100). Data presented as means ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$.

or high vitamin D level (both $P = 0.04$). After adjustment for ionized calcium, the difference disappeared ($P = 0.10$ and 0.09, respectively). Again, the interaction term was significantly associated ($P = 0.004$). Interestingly, men in the highest calcium quartile had a higher T/E ratio compared with two lowest calcium quartiles, also after adjustment of 25-OHD (Fig. 5).

Discussion

The baseline characteristics of this prospective study show that men with vitamin D deficiency had fewer motile spermatozoa, lower SHBG levels and lower testosterone/estradiol ratio, but higher free sex steroid levels than men with adequate vitamin D levels. The relationship between vitamin D and sperm motility is in line with previous data from small cohorts of fertile and infertile men (Blomberg Jensen *et al.*, 2011, 2012a; Yang *et al.*, 2012) and in accordance with human *in vitro* studies (Aquila *et al.*, 2009; Blomberg Jensen *et al.*, 2011; Blomberg Jensen and Dissing, 2012), and support the impaired sperm motility observed in VDR and 1α-hydroxylase knockout mice (Kinuta *et al.*, 2000; Panda *et al.*, 2001; Erben *et al.*, 2002; Blomberg *et al.*, 2013; Sun *et al.*, 2015). Our data are descriptive in nature, but it is plausible that sperm motility could be influenced through VDR-regulated active calcium transport in the male reproductive tract, which is a prerequisite for generating a 2- to 3-fold higher calcium concentration in the seminal fluid compared with serum (Clulow *et al.*, 1994; Blomberg Jensen, 2014). Loss of TRPV6, a VDR-regulated calcium transporter, expressed in the epididymis, leads to impaired sperm motility and infertility in mice due to impaired cellular calcium transport and subsequent changes of the epididymal fluid concentration (Weissgerber *et al.*, 2011). In this large cohort of men, the total number of motile spermatozoa was significantly lower in men with vitamin D deficiency. The low sperm producing capacity in these men is supported by lower inhibin B levels in men with vitamin D deficiency. This finding is novel and highlights that the most pronounced and clinically relevant effects are found in infertile men with real vitamin D deficiency and not in men with insufficient 25-OHD levels. The effects of vitamin D deficiency may be mediated at least in part by secondary changes in serum calcium. Interestingly, serum ionized calcium level was negatively associated with sperm motility, and men with hypocalcemia had higher sperm motility in our study, in contrast to healthy controls (Blomberg Jensen *et al.*, 2011) where albumin corrected calcium was positively associated with sperm motility. The finding is intriguing because it indicates that the effects of vitamin D on sperm motility may differ between normal and infertile men. Obviously, our study is descriptive and cannot be used to determine causality, but our finding is in line with previous reports on lower expression and responsiveness to active vitamin D in spermatozoa from infertile men compared with normal men (Blomberg Jensen *et al.*, 2012a). We cannot explain the negative correlation between calcium and motility; however, it is likely that calcium concentration in the seminal fluid is of greater importance than serum calcium levels for sperm motility. Moreover, the negative relationship with inhibin B and inhibin B/FSH ratio indicate a role for calcium in the regulation of spermatogenesis. RCTs are needed to determine whether supplementation of vitamin D with or without calcium can influence sperm production, motility and other testicular functions in infertile men.

The VDR has also been suggested to stimulate testosterone production directly in human Leydig cells (Hofer *et al.*, 2014). In our cohort, no significant link between 25-OHD and Ca^{2+} with total testosterone or testosterone/LH ratio was found. Previous studies of healthy young men and a small RCT also failed to show a positive link between

25-OHD and testosterone (Jorde *et al.*, 2013; Valimaki *et al.*, 2004; Ramlau-Hansen *et al.*, 2011), while studies of older men with serious co-morbidities found positive associations with testosterone (Blomberg Jensen, 2012). The discrepancy between cohorts may be influenced by an SHBG-effect as indicated by the positive link between 25-OHD and SHBG in our cohort. The positive association with SHBG has been reported previously in young men (Valimaki *et al.*, 2004; Ramlau-Hansen *et al.*, 2011) and may lead to changes in androgen bioavailability. The observed relationship between 25-OHD and SHBG is the best explanation for the negative associations between 25-OHD and FAI, FT and the free fraction of testosterone (FT%). Similarly, men with the highest serum levels of ionized calcium had lower FT than men with low ionized calcium. This implies that men with high vitamin D and/or Ca^{2+} levels may have less biologically active testosterone (although the level of dihydrotestosterone is unknown). Also, serum estradiol was negatively associated with 25-OHD and ionized calcium. VDR suppresses aromatase function in adipose tissue (Krishnan *et al.*, 2010) and $1,25(OH)_2D_3$ may thus decrease bioavailable estradiol through regulation of SHBG or by repressing aromatase activity since more than 80% of circulating estradiol in men is derived from peripheral aromatization of testosterone (Longcope *et al.*, 1969). The novel link with estradiol is intriguing because the consequences of male hypogonadism are not only related to androgen deficiency but also caused by a skewed androgen/estrogen balance (Finkelstein *et al.*, 2013). The EMAS study also showed a higher estradiol level in men with the lowest vitamin D level (Lee *et al.*, 2012). A high number of infertile men present with a low T/E ratio (Andersson *et al.*, 2004a), and elevated or very low estradiol levels in older men are associated with increased mortality (Jankowska *et al.*, 2009). Noteworthy, differences in sex steroid concentrations between vitamin D and calcium groups are modest and probably not of clinical relevance. RCTs are needed to determine whether vitamin D and calcium supplementation suppresses aromatase function and thereby potentially increases the T/E ratio.

Our study is compromised by its descriptive nature and limited by the use of substandard methodology, i.e. ELISA and RIA instead of LC–MS for sex steroid levels, a standardized albumin level instead of direct measurements, and multiple undetermined factors that influence gonadal function and calcium homeostasis in infertile men. Major confounders are the high frequency of serious co-morbidities in our cohort, the variation in age and use of treatment that could influence 25-OHD, calcium homeostasis, semen quality, steroidogenesis or peripheral conversion of androgens. Therefore, we performed the analyses in a subgroup of men without serious co-morbidities, which allows comparison with all men. The endocrine profile of infertile men shares some characteristics with age-related changes such as decreasing testosterone, elevated LH and lower T/E ratio due to an age-associated increased aromatization of testosterone (Vermeulen and Kaufman, 2002). Vitamin D and calcium could in theory act to oppose this effect as both factors were inversely associated with total and FT and free estradiol; while 25-OHD only was associated with the free fraction of testosterone and estradiol. Calcium may exert its influence in the gonad and adipose tissue (Kato *et al.*, 2002), while vitamin D predominantly may influence binding proteins and peripheral actions. Aromatization of circulating androgens is important for feedback regulation in the hypothalamus. Our data support the suggestion that circulating estrogens are of lesser importance in the suppression of gonadotrophin levels compared with androgens undergoing local aromatization in the brain (Finkelstein *et al.*, 1991; Pitteloud *et al.*, 2008). Still, differences in free sex steroids levels are low between vitamin D and calcium groups, which may explain why these changes are not fully reflected in serum gonadotrophin levels.

In conclusion, this observational study indicates that vitamin D deficiency and ionized calcium may influence sex steroid bioavailability and semen quality in infertile men.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

M.B.J.: study conception; N.J., A.J. and M.B.J.: study design; all authors: acquisition, analysis and interpretation of data, draft and revision of the manuscript, and final approval of the version to be published.

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Conflict of interest

None declared.

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