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Preclinical safety assessment of glycosides based standardized fenugreek seeds extract: Acute, subchronic toxicity and mutagenicity studies

Pallavi Deshpande, Vishwaraman Mohan, Prasad Thakurdesai*

Indus Biotech Private Limited, Kondhwa, Pune, Maharashtra, 411048, India.

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ABSTRACT

Objective: To evaluate acute oral toxicity (AOT), subchronic toxicity, and mutagenic potential of glycosides based standardized fenugreek (*Trigonella foenum graecum* L.) seeds extract (SFSE-G). ***Materials and Methods***: The AOT, subchronic (90-day repeated dose) toxicity and mutagenicity (reverse mutation test) of oral administration of SFSE-G were evaluated using Sprague-Dawley (SD) rats as per OECD guideline no. 423, No. 408 and 471 respectively. ***Results***: The SFSE-G did not show mortality or treatment-related adverse signs during acute (limit dose of 2000 mg/kg) and subchronic (90-days repeated dose of 250, 500 and 1000 mg/kgwith 28 days of recovery period) administration. The SFSE-G showed oral median lethal dose (LD_{50}) more than 2000 mg/kg during AOT study. The no-observed adverse effect level (NOAEL) of SFSE-G was 1000 mg/kg in male rats and 500 mg/kg in female rats during subchronic toxicity study. Furthermore, SFSE-G did not show mutagenic potential *in vitro*. ***Conclusions***: SFSE-G was found safe for acute and subchronic (90 days repeated dose) administration in rats with no mutagenic potential.

INTRODUCTION

There is increasing awareness and general acceptability of the use of herbal drugs in today's medical practice (Upton *et al*., 2016) Most of the natural products or herbal medicines contain a diverse variety of phytoconstituents because of variation in growth pattern, geographical location, time of harvesting and storage (Yau *et al*., 2015). Correct identification of composition of herbal medicine is essential to ensure consistent quality, safety and efficacy (Upton *et al*., 2016). The process of standardization is used to prescribe a set of standards or inherent characteristics with definitive qualitative and quantitative values thatare indicative of consistent quality and reproducibility. Apart from consistent quality, all medicines need to fulfill the basic requirements of safety. However, the safety of herbal medicines has become a major concern to both regulatory health authorities and the general public (Kalaiselvan *et al*., 2015, Starr, 2015). Therefore, safety or toxicological studies on herbal medicines needs to be conducted using well accepted international guidelines to ensure safe use for long-term human consumption. One of the potential natural sources of nutritional and medicinal applications is fenugreek (*Trigonella foenum-graecum* L.) seed. Fenugreek seeds are being used by people in Asia, Africa and Mediterranean countries as one of the ingredients in daily diets (Ulbricht *et al*., 2008).

Past reports on fenugreek seeds highlighted a variety of medicinally active phytoconstituents namely alkaloids and polysaccharides (Petropoulos, 2003), flavonoids (Huang and Liang, 1999, Wagner *et al*., 1973), triterpenoids (Shang *et al*., 1998) and steroidal sapogenins (Sauvaire *et al*., 1991).Safety of fenugreek seeds in powder form in human has been established in many clinical trials and reviews (Basch *et al*., 2003). Fenugreek seeds are certified as GRAS (Generally recognized as safe) item under clause §182.20 (Essential oils, oleoresins and natural extractives including distillates) by US Food and Drug Administration (US FDA).

* *Corresponding Author*
Prasad Thakurdesai, Indus Biotech Private Limited, Kondhwa, Pune, Maharashtra, 411048, India. Email: prasad @ indusbiotech.com

One of the important phytoconstituent of fenugreek seeds is glycosides content. These include a variety of furostanol (Gupta *et al.*, 1985, Kang *et al.*, 2013, Murakami *et al.*, 2000, Yoshikawa *et al.*, 1997) and flavonol (Han *et al.*, 2001, Pang *et al.*, 2012b, Rayyan *et al.*, 2010, Taylor *et al.*, 2000) glycosides. Fenugreek seeds glycosides are known to have androgenic and anabolic (Aswar *et al.*, 2010), anti-inflammatory and anti-melanogenic (Kawabata *et al.*, 2011), platelet aggregation inhibition (Pang *et al.*, 2012a) and anti-oxidant (Kenny *et al.*, 2013) properties. The androgenic and anabolic activity (Aswar *et al.*, 2014) and subacute toxicity (Kandhare *et al.*, 2015) of glycosides based standardized fenugreek seeds extract (SFSE-G) in male rats has been reported . Androgenic benefits of SFSE-G capsule supplementation is reported in sedentary (Mokashi *et al.*, 2014) and college-age men (Wilborn *et al.*, 2010). However, the evidence of long-term safety for SFSE-G is not being available.

In order to explore clinical potential of SFSE-G for long-term human consumption, toxicology testing in laboratory animals using well-accepted international guidelines is required. Therefore, the present study aimed to evaluate acute oral toxicity (AOT), repeated dose (90-days, subchronic) toxicity in laboratory rats and mutagenicity testing using OECD guidelines

MATERIALS AND METHODS

Animals

Male and female Sprague-Dawley (SD) rats were used for the acute and subchronic toxicology studies. The rats were obtained from the animal house of Indian Institute of Toxicology, Pune, India. The animals were acclimatized to laboratory conditions for 7 days prior to the experiments. The rats were maintained at a room temperature of 22–24°C, relative humidity between 30% and 70%; 10-15 air changes per hour with a 12 h light/dark cycle.

During acclimatization, the animals were housed in polypropylene cages provided with a standard pellet diet (M/s. Nav Maharashtra Chakan Oil Mills Ltd., Pune) and water *ad libitum*. All protocol was approved by Institutional Animal Ethics Committee of Indian Institute of Toxicology, Pune, India.

All the animal experimentations were performed as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

The test compound, SFSE-G

The test compound, SFSE-G, is a standardized extract of fenugreek seeds (containing 89.42% of glycosides), prepared and standardized by HPLC using earlier reported procedure (Kandhare *et al.*, 2015)and provided for the study by Indus Biotech Private Limited (Pune, India). The solution of SFSE-Gwas freshly prepared in distilled water to obtain dose volume of 10 ml/kg body weight of rats. SFSE-G solution in sterile water for injection was used for mutagenicity evaluation.

Acute oral toxicity (AOT) study

The acute toxicity study of SFSE-G was performed according to OECD guideline 423 (Organisation for Economic Co-operation and Development, (Organisation for Economic Co-operation and Development, 2002). The rats were grouped in 2 groups (5 rats/sex/group) and treated with a single dose as follows:G1 – Vehicle control (VC) (double distilled water, 10 mL/kg, oral), and G2 (SFSE-G, 2000 mg/kg, oral.). The rats were observed daily for 14 days following administration for mortality and clinical signs of toxicity. On day 15, all rats were euthanized and underwent gross pathological examination for signs of toxicity via necropsy.

Subchronic (90-day repeated dose) toxicity study

The study complies with the OECD Guideline for the testing of Chemicals No.408 (Organisation for Economic Co-operation and Development., 1998).The separate vehicle control (VC) group was maintained on distilled water as designated as G1. Based on median lethal dose (LD_{50}) (2000 mg/kg) obtained from AOT study, animals were orally administered daily once with following treatments:

G1 – VC (Distilled water, 10 mL/kg, 90 days) - 15 animals per sex
G2 – SFSE-G-250 (SFSE-G, 250 mg/kg, 90 days) - 15 animals per sex
G3 – SFSE-G-500 (SFSE-G, 500 mg/kg, 90 days) - 15 animals per sex
G4 - SFSE-G -1000 (SFSE-G, 1000 mg/kg, 90 days) - 15 animals per sex
G1R – VC reversal group -VC-R - (Distilled water, 10 mL/kg, 119 days) - 10 animals per sex
G4R – SFSE-G -1000-Reversal group (SFSE-G-1000-R) (SFSE-G, 1000 mg/kg for 90 days) followed by (distilled water, 10 mL/kg (from day 91 to day 119) - 10 animals per sex

All treated rats were observed daily for mortality and clinical signs during the 90-day study period whereas reversal groups (G1R and G4R) were observed for upto day 119 (Reversal period of 28 days). The eyes of control and all the treated dose group animals were examined prior to the initiation of the dosing and on day 91 and 119 (for G1R and G4R) of the study. Eye examination was carried out using an ophthalmoscope (mini 2000, HEINE Optotechnik, Herrsching, Germany) after induction of mydriasis with 0.5% solution of tropicamide. The body weight and feed consumption of each rat were recorded at weekly intervals throughout the study period. Towards the end of the exposure period (day 91), functional observations (by grading different sensory reactivity to stimuli of different types (auditory, visual and proprioceptive stimuli), assessment of grip strength (digital grip

strength meter (Columbus Instruments International, Columbus, OH, USA) and motor activity measurement were performed. The animals were placed in metabolic cages overnight and urine excreted by each animal was collected on day 91 and on day 119 (G1R and G4R) of the study for urinalysis. The parameters for urinalysis included: specific gravity, pH, occult blood, protein, bilirubin, ketones, glucose, nitrite, and urobilinogen. Using whole blood, hematological and coagulation analyzes were carried out. The parameters for hematological analysis included: red blood cell count (RBC), reticulocyte count, hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) and total leukocyte count (TLC), % cells in differential leukocyte count (DLC) (including lymphocyte (L), monocyte (M), basophils (B), eosinophils (E), and Prothrombin time (PT). Additionally, clinical chemistry was evaluated including Blood Urea Nitrogen (BUN), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), Gamma Glutamyl Transferase (GGT), Creatine Phosphokinase (CK), Lactate Dehydrogenase (LDH), Fasting plasma glucose (FPG), Calcium (Ca), Phosphorus (P), Bilirubin, Albumin, creatinine (CR), Sodium (Na), Potassium (K), Chlorine (Cl), Cholesterol, and Triglycerides (Trig). On day 91, all animals were euthanized and underwent gross pathological examination for signs of toxicity via necropsy.

All organs, mucosa, body cavities, etc. were examined for gross pathological changes. Major organs and major endocrine glands (pituitary, adrenal, thymus, thyroid, sex, etc.) were weighed and absolute and relative weights (i.e. percent of the body weight) were calculated. Tissue samples from selected organs (heart, kidney, liver, lung, spleen, stomach, pancreas and skeletal muscle) from the VC (G1 and G1R) and 1000 mg/kg (G4 and G4R) were preserved, fixed, and stained for histopathological evaluation via light microscopy. The remaining tissues/organs were preserved in 10% neutral buffered formalin from control and different dose groups.

Mutagenicity study

Mutagenicity was evaluated by bacterial reverse mutation test (AMES test) was performed in full compliance with the OECD guidelines for mutagenicity testing namely Test No: 471 (Organisation for Economic Co-operation and Development, 1997). As no significant cytotoxic effect was observed, the five highest doses were then used in the subsequent mutagenicity evaluation.

To evaluate mutagenicity, five strains of histidine-dependent *Salmonella typhimurium* (TA97a, TA98, TA100, TA1535 and TA102) were tested in triplicate at the five highest doses (5000.00, 1666.67, 555.55, 185.18 and 61.72 µg/plate) of SFSE-G. Rat liver homogenate tested with mutagen 2-aminofluorene before use. Metabolic activation was performed using a cofactor-supplemented post-mitochondrial fraction (S9 fraction). Positive controls (2-aminofluorene, 2-aminoanthracene, methyl methanesulfonate, 4-Nitroquinolene-N-Oxide, danthron and sodium azide) with and without S9 activator and negative controls (VC and phosphate buffer) with and without S9 activator were included in the evaluation. This was done to ensure the test system was functioning properly (positive controls) and to obtain baseline revertant frequencies for the various strains of bacteria used in the study (negative controls). The plates were counted after 48 h of incubation at 37 °C. The mutagenic activity of the test substance was considered for positive in case of increased concentration over the range tested and a reproducible increase at one or more concentrations in a number of revertant colonies per plate in at least one strain with or without metabolic activation system.

The test substance was considered to be toxic if there was a decrease in the number of revertants and/or thinning or absence of background lawn.

Statistical analysis

Statistical analysis was performed using SPSS analysis program for windows Version 17.0 (SPSS Inc, USA). All the data were checked for normality and values were represented as mean ± standard deviation (SD). Data of body weight and food intake was analyzed by two-way ANOVA followed by unpaired 't' test. Remaining parameters were analyzed by separate One way ANOVAs.

Dunnett's test was used to analyze the differences between treated groups with respective VC groups (G2, G3, G4 v/s. G1 and G4R v/s.G1R). The value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Acute oral toxicity (AOT)

None of the rats died during the observation period. No clinical signs of toxicity, such as ill health or abnormal behavior, occurred during the study. There were no abnormal observations in body weight of rats. No abnormalities or pathological changes were observed in the necropsy. Under the experimental conditions, the results indicated that the median lethal dose (LD_{50}) of SFSE-G is greater than 2000 mg/kg of body weight.

Subchronic (90-day repeated dose) toxicity

Daily oral administration of SFSE-G for 90 days did not induce any obvious symptom of toxicity in rats of any sexes, up to the highest tested dose (1000 mg/kg). No deaths or obvious clinical signs were found in any groups throughout the study period. Body weights of male and female rats did not show significant difference during treatment period except for SFSE-G-1000 group in female rats on 90 days period and SFSE-G-1000-R group (v/s. G1R group) (Table 1). No gross abnormalities were observed in necropsies of any of the rats. The food and water consumptions were measured throughout the study and did not significantly change as compared to the VC rats (Figure 1). Ophthalmoscopic examination and functional observation tests revealed no abnormalities attributable to the treatment.

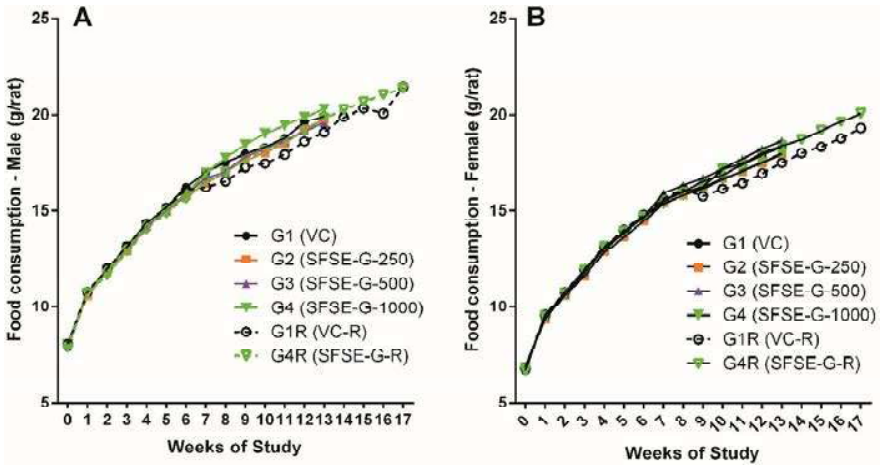

Fig. 1: Effect of SFSE-G food consumption in (A) Male and (B) Female rats during 90 days repeated dose toxicity study. Data are expressed as mean ± standard deviation (SD).

Table 1: Effect of SFSE-G on body weights of rats during 90 days repeated dose toxicity study.

Weeks	Group					
	G1	G2	G3	G4	G1R	G4R
	VC	SFSE-G-250	SFSE-G-500	SFSE-G-1000	VC-R	SFSE-G-1000-R
Male						
0	85.67 ± 7.17	83.06 ± 3.41	83.55 ± 3.82	84.47 ± 5.08	83.94 ± 6.20	85.26 ± 6.00
12	374.11 ± 29.51	376.29 ± 38.54	372.69 ± 38.28	352.49 ± 37.58	364.78 ± 30.37	363.53 ± 31.67
13	381.14 ± 28.45	382.15 ± 37.47	377.23 ± 35.91	358.42 ± 37.42	371.31 ± 29.78	369.30 ± 30.04
17					391.72 ± 25.46	374.86 ± 29.63
Female						
0	88.09 ± 7.45	85.85 ± 5.38	86.47 ± 5.60	87.40 ± 6.06	85.97 ± 6.66	88.00 ± 6.54
12	260.35 ± 17.67	251.54 ± 11.91	247.59 ± 21.34	246.85 ± 12.00	257.96 ± 10.36	242.37 ± 14.00
13	266.65 ± 17.06	257.29 ± 12.04	252.69 ± 21.34	252.47 ± 11.99*	263.88 ± 10.21	246.83 ± 13.20
17					267.14 ± 7.43	254.22 ± 10.83*

Data was represented as Mean ± Standard Deviation (SD). Data was analyzed by unpaired 't' test, * = P < 0.05as compared torespective VC groups.

Table 2: Effect of SFSE-G on hematological parameters during 90 days repeated dose toxicity study (Male rats).

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	SFSE-G-250	SFSE-G-500	SFSE-G-1000	VC-R	SFSE-G-1000-R
Hb (g %)	15.86 ± 0.84	15.01± 0.91**	15.76 ± 0.60	16.09 ± 0.66	17.94 ± 1.48	16.02 ± 0.64**
RBC (x106 /µL)	8.61 ± 0.52	7.97 ± 0.50**	8.67 ± 0.43	8.85 ± 0.55	10.11 ± 0.70	8.89 ± 0.37**
Reticulocytes (%)	1.90 ± 0.44	1.65 ± 0.39	1.69 ± 0.44	1.73 ± 0.38	1.52 ± 0.58	1.50 ± 0.43
HCT (%)	43.15 ± 2.07	40.60 ± 2.79*	43.59 ± 1.83	44.91 ± 2.28	49.12 ± 3.36	43.00 ± 1.35**
MCV (mm^3)	50.17 ± 1.51	50.95 ± 1.15	50.29 ± 1.39	50.79 ± 1.34	48.56 ± 1.45	48.44 ± 1.85
MCH (pg)	18.47 ± 0.69	18.81 ± 0.39	18.19 ± 0.58	18.22 ± 0.58	17.72 ± 0.33	18.02 ± 0.91
MCHC (%)	36.79 ± 0.49	36.95 ± 0.55	36.19± 0.46*	35.87 ± 0.69**	36.52 ± 1.08	37.26 ± 0.58
Platelets (x 10^3 /µL)	416.20 ± 66.93	389.60 ± 49.72	436.47 ± 72.72	425.73 ± 50.94	391.20 ± 69.19	473.80 ± 37.59*
PT (sec)	15.53 ± 3.23	14.93 ± 4.03	13.93 ± 3.33	15.13 ± 3.25	16.20 ± 3.42	15.20 ± 3.03
TLC (x 10^3 /µL)	11.73 ± 3.36	10.49 ± 2.11	10.73 ± 3.19	10.61 ± 2.14	10.58 ± 3.03	10.58 ± 1.16
Differential Leucocyte Count						
N (%)	21.33 ± 2.69	21.20 ± 3.95	21.13 ± 4.14	22.53 ± 4.58	20.80 ± 3.27	20.80 ± 4.15
L (%)	74.93 ± 2.79	75.60 ± 3.87	75.27 ± 3.92	73.87 ± 4.52	75.60 ± 3.21	76.00 ± 3.81
E (%)	1.13 ± 0.74	0.87 ± 0.74	1.27 ± 0.70	1.07 ± 0.80	1.20 ± 0.84	1.20 ± 0.84
M (%)	2.60 ± 0.91	2.33 ± 0.72	2.33 ± 0.72	2.53 ± 0.83	2.40 ± 1.14	2.00 ± 0.71
B (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data was represented as Mean ± Standard Deviation, Data was analyzed by unpaired 't' test, * = P < 0.05, ** P < 0.01 as compared of respective parameter value of corresponding VC group. Hb: Hemoglobin; RBC: Red Blood Corpuscles, HCT : Hematocrit, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, TLC: Total leukocyte (White Blood Corpuscles) count, PT.: Prothrombin time, N: Neutrophils, L : Lymphocytes, E: Eosinophils, M : Monocytes, B : Basophils

Hematological (Table 2 and Table 3) and biochemical observations (Table 4 and Table 5) and urinalysis were recorded in male and female rats. All the values hematological and biochemical values were within normal biological and laboratory limits and the differences between the values were not consistent between doses or periods of observation (treatment period / reversal period) and considered incidental. For example, hematology values in SFSE-G treated groups of rats were significantly different from corresponding VC groups in terms of Hb, RBC, HCT, MCHC, TLC during 90 day treatment period and

Hb, RBC, HCT and platelets during the recovery period (Table 2 and Table 3). Biochemical parameters related to kidney function test (CR, CK and BUN) did not show significant change as compared to respective VC group in female rats whereas BUN values show significant ($P < 0.05$) decrease in G2 (SFSE-G-250 mg/kg treated) group (Table 4).

Similarly, all liver function related biochemical parameters (ALT, AST, ALP, GGT, Bilirubin, Albumin) were unchanged in female rats whereas ALT values in G2 (SFSE-G-250 treated group) showed significant ($P <0.05$) increase in male rats (Table 4 and Table 5). The electrolyte concentrations (Ca, P, Na, K and Cl) levels in male rats did not show significant changes as compared to respective VC groups except Ca levels (SFSE-G-1000 group, $P < 0.01$) and Cl levels (SFSE-G-250, $P < 0.01$). However, female rats showed significant changes in electrolyte concentration related parameters such as Na levels (SFSE-G-250 and SFSE-G-500 but not SFSE-G-1000 group), Ca and K levels (SFSE-G-500 and SFSE-G-1000 group), Cl levels (all SFSE-G treated groups) with no significant change in P levels. The plasma lipid-related parameters (FPG, cholesterol, triglyceride, and LDH levels) in SFSE-G treated male and female rats remained unchanged except G2 (SFSE-G-250 treated) group which showed significant ($P < 0.05$) decrease in triglyceride levels in female rats.

Table 3: Effect of SFSE-G on hematological parameters during 90 days repeated dose toxicity study (Female rats).

Parameters	G1 VC	G2 SFSE-G-250	G3 SFSE-G-500	G4 SFSE-G-1000	G1R VC-R	G4R SFSE-G-1000-R
Hb (g %)	16.06 ± 1.67	15.41 ± 0.65	15.93 ± 2.24	15.10 ± 0.82	16.10 ± 0.31	15.66 ± 0.98
RBC (x106 /µL)	8.27 ± 0.87	7.75 ± 0.46	8.16 ± 1.16	7.69 ± 0.43*	8.39 ± 0.31	8.10 ± 0.46
Reticulocytes (%)	1.83 ± 0.35	1.67 ± 0.44	1.63 ± 0.41	1.62 ± 0.48	1.48 ± 0.48	1.80 ± 0.68
HCT (%)	43.68 ± 4.64	41.25 ± 1.76	43.09 ± 6.34	40.77 ± 2.21*	43.48 ± 0.96	41.82 ± 2.58
MCV (mm^3)	52.84 ± 1.92	53.29 ± 2.28	52.81 ± 1.32	53.01 ± 1.35	51.80 ± 1.04	51.62 ± 0.99
MCH (pg)	19.43 ± 0.83	19.90 ± 0.88	19.56 ± 0.57	19.63 ± 0.56	19.22 ± 0.83	19.36 ± 0.62
MCHC (%)	36.80 ± 0.89	37.34 ± 0.38*	37.04 ± 1.24	37.03 ± 0.40	37.10 ± 1.02	37.48 ± 0.50
Platelets (x 10^3 /µL)	410.80 ± 111.28	441.47 ± 59.52	425.00 ± 111.57	409.80 ± 80.62	392.60 ± 79.32	440.00 ± 86.80
PT (sec)	15.00 ± 3.85	13.93 ± 3.84	13.87 ± 3.87	15.20 ± 3.47	15.60 ± 3.91	16.40 ± 3.58
TLC (x 10^3 /µL)	12.44 ± 4.06	12.31 ± 3.86	9.63 ± 3.20*	10.76 ± 1.91	9.46 ± 2.88	8.62 ± 2.83
Differential Leukocyte count						
N (%)	20.93 ± 4.06	21.67 ± 3.68	21.47 ± 4.12	20.80 ± 3.88	21.00 ± 3.39	20.00 ± 5.15
L (%)	75.47 ± 3.60	74.80 ± 3.00	75.27 ± 3.49	75.73 ± 3.39	75.40 ± 2.51	76.40 ± 4.10
E (%)	1.13 ± 0.83	1.00 ± 0.85	1.00 ± 0.76	1.13 ± 0.74	1.20 ± 0.84	1.20 ± 0.84
M (%)	2.47 ± 0.64	2.53 ± 0.64	2.27 ± 0.80	2.33 ± 0.72	2.40 ± 0.55	2.40 ± 0.55
B (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data was represented as Mean ±Standard Deviation, Data was analyzed by unpaired 't' test, * = $P < 0.05$, as compared with respective parameter value of corresponding VC group Hb: Hemoglobin; RBC: Red Blood Corpuscles, HCT : Hematocrit, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC:Mean Corpuscular Hemoglobin Concentration, TLC: Total leukocyte(White Blood Corpuscles) count, PT.: Prothrombin time, N: Neutrophils, L : Lymphocytes, E: Eosinophils, M : Monocytes, B : Basophils.

Table 4: Effect of SFSE-G on blood chemistry on during 90 days repeated dose toxicity study (Male rats).

Parameters	G1 VC	G2 SFSE-G-250	G3 SFSE-G-500	G4 SFSE-G-1000	G1R VC-R	G4R SFSE-G-1000-R
Liver function Tests						
ALT(IU/L)	38.33 ± 6.80	43.47 ± 4.00*	39.93 ± 5.61	40.20 ± 5.76	37.20 ± 6.22	33.40 ± 2.19
AST (IU/L)	63.40 ± 3.48	64.47 ± 4.24	63.53 ± 3.29	63.33 ± 3.81	60.60 ± 3.78	63.60 ± 3.51
ALP (IU/L)	72.93 ± 4.10	73.67 ± 6.52	71.33 ± 6.28	73.93 ± 5.19	70.00 ± 4.64	71.40 ± 5.22
GGT (U/L)	14.73 ± 4.45	15.40 ± 3.48	18.00 ± 3.23	17.13 ± 3.87	15.20 ± 3.56	13.20 ± 3.35
Bilirubin (mg%)	0.67 ± 0.07	0.67 ± 0.07	0.65 ± 0.09	0.65 ± 0.07	0.69 ± 0.08	0.67 ± 0.06
Total Protein (g%)	7.68 ± 0.64	8.07 ± 0.32	7.88 ± 0.41	7.85 ± 0.51	7.50 ± 0.60	7.53 ± 0.77
Albumin (g%)	3.62 ± 0.29	3.79 ± 0.24	3.57 ± 0.19	3.56 ± 0.26	3.72 ± 0.36	3.59 ± 0.32
Kidney Function test						
CR (mg%)	0.96 ± 0.11	0.90 ± 0.08	0.95 ± 0.09	0.94 ± 0.11	0.95 ± 0.06	1.00 ± 0.12
CK (IU/L)	62.00 ± 4.19	61.20 ± 4.93	61.27 ± 3.75	65.13 ± 4.70	64.80 ± 5.02	64.20 ± 1.64
BUN (mg%)	36.20 ± 5.54	32.00 ± 2.88*	35.20 ± 5.13	34.87 ± 4.37	33.20 ± 3.03	29.60 ± 5.55
Serum Electrolytes						
Ca (mg%)	2.11 ± 0.10	2.16 ± 0.10	2.13 ± 0.19	2.27 ± 0.08**	2.36 ± 0.11	2.29 ± 0.05
P (mg%)	4.25 ± 0.39	4.07 ± 0.45	4.40 ± 0.45	4.02 ± 0.45	4.04 ± 0.32	4.28 ± 0.22
Na (mmol/l)	139.56 ± 1.81	139.81 ± 0.84	139.79 ± 0.95	140.42 ± 1.18	138.00 ± 0.97	140.19 ± 0.30**
K (mmol/l)	4.55 ± 0.24	4.43 ± 0.19	4.42 ± 0.30	4.61 ± 0.27	4.30 ± 0.21	4.29 ± 0.16
Cl (mmol/l)	103.95 ± 4.39	99.24 ± 5.15**	107.37 ± 3.36	102.24 ± 3.35	107.20 ± 1.61	106.18 ± 2.73
Metabolic function tests						
FPG (mg%)	98.27 ± 5.78	95.40 ± 9.41	93.67 ± 9.04	97.80 ± 9.96	88.20 ± 6.14	84.40 ± 10.69
Cholesterol (mg%)	61.07 ± 6.37	63.87 ± 6.45	63.40 ± 6.03	63.27 ± 3.53	64.60 ± 3.51	64.80 ± 3.56
Triglyceride (mg%)	107.53 ± 5.63	107.47 ± 9.36	110.20 ± 9.16	106.87 ± 6.82	111.40 ± 10.01	111.40 ± 6.99
LDH (IU/L)	367.53 ± 26.79	363.07 ± 37.67	368.87 ± 27.63	359.60 ± 23.46	362.80 ± 36.35	340.40 ± 22.68

Data was represented as Mean ±Standard Deviation, Data was analyzed by unpaired 't' test, * = $P < 0.05$, ** $P < 0.01$ as compared of respective parameter value of corresponding VC group. ALT: Alanine Aminotransferase,AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, GGT: Gamma Glutamyl Transferase, CR: Creatinine, CK: Creatine Phospho-Kinase, BUN : Blood Urea Nitrogen, Ca: Calcium, P: Phosphorus, Na: Sodium, K: Potassium, Cl: Chlorine, FPG: Fasting plasma glucose, LDH: Lactate De-Hydrogenase.

Table 5: Effect of SFSE-G on blood chemistry on during 90 days repeated dose toxicity study (Female rats).

Parameters	G1	G2	G3	G4	G1R	G4R
	VC	**SFSE-G-250**	**SFSE-G-500**	**SFSE-G-1000**	**VC-R**	**SFSE-G-1000-R**
Liver function Tests						
ALT(IU/L)	40.93 ± 4.88	41.00 ± 4.74	37.93 ± 5.97	40.53 ± 5.93	37.60 ± 6.91	41.60 ± 5.13
AST (IU/L)	62.60 ± 3.16	63.80 ± 4.28	61.27 ± 4.95	62.93 ± 4.59	58.20 ± 6.18	63.40 ± 3.36*
ALP (IU/L)	73.27 ± 5.08	73.27 ± 8.04	71.47 ± 7.85	70.53 ± 6.27	70.00 ± 5.20	72.40 ± 4.67
GGT (U/L)	18.47 ± 3.02	17.67 ± 4.47	16.93 ± 3.45	15.93 ± 3.97	17.20 ± 5.26	13.40 ± 4.67
Bilirubin (mg%)	0.67 ± 0.07	0.66 ± 0.08	0.67 ± 0.08	0.66 ± 0.08	0.67 ± 0.07	0.63 ± 0.05
Total Protein (g%)	7.56 ± 0.48	7.94 ± 0.38	7.68 ± 0.58	7.49 ± 0.66	7.33 ± 0.56	7.78 ± 0.59
Albumin (g%)	3.49 ± 0.35	3.59 ± 0.34	3.60 ± 0.23	3.53 ± 0.21	3.66 ± 0.20	3.57 ± 0.35
Kidney Function test						
CR (mg%)	1.00 ± 0.08	1.01 ± 0.10	0.96 ± 0.08	0.98 ± 0.08	0.95 ± 0.07	0.96 ± 0.05
CK (IU/L)	62.80 ± 4.66	64.00 ± 3.87	62.27 ± 4.32	61.87 ± 4.45	61.80 ± 4.60	61.80 ± 4.49
BUN (mg%)	35.27 ± 5.65	32.93 ± 4.08	34.07 ± 4.15	36.53 ± 6.17	33.60 ± 1.82	34.60 ± 5.13
Serum Electrolytes						
Ca (mg%)	2.45 ± 0.14	2.55 ± 0.11	2.26 ± 0.14*	2.27 ± 0.13*	2.29 ± 0.12	2.42 ± 0.05*
P (mg%)	4.12 ± 0.41	4.33 ± 0.41	4.13 ± 0.45	4.27 ± 0.44	3.98 ± 0.38	4.12 ± 0.22
Na (mmol/l)	138.13 ± 1.81	136.32 ± 1.70**	136.06 ± 1.41**	137.68 ± 1.28	136.74 ± 0.84	137.90 ± 0.87*
K (mmol/l)	5.20 ± 0.21	5.22 ± 0.39	4.51 ± 0.23**	4.70 ± 0.27**	4.17 ± 0.36	4.09 ± 0.34
Cl (mmol/l)	96.97 ± 15.59	107.47 ± 8.95*	128.12 ± 9.18**	131.31 ± 15.78**	110.54 ± 1.72	111.53 ± 2.03
Metabolic function tests						
FPG (mg%)	95.53 ± 7.45	96.80 ± 8.77	98.53 ± 13.18	98.87 ± 10.97	90.00 ± 9.59	96.80 ± 13.16
Cholesterol (mg%)	64.47 ± 4.44	65.27 ± 3.97	63.53 ± 5.07	64.53 ± 4.12	66.60 ± 2.88	64.60 ± 4.39
Triglyceride(mg%)	110.87 ± 6.36	105.73 ± 4.92*	112.33 ± 5.18	110.13 ± 10.20	111.80 ± 8.41	114.20 ± 6.22
LDH (IU/L)	372.07 ± 26.35	357.07 ± 27.16	363.60 ± 22.76	375.07 ± 13.32	367.60 ± 25.66	383.00 ± 19.52

Data was represented as Mean ±Standard Deviation, Data was analyzed by unpaired 't' test, * = P < 0.05, ** P < 0.01 as compared of respective parameter value of corresponding VC group. ALT: Alanine Aminotransferase,AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, GGT: Gamma Glutamyl Transferase, CR: Creatinine, CK: Creatine Phospho-Kinase, BUN : Blood Urea Nitrogen, Ca: Calcium, P: Phosphorus, Na: Sodium, K: Potassium, Cl: Chlorine, FPG: Fasting plasma glucose, LDH: Lactate De-Hydrogenase.

Table 6: Effect of SFSE-G on relative organ weights of during 90 days repeated dose toxicity study (Male rats).

Organ	G1	G2	G3	G4	G1R	G4R
	VC	**SFSE-G-250**	**SFSE-G-500**	**SFSE-G-1000**	**VC-R**	**SFSE-G-1000-R**
Brain	0.561 ± 0.037	0.561 ± 0.068	0.561 ± 0.042	0.590 ± 0.045	0.529 ± 0.027	0.560 ± 0.039
Liver	2.853 ± 0.255	2.835 ± 0.279	2.982 ± 0.233	2.957 ± 0.164	2.996 ± 0.197	3.033 ± 0.278
Kidneys	0.725 ± 0.053	0.717 ± 0.081	0.724 ± 0.056	0.730 ± 0.058	0.731 ± 0.049	0.763 ± 0.044
Adrenals	0.014 ± 0.0013	0.0131 ± 0.0031	0.0132 ± 0.0018	0.0140 ± 0.0016	0.013 ± 0.002	0.013 ± 0.002
Testes	0.858 ± 0.087	0.794 ± 0.104	0.826 ± 0.123	0.896 ± 0.087	0.846 ± 0.169	0.823 ± 0.057
Heart	0.335 ± 0.031	0.334 ± 0.027	0.333 ± 0.027	0.338 ± 0.022	0.322 ± 0.014	0.325 ± 0.014
Spleen	0.336 ± 0.055	0.368 ± 0.034	0.358 ± 0.065	0.364 ± 0.041	0.323 ± 0.031	0.357 ± 0.083
Lungs	0.466 ± 0.055	0.507 ± 0.077	0.486 ± 0.068	0.477 ± 0.059	0.455 ± 0.057	0.418 ± 0.034
Thymus	0.049 ± 0.010	0.055 ± 0.015	0.055 ± 0.014	0.062 ± 0.023	0.031 ± 0.007	0.044 ± 0.017
Epididymis	0.336 ± 0.037	0.325 ± 0.033	0.327 ± 0.052	0.360 ± 0.036	0.365 ± 0.039	0.365 ± 0.023

Data was represented as Mean ±Standard Deviation, Data was analyzed by unpaired 't' test, None of the values were significant for G2,G3 and G4 V/s. G1 and G4R v/s. G1R.

Table 7: Effect of SFSE-G on relative organ weights of during 90 days repeated dose toxicity study (Female rats)

Organ	G1	G2	G3	G4	G1R	G4R
	VC	**SFSE-G-250**	**SFSE-G-500**	**SFSE-G-1000**	**VC-R**	**SFSE-G-1000-R**
Brain	0.749 ± 0.040	0.780 ± 0.047	0.785 ± 0.052	0.777 ± 0.031	0.764 ± 0.032	0.785 ± 0.028
Liver	3.156 ± 0.270	3.235 ± 0.343	3.276 ± 0.190	3.372 ± 0.345	3.155 ± 0.246	3.255 ± 0.335
Kidneys	0.652 ± 0.043	0.634 ± 0.090	0.690 ± 0.059	0.678 ± 0.040	0.685 ± 0.059	0.695 ± 0.049
Adrenals	0.0210 ± 0.0025	0.0228 ± 0.0039	0.0225 ± 0.0023	0.0221 ± 0.0025	0.022 ± 0.003	0.021 ± 0.002
Ovaries	0.0382 ± 0.0054	0.0358 ± 0.0052	0.0412 ± 0.0035	0.0384 ± 0.0056	0.0322 ± 0.0052	0.0357 ± 0.0037
Heart	0.336 ± 0.017	0.346 ± 0.032	0.347 ± 0.027	0.346 ± 0.023	0.342 ± 0.039	0.354 ± 0.023
Spleen	0.423 ± 0.073	0.419 ± 0.076	0.437 ± 0.083	0.433 ± 0.068	0.354 ± 0.065	0.350 ± 0.054
Lungs	0.566 ± 0.084	0.604 ± 0.054	0.578 ± 0.044	0.592 ± 0.052	0.582 ± 0.045	0.609 ± 0.053
Thymus	0.084 ± 0.023	0.098 ± 0.016	0.101 ± 0.025	0.098 ± 0.022	0.065 ± 0.009	0.068 ± 0.014
Uterus	0.141 ± 0.033	0.163 ± 0.041	0.134 ± 0.037	0.160 ± 0.031	0.151 ± 0.044	0.160 ± 0.035

Data was represented as Mean ±Standard Deviation, Data was analyzed by unpaired 't' test, None of the values were significant for G2,G3 and G4 V/s. G1 and G4R v/s. G1R.

The data of weights of organs in SFSE-G treated rats during treatment and reversal period in male and female rats is presented in Table 6 and Table 7. None of the SFSE-G treated group show significant difference during treatment or reversal period as compared with corresponding VC group.No treatment-related gross pathological changes were observed in any organs of the test animals during necropsy (Table 8).

Table 8: Summary of gross pathology findings.

Parameter	G1		G2		G3		G4		G1R		G4R	
	VC		SFSE-G-250		SFSE-G-500		SFSE-G-1000		VC-R		SFSE-G-1000-R	
Sex	M	F	M	F	M	F	M	F	M	F	M	F
Organs/lesions	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

M = Male, F = Female, NAD = No Abnormality Detected.

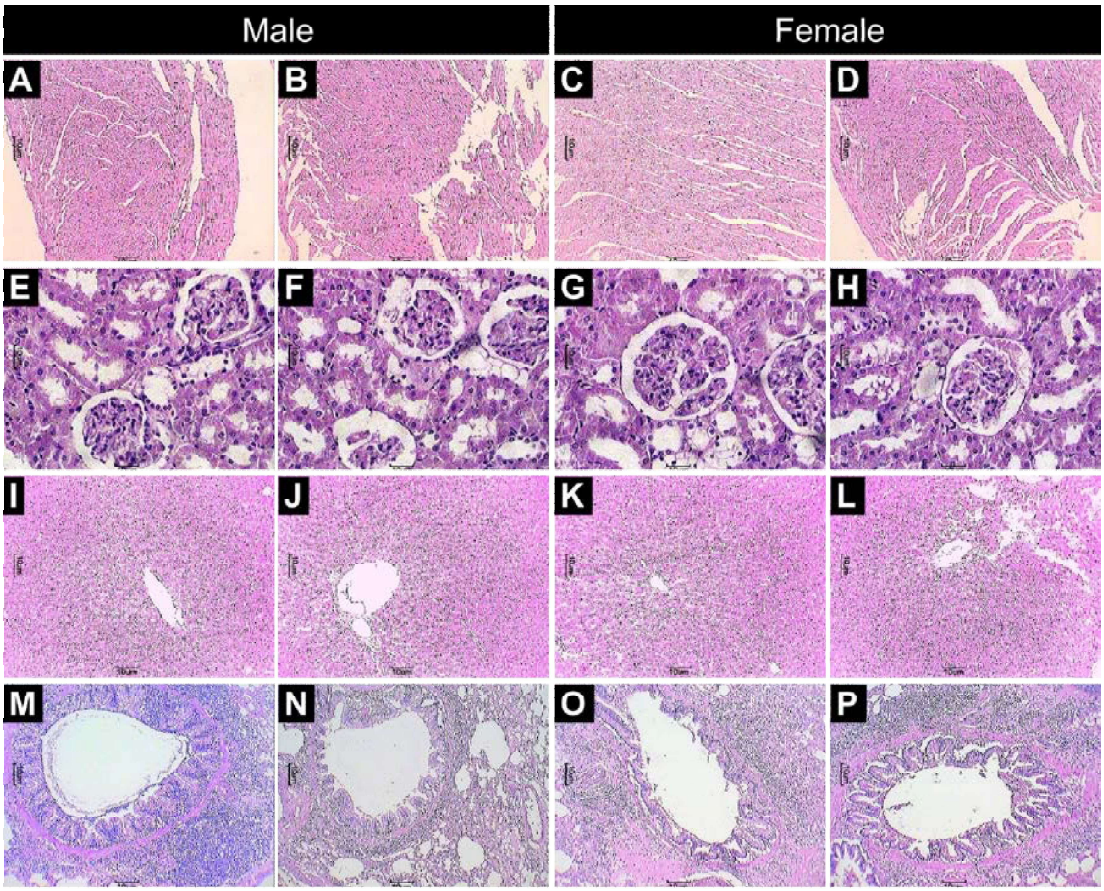

Fig. 2: Effects of SFSE-G on histological findings of heart (A–D), kidney (E–H), liver (I–L) and lung (M–P) tissue in rats during 90- days repeated dose toxicity study. Photomicrographs from representative rats from respective groups: VC (A, C, E, G, I, K, M, and O) and SFSE-G-1000 (B, D, F, H, J, L, N and P) (H&E stain) at 40X.

The finding from histological examination of sections of organs is presented in Table 9 and representative photomicrographs are presented as Figure 2. Histopathological examination of the VC group and SFSE-G treated rats showed anormal structure with mild changes and absence of any gross pathological lesion in organs. The changes observed in both VC and SFSE-G-1000 treatment groups were similar and comparable in both sexes and hence considered as incidental, congenital, and spontaneous. Based on the results of present subchronic toxicity study of SFSE-G, the no observed adverse effect level (NOAEL) is 1000 mg/kg per day in male rats and 500 mg/kg per day in female rats.

Mutagenicity

The numbers of reverse mutation colonies for the positive control substances such as sodium azide (NaN3), 2-acetamidofluorene (2-AF), 4-Nitroquinolene-N-Oxide (NQNO), Methyl methane sulphonate (MMS), 2 - Aminoanthracene (2-AA) and danthron showed significant increases in the revertant count than the negative control.

Thus, the test strains fulfilled the quality check criteria. No substantial increases in the revertant colony count in any of the five strains were reported in the SFSE-G treated plates in the presence or absence of metabolic activation (S9 mix).

The spontaneous reversion rates in the negative and positive controls were within the range of historical data. The results of these investigations suggest that under the experimental conditions, SFSE-G did not induce gene mutation by pair changes or frame-shifts in the genome of the strains used. Thus, SFSE-G can be considered as non-mutagenic.

Table 9: Summary of histopathology findings.

Sex	Male		Female	
Parameters	**VC**	**SFSE -G- 1000**	**VC**	**SFSE -G- 1000**
Number of Animals examined :	15	15	15	15
Adrenals	NA	NA	NA	NA
Dilation, sinusoidal	3	2	3	2
Aorta	0	0	0	0
Brain	NA	NA	NA	NA
Lymphocytic Infiltration	0	0	1	0
Necrosis	2	0	1	1
Caecum	0	0	0	0
Colon	0	0	0	0
Duodenum	0	0	0	0
Epididymis	0	0	NA	NA
Eyes	0	0	0	0
Heart	0	0	0	0
Ileum	0	0	0	0
Jejunum	0	0	0	0
Kidneys	NA	NA	NA	NA
Lymphocyte Infiltration, interstitial	1	1	3	4
Necrosis	0	1	2	0
Liver	NA	NA	NA	NA
Lymphocyte Infiltration, periportal	14	13	15	15
Necrosis	1	0	0	1
Lungs	NA	NA	NA	NA
Pneumonitis	15	15	15	15
Haemorrhages	4	6	7	3
Histiocytosis	1	2	0	2
Lymph Node	NA	NA	NA	NA
Cyst	0	0	0	1
Ovaries	NA	NA	NA	NA
Skin	0	0	0	0
Oesophagus	0	0	0	0
Pancreas	NA	NA	NA	NA
LymphocyteInfiltration	0	0	0	1
Pituitary	NA	NA	NA	NA
Cysts	3	0	2	1
Prostate	0	0	NA	NA
Rectum	0	0	0	0
Sciatic nerve	0	0	0	0
Seminal vesicles	0	0	NA	NA
Skeletal muscle	0	0	0	0
Spleen	0	0	0	0
Spinal cord	0	0	0	0
Sternum with bone marrow	0	0	0	0
Stomach	NA	NA	NA	NA
Dilatation, glandular	0	0	0	1
Testes	0	0	NA	NA
Trachea	NA	NA	NA	NA
Lymphocytic Infiltration, submucosal	6	10	6	8
Thymus	0	0	0	0
Thyroid / Parathyroid	NA	NA	NA	NA
Ultimobranchial Cysts	3	6	1	2
Urinary Bladder	0	0	0	0
Uterus	NA	NA	NA	NA
Eosinophilic infiltration	NA	NA	14	15

NA = Not applicable.

DISCUSSION

Toxicology studies are the platform for hazard identification stage of safety assessment (Wallace, 2011). The preclinical toxicity testing with safe dose level determination is needed to initiate the clinical evaluation of investigational products (Setzer and Kimmel, 2003). Choosing the appropriate tests and dosing regimens that will demonstrate an adequate margin of exposure is a critical step in establishing human safety. Toxicity data from studies following well-accepted international guidelines in animals is required by regulatory agencies throughout the world to support regulatory applications and marketing approval of natural products.

During the evaluation of the safety information of plants derived natural products, the determination of median lethal dose (LD_{50}) is usually an initial step to be conducted. Data from the acute toxicity study may provide initial information on the mode of toxic action of a substance, help in dose determination in animal studies (Ukwuani *et al.*, 2012). In the present study, acute administration of SFSE-G at a dose of 2000 mg/kg, showed no adverse effects up to 14 days and so considered as the median lethal dose (LD_{50}).

Based on results of AOT, subchronic (90-days repeated dose) toxicity study of SFSE-G was conducted in rats. Subchronic studies assess the undesirable effects of repeated long-term exposure of test product over a portion of the average life span of experimental animals, such as rodents. Therefore, they provide useful information on target organ toxicity and help to determine appropriate dose regimens for long-term use. Administration of SFSE-G for 90 days produced no clinical signs of toxicity or mortality in either sex.

The body weight changes serve as a sensitive indication of the general health status of animals (El Hilaly *et al.*, 2004). Loss of appetite is often synonymous with weight loss due to disturbances in carbohydrate, protein or fat metabolisms (Klaassen, 2013). At higher doses, plant extracts may metabolize to a toxic end product, which could interfere with gastric function and decreased food conversion efficiency (Chokshi, 2007). In the present study, SFSE-G treated groups of rats showed body weight gain (although statistically not significant). Furthermore, the food consumption of SFSE-G treated rats remained unaltered during the 90-day treatment period. These results indicated the absence of adverse effects on the normal metabolism of food components during the treatment period and maintained expected nutritional benefits of normal food and water, such as weight gain and stability of the appetite.

Evaluation of hematological parameters can be used to determine the extent of the deleterious effect of SFSE-G on the blood of an animal(Chukwuemeka *et al.*, 2015).Furthermore, such analysis is relevant to risk evaluation as changes in the hematological system have higher predictive value for human toxicity when the data are translated from animal studies (Chanda *et al.*, 2015, Diaz *et al.*, 2016).

A hematology data of SFSE-G treated rats did not show treatment-related changes as compared to VC rats. The results of hematology indicated the safety of SFSE-G on erythropoiesis, morphology or osmotic fragility of the red blood cells (Hall, 2015). Leukocytes are the first line of cellular defense that respond to infectious agents, tissue injury, or inflammatory process. The absence of significant treatment-related changes on TLC or differential leukocyte parameters in SFSE-G treated rats in the present study confirmed the safety of SFSE-G.

A normal hematological profile of SFSE-G was further supported by the absence of adverse effects on biochemical parameters. Being vital to the survival of an organism, liver and kidney function tests are important in any toxicity evaluation program of drugs and plant extracts (Avigan *et al.*, 2016, Stickel and Shouval, 2015). High levels of ALT, AST, and ALP indicate hepatotoxicity and possibility of liver diseases (Simpson and Freshwater, 2015). A decrease in total protein and albumin is a sign of the reduced protein synthesis, impaired hepatocellular function, infection or continuous loss of albumin (Cabrerizo *et al.*, 2015, Waidely *et al.*, 2016).

The absence of treatment-related adverse effects on liver function parameters in SFSE-G treated rats in the present study suggests safety of subchronic administration of SFSE-G treatment on liver or hepatocyte functions in the rats.

The weighing dissected organs in toxicity studies are useful to assess their sensitivity to test compound and a target organ of toxicity in terms of possible enzyme induction, physiologic perturbations, and acute injury (Michael *et al.*, 2007). The results of present study revealed the absence of target organ toxicity by subchronic administration of SFSE-G. The absolute and relative weights of the essential organs (heart, liver, spleen, kidneys, and lungs) were neither adversely affected nor showed clinical signs of toxicity throughout the subchronic treatment of SFSE-G in the tested dose range. The safety of SFSE-G on essential organ toxicity correlates well with histological findings with little inter-animal variability. The photomicrographs from sections of vital organs (heart, liver, spleen, kidney, and lung) did not show any alterations in cell structure, unfavorable effects or pathological changes.

In the present study, recovery group, SFSE-G-1000-R (treated with limit dose, 1000 mg/kg) did not show the occurrence of systemic toxic effects during recovery period (28 days after treatment period of 90 days) as compared to VC-R group. The absence of toxic effects or any reversibility in SFSE-1000-R group confirmed nontoxic nature of SFSE-G.

To evaluate mutagenicity potential of SFSE-G, the reverse mutation assay (AMES test) (Mortelmans and Zeiger, 2000)was used in the present study. The SFSE-G, over a broad concentration range (61.72–5000 µg/plate, did not show mutagenicity either presence or absence of a metabolic activator.

In the past, subchronic toxicity safety studies of glycosides from other plant sources were reported. For example, glycosides from *Stevia rebaudiana* were found safe during subchronic toxicity study in Wistar rats (Curry and Roberts, 2008). Furthermore, purified fraction of rhamnoside, a glycoside, from *Pteranthus dichotomus* found to be safe for acute administration in rats (Atta *et al.*, 2013). The results of the present study are in line with reports of the safety of glycosides isolated from other sources. Human equivalent dose (HED) can be derived from NOAEL by using USFDA guidance for Industry (Center for Drug Evaluation and Research, 2005). Based on results of the present study (NOAEL of 500 mg/kg in rats, HED of SFSE-G is can be calculated to 4.8 g/day (for the average human weight of 60 kg). The HED of 4.8 g /day is 8 times higher than efficacy dose of 600 mg/day during a clinical study in male healthy subjects (Mokashi *et al.*, 2014) confirming broad margin of safety of SFSE-G.

CONCLUSION

The present study demonstrated the preclinical safety of SFSE-G during acute and subchronic (90-days repeated dose) administration in laboratory rats without mutagenicity potential *in vitro*. The median lethal dose cut-off value of SFSE-G was more than 2000 mg/kg and NOAEL of 1000 mg/kg and 500 mg/kg in male and female rats respectively.

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REFERENCES

Aswar U, Bodhankar S, Thakurdesai P, Mohan V. Androgenic and anabolic activity of glycoside based standardized fenugreek seeds extract on male Wistar rats. APTICON2014- 19th Annual National Convention of Association of Pharmaceutical Teachers of India. Pune, India: APTI, India; 2014.

Aswar U, Bodhankar SL, Mohan V, Thakurdesai PA. Effect of furostanol glycosides from *Trigonella foenum-graecum* on the reproductive system of male albino rats. Phytother Res. 2010; 24:1482–8.

Atta EM, Nassar AA, Hasan NM, Hasan AR. New flavonoid glycoside and pharmacological activities of Pteranthus dichotomus Forssk. Records of Natural Products. 2013; 7.

Avigan MI, Mozersky RP, Seeff LB. Scientific and Regulatory Perspectives in Herbal and Dietary Supplement Associated Hepatotoxicity in the United States. Int J Mol Sci. 2016; 17:331.

Basch E, Ulbricht C, Kuo G, Szapary P, Smith M. Therapeutic applications of fenugreek. Altern Med Rev. 2003; 8:20-7.

Cabrerizo S, Cuadras D, Gomez-Busto F, Artaza-Artabe I, Marin-Ciancas F, Malafarina V. Serum albumin and health in older people: Review and meta analysis. Maturitas. 2015; 81:17-27.

Center for Drug Evaluation and Research. Guidance for Industry: Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. US Department of Health and Human Services, Food and Drug Administration. 2005.

Chanda S, Parekh J, Vaghasiya Y, Dave R, Baravalia Y, Nair R. Medicinal plants-from traditional use to toxicity assessment: A Review. International Journal of Pharmaceutical Sciences and Research. 2015; 6:2652.

Chokshi D. Subchronic oral toxicity of a standardized white kidney bean (Phaseolus vulgaris) extract in rats. Food Chem Toxicol. 2007; 45:32-40.

Curry LL, Roberts A. Subchronic toxicity of rebaudioside A. Food Chem Toxicol. 2008; 46 Suppl 7:S11-20.

Diaz D, Hartley DP, Kemper R. Issue Investigation and Practices in Discovery Toxicology. Drug Discovery Toxicology: From Target Assessment to Translational Biomarkers. 2016:530-9.

El Hilaly J, Israili ZH, Lyoussi B. Acute and chronic toxicological studies of Ajuga iva in experimental animals. Journal of ethnopharmacology. 2004; 91:43-50.

Gupta RK, Jain DC, Thakur RS. Furostanol glycosides from *Trigonella foenum-graecum* seeds. Phytochemistry. 1985; 24:2399-401.

Hall JE. Guyton and Hall textbook of medical physiology. 13th ed: Elsevier Health Sciences; 2015.

Han Y, Nishibe S, Noguchi Y, Jin Z. Flavonol glycosides from the stems of Trigonella foenum-graecum. Phytochemistry. 2001; 58:577-80.

Huang W, Liang X. Determination of two flavone glycosides in the seeds of Trigonella foenum-graecum L. from various production locality. Journal of Plant Resources and Environment. 1999; 9:53-4.

Kalaiselvan V, Saurabh A, Kumar R, Singh GN. Adverse reactions to herbal products: An analysis of spontaneous reports in the database of the Pharmacovigilance Programme of India. Journal of herbal medicine. 2015; 5:48-54.

Kandhare AD, Bodhankar SL, Mohan V, Thakurdesai PA. Acute and repeated doses (28 days) oral toxicity study of glycosides based standardized fenugreek seed extract in laboratory mice. Regul Toxicol Pharmacol. 2015; 72:323-34.

Kang L-p, Zhao Y, Pang X, Yu H-s, Xiong C-q, Zhang J, *et al*. Characterization and identification of steroidal saponins from the seeds of Trigonella foenum-graecum by ultra high-performance liquid chromatography and hybrid time-of-flight mass spectrometry. J Pharm Biomed Anal. 2013; 74:257-67.

Kawabata T, Cui MY, Hasegawa T, Takano F, Ohta T. Anti-inflammatory and anti-melanogenic steroidal saponin glycosides from Fenugreek (*Trigonella foenum-graecum* L.) seeds. Planta Medica. 2011; 77:705-10.

Kenny O, Smyth TJ, Hewage CM, Brunton NP. Antioxidant properties and quantitative UPLC-MS analysis of phenolic compounds from extracts of fenugreek (Trigonella foenum-graecum) seeds and bitter melon (Momordica charantia) fruit. Food chemistry. 2013; 141:4295-302.

Klaassen CD. Casarett and Doull's toxicology: the basic science of poisons: McGraw-Hill New York (NY); 2013.

Michael B, Yano B, Sellers RS, Perry R, Morton D, Roome N, *et al*. Evaluation of organ weights for rodent and non-rodent toxicity studies: a review of regulatory guidelines and a survey of current practices. Toxicol Pathol. 2007; 35:742-50.

Mokashi M, Singh-Mokashi R, Mohan V, Thakurdesai PA. Effects of glycosides based fenugreek seed extract on serum testosterone levels of healthy sedentary male subjects: A exploratory double blind, placebo controlled, crossover study. Asian Journal of Pharmaceutical and Clinical Research. 2014; 7:177-81.

Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. Mutat Res. 2000; 455:29-60.

Murakami T, Kishi A, Matsuda H, Yoshikawa M. Medicinal foodstuffs. XVII. Fenugreek seed. (3): structures of new furostanol-type steroid saponins, trigoneosides Xa, Xb, XIb, XIIa, XIIb, and XIIIa, from the seeds of Egyptian Trigonellafoenum-graecum L. Chem Pharm Bull (Tokyo). 2000; 48:994-1000.

Organisation for Economic Co-operation and Development. Test No. 471: Bacterial Reverse Mutation Test. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris: OECD Publishing; 1997.

Organisation for Economic Co-operation and Development. Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris: OECD Publishing; 2002.

Organisation for Economic Co-operation and Development. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris: OECD Publishing; 1998. p. 1 online resource (v.).

Pang X, Cong Y, Yu H-S, Kang L-P, Feng B, Han B-X, *et al*. Spirostanol saponins derivated from the seeds of *Trigonella foenum-graecum* by β-glucosidase hydrolysis and their inhibitory effects on rat platelet aggregation. Planta Med. 2012a; 78:276-85.

Pang X, Kang L-P, Yu H-S, Zhao Y, Xiong C-Q, Zhang J, *et al*. New kaurene diterpenoid glycosides from fenugreek seeds. Nat Prod Res. 2012b.

Petropoulos GA. Fenugreek: the genus Trigonella: CRC Press; 2003.

Rayyan S, Fossen T, Andersen ØM. Flavone C-glycosides from seeds of fenugreek, Trigonella foenum-graecum L. J Agric Food Chem. 2010; 58:7211-7.

Sauvaire Y, Ribes G, Baccou JC, Loubatieeres-Mariani MM. Implication of steroid saponins and sapogenins in the hypocholesterolemic effect of fenugreek. Lipids. 1991; 26:191-7.

Setzer RW, Kimmel CA. Use of NOAEL, benchmark dose, and other models for human risk assessment of hormonally active substances. Pure and applied chemistry. 2003; 75:2151-8.

Shang M, Cai S, Han J, Li J, Zhao Y, Zheng J, *et al*. [Studies on flavonoids from Fenugreek (Trigonella foenumgraecum L.)]. Zhongguo Zhong Yao Za Zhi. 1998; 23:614-6, 39.

Simpson MA, Freshwater DA. The interpretation and management of abnormal liver function tests. J R Nav Med Serv. 2015; 101:74-9.

Starr RR. Too little, too late: ineffective regulation of dietary supplements in the United States. Am J Public Health. 2015; 105:478-85.

Stickel F, Shouval D. Hepatotoxicity of herbal and dietary supplements: an update. Arch Toxicol. 2015; 89:851-65.

Taylor WG, Elder JL, Chang PR, Richards KW. Microdetermination of diosgenin from fenugreek (Trigonella foenum-graecum) seeds. J Agric Food Chem. 2000; 48:5206-10.

Ukwuani A, Abubakar M, Hassan S, Agaie B. Toxicological studies of hydromethanolic leaves extract of *Grewia crenata*. Int J Pharm Sci Drug Res. 2012; 4:245-9.

Ulbricht C, Basch E, Burke D, Cheung L, Ernst E, Giese N, *et al*. Fenugreek (*Trigonella foenum-graecum* L. Leguminosae): an evidence-based systematic review by the natural standard research collaboration. J Herbal Pharmacother. 2008; 7:143-77.

Upton R, Graff A, Jolliffe G, Länger R, Williamson E. American Herbal Pharmacopoeia: Botanical Pharmacognosy-Microscopic Characterization of Botanical Medicines: CRC Press; 2016.

Wagner H, Iyengar MA, Hörhammer L. Vicenin-1 and-2 in the seeds of Trigonella foenumgraecum. Phytochemistry. 1973; 12:2548.

Waidely E, Al-Yuobi AR, Bashammakh AS, El-Shahawi MS, Leblanc RM. Serum protein biomarkers relevant to hepatocellular carcinoma and their detection. Analyst. 2016; 141:36-44.

Wallace HM. Risk Perception in Toxicology—Part II: Toxicology Must Be the Solution Not the Problem. Toxicol Sci. 2011; 121:7-10.

Wilborn C, Taylor L, Poole C, Foster C, Willoughby D, Kreider R. Effects of a Purported Aromatase and 5 α-Reductase Inhibitor on Hormone Profiles in College-Age Men. Int J Sport Nutr. 2010; 20:457.

Yau WP, Goh CH, Koh HL. Quality Control and Quality assurance of Phytomedicines: Key Considerations, methods, and analytical challenges. Phytotherapies: Efficacy, Safety, and Regulation. 2015:18.

Yoshikawa M, Murakami T, Komatsu H, Murakami N, Yamahara J, Matsuda H. Medicinal foodstuffs. IV. Fenugreek seed. (1): structures of trigoneosides Ia, Ib, IIa, IIb, IIIa, and IIIb, new furostanol saponins from the seeds of Indian Trigonella foenum-graecum L. Chem Pharm Bull (Tokyo). 1997; 45:81-7.

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REVIEW

Associations of Testosterone and Related Hormones With All-Cause and Cardiovascular Mortality and Incident Cardiovascular Disease in Men

Individual Participant Data Meta-analyses

Bu B. Yeap, MBBS, PhD; Ross J. Marriott, PhD; Girish Dwivedi, MD, PhD; Robert J. Adams, MBBS, MD; Leen Antonio, MD, PhD; Christie M. Ballantyne, MD; Douglas C. Bauer, MD; Shalender Bhasin, MD; Mary L. Biggs, PhD, MPH; Peggy M. Cawthon, PhD, MPH; David J. Couper, PhD; Adrian S. Dobs, MD, MHS; Leon Flicker, MBBS, PhD; David J. Handelsman, MBBS, PhD; Graeme J. Hankey, MBBS, MD; Anke Hannemann, PhD; Robin Haring, PhD; Benjumin Hsu, PhD; Sean A. Martin, PhD; Alvin M. Matsumoto, MD; Dan Mellström, MD, PhD; Claes Ohlsson, MD, PhD; Terence W. O'Neill, MD, MSc; Eric S. Orwoll, MD; Matteo Quartagno, PhD; Molly M. Shores, MD; Antje Steveling, MD; Åsa Tivesten, MD, PhD; Thomas G. Travison, PhD; Dirk Vanderschueren, MD, PhD; Gary A. Wittert, MBBCh, MD; Frederick C.W. Wu, MBChB, MD; and Kevin Murray, PhD

Background: Whether circulating sex hormones modulate mortality and cardiovascular disease (CVD) risk in aging men is controversial.

Purpose: To clarify associations of sex hormones with these outcomes.

Data Sources: Systematic literature review to July 2019, with bridge searches to March 2024.

Study Selection: Prospective cohort studies of community-dwelling men with sex steroids measured using mass spectrometry and at least 5 years of follow-up.

Data Extraction: Independent variables were testosterone, sex hormone–binding globulin (SHBG), luteinizing hormone (LH), dihydrotestosterone (DHT), and estradiol concentrations. Primary outcomes were all-cause mortality, CVD death, and incident CVD events. Covariates included age, body mass index, marital status, alcohol consumption, smoking, physical activity, hypertension, diabetes, creatinine concentration, ratio of total to high-density lipoprotein cholesterol, and lipid medication use.

Data Synthesis: Nine studies provided individual participant data (IPD) (255 830 participant-years). Eleven studies provided summary estimates (n = 24 109). Two-stage random-effects IPD meta-analyses found that men with baseline testosterone concentrations below 7.4 nmol/L (<213 ng/dL), LH concentrations above 10 IU/L, or estradiol concentrations below 5.1 pmol/L had higher all-cause mortality, and those with testosterone concentrations below 5.3 nmol/L (<153 ng/dL) had higher CVD mortality risk. Lower SHBG concentration was associated with lower all-cause mortality (median for quintile 1 [Q1] vs. Q5, 20.6 vs. 68.3 nmol/L; adjusted hazard ratio [HR], 0.85 [95% CI, 0.77 to 0.95]) and lower CVD mortality (adjusted HR, 0.81 [CI, 0.65 to 1.00]). Men with lower baseline DHT concentrations had higher risk for all-cause mortality (median for Q1 vs. Q5, 0.69 vs. 2.45 nmol/L; adjusted HR, 1.19 [CI, 1.08 to 1.30]) and CVD mortality (adjusted HR, 1.29 [CI, 1.03 to 1.61]), and risk also increased with DHT concentrations above 2.45 nmol/L. Men with DHT concentrations below 0.59 nmol/L had increased risk for incident CVD events.

Limitations: Observational study design, heterogeneity among studies, and imputation of missing data.

Conclusion: Men with low testosterone, high LH, or very low estradiol concentrations had increased all-cause mortality. SHBG concentration was positively associated and DHT concentration was nonlinearly associated with all-cause and CVD mortality.

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Older men have lower testosterone concentrations on average than younger or middle-aged men, particularly after age 70 years (1). The decrease in testosterone after age 70 years is accompanied by increased luteinizing hormone (LH) levels, consistent with impaired testicular Leydig cell function (2). Obesity and other medical comorbidities can contribute to lower testosterone concentrations in men by reducing the activity of the hypothalamic–pituitary–testicular (HPT) axis,

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even when it is structurally intact (2–4). Given the effects of testosterone on male libido, virilization, and body composition (enhanced muscle mass and reduced fat) (5), an important question has been whether and when lower endogenous testosterone concentrations are related to poorer health outcomes. In the circulation, testosterone is bound to carrier proteins, primarily sex hormone–binding globulin (SHBG), which modulate its availability to tissues (6). Testosterone and SHBG concentrations are related, and men may have low testosterone concentrations in the presence of low SHBG levels without having organic disease affecting the HPT axis (5). Low testosterone in the presence of high LH indicates primary testicular impairment, whereas low testosterone with low or normal LH may be consistent with centrally mediated disease or with suppression of HPT axis function due to obesity and other nongonadal conditions (5, 7). Testosterone is further metabolized to dihydrotestosterone (DHT), which, like testosterone, activates androgen receptors, and to estradiol, which activates estrogen receptors to mediate actions of testosterone in tissues such as bone (8, 9). Understanding the relationship between testosterone and SHBG and these other hormones can inform interpretation of health outcomes associated with differences in hormone concentrations.

Observational studies have reported either null or inverse associations of circulating total testosterone with all-cause and cardiovascular disease (CVD)–related mortality (10). There was a nonlinear association of lower total testosterone with all-cause mortality but no association with CVD deaths or CVD events in men aged 40 to 69 years from the UK Biobank (11, 12). However, the UK Biobank population is relatively healthy, and testosterone was measured by immunoassay rather than mass spectrometry, which provides more accurate and less variable results (13, 14). In studies of older men, higher total testosterone concentrations measured using mass spectrometry were associated with lower risk for CVD events, particularly stroke rather than myocardial infarction (MI) (15, 16). However, a large cardiovascular safety trial in middle-aged and older men with overweight or obesity and cardiovascular risk factors or disease (TRAVERSE [Testosterone Replacement Therapy for Assessment of Long-term Vascular Events and Efficacy Response in Hypogonadal Men]) found that 22 months of testosterone treatment did not increase risk for major cardiovascular events or mortality (17). Therefore, a meta-analysis of prospective cohort studies that measured total testosterone using mass spectrometry in community-dwelling men spanning the age range and different geographic regions could address these contrasting findings. Likewise, mass spectrometry provides greater accuracy and precision than immunoassays for DHT and estradiol, which circulate at much lower concentrations than testosterone in men (1, 13, 14). However, few studies have examined the associations of DHT and estradiol measured using mass spectrometry with mortality and CVD outcomes in men across a range of ages (10).

Our aim was to conduct individual participant data meta-analyses (IPDMAs) of prospective cohort studies of men that measured sex hormones (including total testosterone) using mass spectrometry and had at least 5 years of follow-up for outcomes (18, 19). Our primary hypothesis was that in men, endogenous circulating testosterone concentration is associated with risks for all-cause mortality, CVD death, and incident CVD events. Parallel IPDMAs were conducted for SHBG, LH, DHT, and estradiol to test the secondary hypotheses that any of these variables were associated with these outcomes. Exploratory analyses were conducted with heart failure (HF), MI, and stroke as separate outcomes. Given the relationship between testosterone and SHBG and the implications of high versus low or normal LH concentrations in the setting of lower testosterone concentrations, additional exploratory analyses that jointly considered testosterone and SHBG and testosterone and LH were done.

METHODS

Data Sources and Searches

The AIMS (Androgens In Men Study) protocol was submitted to PROSPERO on 23 July 2019 and registered on 20 November 2019 (CRD42019139668) (18). Eligible studies (data sources) were prospective cohort studies, previously identified in a published systematic review (19), of community-dwelling men with total testosterone concentrations measured using mass spectrometry and at least 5 years of follow-up for CVD events, CVD deaths, all-cause mortality, cancer deaths, cancer diagnoses, cognitive decline, or dementia (**Appendix Figure 1**, available at Annals.org). Cancer- and dementia-related outcomes will be published separately. The original search included literature to July 2019; a bridge search was conducted to update results from the initial search to May 2023, and a second bridge search was done to update results to March 2024 (2). The bridge searches found 2 studies, neither of which reported 5 or more years of follow-up for outcomes of interest; their authors were not approached for IPD (**Supplement Results** and **Supplement Figure 1**, available at Annals.org). This review was done in accordance with the guidance provided in the PRISMA-IPD (PRISMA [Preferred Reporting Items for Systematic reviews and Meta-Analyses] for Individual Patient Data systematic reviews) reporting checklist.

Study Selection

From among 2177 references, we identified 11 eligible studies (19), 9 of which provided IPD-level data (20–32) and 2 of which provided aggregate data (AD) statistics (33, 34). Summary attributes and a flowchart specific to the analyses of all-cause mortality, CVD deaths, and incident CVD events are provided in the **Appendix Table** (available at Annals.org) and **Appendix Figure 1**. Within studies, participants

with a history of orchidectomy, those with use of androgens or antiandrogens, those with missing sex hormone measurements, those who were lost to follow-up at an unknown date, and those for whom outcome event status could not be determined were excluded from analyses (Appendix Methods and **Appendix Figure 1**).

Data Extraction and Quality Assessment

All participants consented to participate in the original cohort studies, and this meta-analysis was approved by the Human Research Ethics Committee of the University of Western Australia. Newcastle-Ottawa quality assessments of the selected studies were performed (Appendix Methods and **Supplement Methods**, available at Annals.org). Variables for the planned IPDMAs covering sociodemographic, lifestyle, and medical factors; hormone-related exposures; and outcomes were agreed on in advance and published in a protocol paper (**Supplement Table 1**, available at Annals.org) (18) and requested from each individual study (Appendix Methods). Data sets from individual studies were securely sent, stored in a central repository, and checked, and baseline variables were harmonized (2). Primary health outcomes of interest were all-cause mortality, death caused by CVD, and incident CVD events. Deaths caused by CVD were defined as deaths from MI, cardiac arrest, HF, or stroke, and incident CVD events were defined as occurrence of fatal or nonfatal MI, HF, or stroke. Prospective time-to-event data determined using existing definitions routinely used by each study were requested and supplied as adjudicated event data or derived from supplied IPD using International Classification of Diseases (ICD) codes (**Supplement Results** and **Supplement Table 2**, available at Annals.org). Information on numbers of participants, ages, testosterone concentrations, outcome events, and duration of follow-up was tabulated (**Appendix Table 1**; see also Supplementary Table 6 in the previously published systematic review [19]). Missing data are described in the Appendix Methods, the **Supplement Results**, and **Supplement Tables 3A** to **3C** (available at Annals.org).

Data Synthesis and Analysis

The IPDMAs summarized relationships between baseline hormone concentrations (total testosterone [primary exposure]; SHBG, LH, DHT, and estradiol [secondary exposures]) and relative risks for all-cause mortality, CVD death, and incident CVD events (outcomes). Random-effects IPDMAs were performed because variations in effect estimates among studies were assumed to be at least partly attributable to differences in local factors (35). The 2-stage approach was adopted to facilitate incorporation of studies with AD (36). This involved fitting the same model to each data set separately (stage 1) and then analyzing the study-specific estimates using a random-effects model (stage 2). The IPDMAs were first conducted using only the 9 IPD-level data sets; then, suitable AD statistics were requested from the other 2 studies. However, some IPDMAs analyzed fewer studies because studies that did not have IPD for both the outcome and hormone exposure variables were not included (**Supplement Table 1**). Analyses were performed in R, version 4.0.2 (R Foundation for Statistical Computing).

Two different Cox proportional hazards models were fitted. The minimal model (model 1) included only the terms for hormone exposure and baseline age. The full model (model 2) included the following additional terms: baseline body mass index, marital status, alcohol consumption, smoking status, physical activity, ratio of total to high-density lipoprotein (HDL) cholesterol, hypertension, diabetes, creatinine concentration, and lipid medication use. This set of model predictors encompasses factors that influence testosterone concentrations and potential confounders and were the ones that were included in the initial plan (18) and were available in all 9 IPD-level data sets (**Supplement Table 1**). In exploratory analyses, additional terms for SHBG category (low, normal, or high) and the interaction between testosterone and SHBG and terms for LH category and the interaction between testosterone and LH were added to model 2. Total–HDL cholesterol ratio was used as an indicator of risk for heart disease (37). For analyses of all-cause mortality, history of cancer at baseline was included as an additional model predictor. For analyses of incident CVD events, participants with a history of CVD were excluded.

Continuous predictors were modeled using restricted cubic splines and used values for centering and knot points that were consistent across studies (**Supplement Methods**). Substantive model compatible fully conditional specification (SMCFCS) was used to generate 40 imputed versions of each data set, with imputations that were congenial with each IPDMA model (**Supplement Methods**) (38). The validity of the proportional hazards assumption was assessed for the first imputed data set using per-variable and global tests and Schoenfeld residual plots. Results from fitting each model to each imputed data set in stage 1 were pooled using Rubin's rules before combining in stage 2 (39).

Summary estimates for associations between each outcome and hormone variable are presented in tables and graphically in summary curves and forest plots. Hazard ratios (HRs) and 95% CIs were calculated from each fitted IPDMA model relative to the median of the fifth quintile (Q5 median [reference value]). Full results are presented for IPDMAs of the 9 IPD-level data sets. The relative extent of heterogeneity was quantified using the I^2 statistic (40). Contour-enhanced funnel plots were constructed to explore publication bias. Sensitivity analyses, analyses exploring associations with specific types of CVD events, and analyses that jointly considered testosterone and SHBG and testosterone and LH were also conducted (Appendix Methods and **Supplement Methods**).

Role of the Funding Source

The funders had no role in the collection, analysis, or interpretation of the data or the writing or submission of this article.

RESULTS

Analysis Population

A detailed description of the analysis population is provided in the Appendix Results and the **Appendix Table**. There were IPD for 20 654 persons for mortality analyses (**Appendix Figure 1**), comprising 255 830 participant-years of risk, with 7241 deaths and 1351 CVD deaths. Aggregate data statistics were provided for 3455 persons, with 1099 deaths and 326 CVD deaths. For CVD event analyses, IPD were available for 12 829 persons and AD were available for another 1956, with 2803 and 326 incident CVD events, respectively.

Testosterone

In an age-adjusted analysis, men with baseline testosterone concentrations at or below the median of Q1 had higher relative risk for all-cause mortality (**Table** [model 1]; **Supplement Figure 2a**, available at Annals.org). In a fully adjusted analysis, the CIs of the HR included 1.0 at all quintile medians (**Table** [model 2]), and only men with low baseline testosterone concentrations (<7.4 nmol/L [<213 ng/dL], where the HR and CI were >1.0) had a higher risk for all-cause mortality (**Figure 1**, *top*).

Age-adjusted CVD mortality risk was higher for men with baseline testosterone concentrations at or below the median of Q1 (**Table** [model 1]; **Supplement Figure 2b**, available at Annals.org). In a fully adjusted analysis, the CIs included 1.0 for all quintile medians (**Table** [model 2]), but the HR and CI were above 1.0 for men with very low testosterone values (<5.3 nmol/L [<153 ng/dL]) (**Figure 1**, *middle*).

Age-adjusted risk for incident CVD events was higher for men with lower baseline testosterone levels (**Table** [model 1]; **Supplement Figure 2c**, available at Annals.org). This was attenuated after all factors were controlled for, with all CIs including 1.0 (**Table** [model 2]; **Figure 1**, *bottom*).

The bounds of the 95% CIs for the I^2 values ranged from 0% to 39.4% for these IPDMAs, demonstrating that although some estimates were imprecise, relative heterogeneity was negligible to moderate (**Table**).

Sex Hormone–Binding Globulin

In age-adjusted and fully adjusted analyses, SHBG was directly associated with all-cause mortality risk for concentrations at and above the Q3 median (37.9 nmol/L) (**Figure 2**, *top*; **Supplement Figure 3a**, available at Annals.org). Age-adjusted HRs and CIs were below 1.0 for Q2 to Q4 medians, and fully adjusted HRs and CIs were below 1.0 for the Q1 to Q4 medians relative to the Q5 median (68.3 nmol/L) (**Table** [model 2]). However, although the age-adjusted risk for all-cause mortality appeared to be J-shaped (**Supplement Figure 3a**), in both the age-adjusted analysis and the fully adjusted analysis, all-cause mortality risk was lowest for the Q3 median (37.9 nmol/L; HR, 0.83 [95% CI, 0.77 to 0.89]) (**Table** [model 2]; **Figure 2**, *top*).

For associations of SHBG with CVD deaths, 95% CIs of the HRs were broad in age-adjusted and fully adjusted analyses (**Figure 2**, *middle*; **Supplement Figure 3b**, available at Annals.org). This is reflected in the comparison of quintile medians (median for Q1 vs. Q5: fully adjusted HR, 0.81 [CI, 0.65 to 1.00]) (**Table** [model 2]). This increased uncertainty may reflect the smaller number of CVD deaths, as shown on the forest plot for HRs at the Q1 median (20.6 nmol/L) (**Figure 2**, *middle*).

In the age-adjusted analysis, men with low baseline SHBG concentrations had higher risk for incident CVD events (**Supplement Figure 3c**, available at Annals.org). This was attenuated in fully adjusted analyses, with all CIs including 1.0 (**Table** [model 2]; **Figure 2**, *bottom*).

The bounds of the 95% CIs for the I^2 values ranged from 0% to 41.1%, demonstrating that relative heterogeneity was negligible to moderate (**Table**).

Luteinizing Hormone

Summary curves of the HR for all-cause mortality, CVD death, or incident CVD events with LH concentration relative to the Q5 median (10.0 IU/L) were similar in age-adjusted and fully adjusted analyses (**Appendix Figure 2** and **Supplement Figure 4**, available at Annals.org). The CIs included 1.0 at all LH quintile medians for all 3 outcomes (**Table**) and for all LH concentrations for CVD deaths and CVD events (**Appendix Figure 2** and **Supplement Figure 4**). However, men with baseline LH concentrations above 10.0 IU/L (where the fully adjusted HR and the CI were >1.0) had elevated relative risk for all-cause death (**Appendix Figure 2**).

The bounds of the 95% CIs for the I^2 values ranged from 0% to 62.2%, demonstrating that relative heterogeneity was negligible to moderate in these IPDMAs (**Table**).

Dihydrotestosterone

In age-adjusted and fully adjusted analyses, baseline DHT concentration was inversely associated with risks for all-cause and CVD death for concentrations relative to but below the Q5 median (2.45 nmol/L) (**Figure 3**, *top* and *middle*; **Supplement Figure 5**, available at Annals.org). A U-shaped association with all-cause mortality was present, with higher risk for DHT at the Q1 median (0.69 nmol/L) (fully adjusted HR, 1.19 [CI, 1.08 to 1.30]) and for DHT concentrations above the Q5 median (2.45 nmol/L) (**Table** [model 2]; **Figure 3**, *top*). Risk for CVD death was higher at the Q1 median relative to the Q5 median (fully adjusted HR, 1.29 [CI, 1.03 to 1.61]) and for DHT concentrations above the Q5 median (**Table** [model 2]; **Figure 3**, *middle*).

Table. Summary Estimates of Hazard Ratios for All-Cause Mortality, CVD Death, and Incident CVD Events, by Quintiles of Total Testosterone, SHBG, LH, DHT, and Estradiol Concentrations, and Estimated Relative Heterogeneity*

Outcome	Q1 (Lowest)	Q2	Q3	Q4	Q5 (Highest)	I^2 (95% CI), %
Total testosterone						
Median concentration						
nmol/L	8.46	11.83	14.68	18.15	24.50	–
ng/dL	244	340	423	523	706	–
Hazard ratio (95% CI)						
All-cause mortality						
Model 1†	1.09 (1.01–1.17)	0.96 (0.88–1.04)	0.93 (0.84–1.03)	0.95 (0.89–1.00)	Reference	0.0 (0.0–30.2)
Model 2‡	1.06 (0.97–1.15)	0.97 (0.90–1.05)	0.96 (0.87–1.05)	0.97 (0.92–1.03)	Reference	0.0 (0.0–35.7)
CVD death						
Model 1†	1.32 (1.06–1.64)	1.17 (0.92–1.49)	1.18 (0.90–1.55)	1.13 (0.96–1.32)	Reference	0.4 (0.0–39.4)
Model 2‡	1.13 (0.86–1.47)	1.05 (0.78–1.40)	1.11 (0.83–1.48)	1.10 (0.95–1.28)	Reference	8.1 (0.0–38.8)
Incident CVD events§						
Model 1†	1.28 (1.14–1.43)	1.13 (1.00–1.28)	1.00 (0.87–1.15)	0.98 (0.90–1.07)	Reference	0.0 (0.0–18.1)
Model 2‡	1.06 (0.92–1.22)	0.99 (0.86–1.13)	0.94 (0.81–1.09)	0.96 (0.88–1.04)	Reference	0.0 (0.0–29.3)
SHBG						
Median concentration, *nmol/L*	20.60	29.80	37.90	48.35	68.30	–
Hazard ratio (95% CI)						
All-cause mortality						
Model 1†	0.94 (0.84–1.05)	0.87 (0.81–0.93)	0.84 (0.77–0.90)	0.88 (0.84–0.93)	Reference	0.0 (0.0–30.8)
Model 2‡	0.85 (0.77–0.95)	0.84 (0.79–0.90)	0.83 (0.77–0.89)	0.88 (0.84–0.93)	Reference	0.0 (0.0–22.0)
CVD death						
Model 1†	0.98 (0.80–1.20)	1.09 (0.85–1.40)	1.10 (0.87–1.39)	1.03 (0.90–1.18)	Reference	10.4 (0.0–41.1)
Model 2‡	0.81 (0.65–1.00)	0.99 (0.78–1.25)	1.06 (0.84–1.34)	1.03 (0.90–1.18)	Reference	0.0 (0.0–37.5)
Incident CVD events§						
Model 1†	1.27 (1.08–1.49)	1.12 (0.98–1.28)	1.07 (0.93–1.22)	1.03 (0.96–1.12)	Reference	0.0 (0.0–36.7)
Model 2‡	0.97 (0.87–1.10)	0.96 (0.86–1.07)	0.96 (0.85–1.08)	0.98 (0.91–1.05)	Reference	0.0 (0.0–25.2)
LH						
Median concentration, *IU/L*	2.19	3.36	4.44	6.00	10.00	–
Hazard ratio (95% CI)						
All-cause mortality						
Model 1†	0.90 (0.73–1.09)	0.85 (0.68–1.06)	0.87 (0.67–1.14)	0.92 (0.76–1.11)	Reference	32.1 (0.0–62.2)
Model 2‡	0.95 (0.79–1.13)	0.90 (0.75–1.07)	0.91 (0.72–1.14)	0.93 (0.79–1.11)	Reference	16.9 (0.0–53.0)
CVD death						
Model 1†	0.88 (0.71–1.09)	0.83 (0.65–1.05)	0.86 (0.60–1.24)	0.92 (0.69–1.21)	Reference	0.0 (0.0–48.1)
Model 2‡	0.85 (0.66–1.10)	0.83 (0.66–1.03)	0.85 (0.65–1.13)	0.91 (0.73–1.13)	Reference	0.0 (0.0–42.5)
Incident CVD events§						
Model 1†	0.89 (0.75–1.07)	0.90 (0.76–1.07)	0.91 (0.76–1.10)	0.94 (0.82–1.08)	Reference	0.0 (0.0–39.5)
Model 2‡	0.92 (0.68–1.24)	0.94 (0.74–1.20)	0.93 (0.74–1.17)	0.95 (0.81–1.11)	Reference	0.0 (0.0–45.2)
DHT						
Median concentration, *nmol/L*	0.69	1.09	1.41	1.78	2.45	–
Hazard ratio (95% CI)						
All-cause mortality						
Model 1†	1.21 (1.11–1.32)	1.09 (1.00–1.20)	1.04 (0.92–1.19)	0.99 (0.93–1.05)	Reference	6.7 (0.0–41.7)
Model 2‡	1.19 (1.08–1.30)	1.10 (1.00–1.21)	1.08 (0.93–1.26)	1.01 (0.93–1.09)	Reference	0.0 (0.0–48.1)
CVD death						
Model 1†	1.40 (1.09–1.79)	1.21 (0.93–1.57)	1.25 (0.93–1.67)	1.05 (0.91–1.22)	Reference	9.5 (0.0–46.0)
Model 2‡	1.29 (1.03–1.61)	1.19 (0.96–1.48)	1.33 (0.97–1.82)	1.09 (0.92–1.27)	Reference	0.0 (0.0–41.1)
Incident CVD events§						
Model 1†	1.34 (1.16–1.54)	1.19 (1.04–1.38)	1.08 (0.92–1.27)	1.02 (0.94–1.11)	Reference	0.0 (0.0–8.4)
Model 2‡	1.18 (0.98–1.41)	1.07 (0.89–1.29)	1.03 (0.85–1.24)	1.01 (0.92–1.10)	Reference	0.0 (0.0–36.4)
Estradiol						
Median concentration, *pmol/L*	40.56	59.86	73.79	90.00	119.12	–
Hazard ratio (95% CI)						
All-cause mortality						
Model 1†	1.03 (0.83–1.29)	0.93 (0.76–1.13)	0.93 (0.79–1.09)	0.96 (0.89–1.03)	Reference	34.6 (0.0–61.4)
Model 2‡	0.98 (0.78–1.25)	0.93 (0.73–1.18)	0.96 (0.80–1.16)	0.98 (0.90–1.06)	Reference	24.1 (0.0–55.5)
CVD death						
Model 1†	1.04 (0.84–1.29)	0.96 (0.72–1.27)	0.88 (0.63–1.22)	0.90 (0.76–1.05)	Reference	23.2 (0.0–54.9)
Model 2‡	1.02 (0.81–1.27)	1.01 (0.76–1.32)	0.98 (0.71–1.35)	0.96 (0.80–1.14)	Reference	28.4 (0.0–58.0)

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Table–Continued

Outcome	Q1 (Lowest)	Q2	Q3	Q4	Q5 (Highest)	I^2 (95% CI), %
Incident CVD events§						
Model 1†	1.04 (0.90–1.19)	0.98 (0.85–1.13)	0.89 (0.69–1.14)	0.96 (0.83–1.10)	Reference	0.0 (0.0–43.1)
Model 2‡	1.06 (0.92–1.22)	0.99 (0.85–1.14)	0.91 (0.71–1.17)	0.98 (0.85–1.12)	Reference	0.0 (0.0–41.9)

CVD = cardiovascular disease; DHT = dihydrotestosterone; LH = luteinizing hormone; Q = quintile; SHBG = sex hormone–binding globulin.
* Hazard ratios were calculated for the medians of testosterone, SHBG, LH, DHT, and estradiol concentrations within each sample quintile (Q1 to Q5) relative to the median for Q5 as calculated from the summary curve. Quintile boundaries as determined from all individual participant data across studies were as follows. Total testosterone: Q1/2, 10.4 nmol/L (300 ng/dL); Q2/3, 13.2 nmol/L (380 ng/dL); Q3/4, 16.3 nmol/L (470 ng/dL); and Q4/5, 20.5 nmol/L (591 ng/dL). SHBG: Q1/2, 25.5 nmol/L; Q2/3, 33.8 nmol/L; Q3/4, 42.6 nmol/L; and Q4/5, 55.7 nmol/L. LH: Q1/2, 2.9 IU/L; Q2/3, 3.9 IU/L; Q3/4, 5.1 IU/L; and Q4/5, 7.2 IU/L. DHT: Q1/2, 0.9 nmol/L; Q2/3, 1.3 nmol/L; Q3/4, 1.6 nmol/L; and Q4/5, 2.0 nmol/L. Estradiol: Q1/2, 51.4 pmol/L; Q2/3, 66.7 pmol/L; Q3/4, 80.4 pmol/L; and Q4/5, 100.0 pmol/L.
† Model 1 included terms for the respective hormones and age.
‡ Model 2 included the model 1 terms plus body mass index, marital or de facto relationship status, education, alcohol consumption, smoking status, physical activity, ratio of total to high-density lipoprotein cholesterol, hypertension, diabetes, creatinine, and use of lipid medications (a proxy for hyperlipidemia). History of cancer was also included as a predictor in analyses of all-cause mortality. Continuous variables (total testosterone, SHBG, LH, DHT, estradiol, age, body mass index, ratio of total to high-density lipoprotein cholesterol, creatinine) were modeled using restricted cubic splines.
§ Participants with a history of CVD were excluded from analyses of incident CVD events.

In the age-adjusted analysis, men with a baseline DHT concentration at or below the Q2 median had increased risk for incident CVD events (**Table** [model 1]; **Supplement Figure 5**). In fully adjusted analyses, this inverse association was attenuated (**Table** [model 2]), with the HR and CI above 1.0 only in men with very low DHT concentrations (<0.59 nmol/L) (**Figure 3**, *bottom*).

The bounds of the 95% CIs for the I^2 values ranged from 0% to 48.1%, demonstrating that relative heterogeneity was negligible to moderate in these IPDMAs (**Table**).

Estradiol

Summary curves of the HR for all-cause death, CVD death, and incident CVD events with estradiol concentration relative to the Q5 median (119.1 pmol/L) were similar in age-adjusted and fully adjusted analyses (**Appendix Figure 3** and **Supplement Figure 6**, available at Annals.org). The CIs for estradiol quintile medians included 1.0 for all 3 outcomes (**Table**). Only men with very low baseline estradiol concentrations (<5.1 pmol/L, for which the fully adjusted HR and CI were >1.0) had higher risk for all-cause mortality relative to the Q5 median, with no associations for CVD deaths or CVD events in the age-adjusted or fully adjusted analyses (**Appendix Figure 3**; **Supplement Figure 6**).

The bounds of the 95% CIs for the I^2 values ranged from 0% to 61.4%, demonstrating that relative heterogeneity was negligible to moderate in these IPDMAs (**Table**).

Other Analyses

Sensitivity analyses and analyses exploring associations with specific types of CVD events were done (Appendix Results; **Supplement Figures 7** to **17**, available at Annals.org). Estimates of summary HRs from IPDMAs incorporating AD from CHAMP (Concord Health and Ageing in Men Project) and the MrOS (Osteoporotic Fractures in Men) study in Sweden (11 studies in total) did not differ substantively from those that used IPD only (9 studies) and had low to negligible heterogeneity, suggesting that the AD from those studies were comparable to others and that availability bias from studies not providing IPD was negligible (**Appendix Figure 4**, available at Annals.org). Furthermore, no important asymmetry was apparent in funnel plots (**Supplement Results** and **Supplement Figure 18**, available at Annals.org). Exploratory analyses indicated that the association of lower testosterone with higher all-cause mortality risk was attenuated for men with lower SHBG concentrations, whereas risk was largely consistent across categories of LH (**Supplement Results**, **Supplement Table 4**, and **Supplement Figure 19**, available at Annals.org).

Discussion

This is, to our knowledge, the first IPDMA of major prospective cohort studies using mass spectrometry sex steroid assays, which clarifies previous inconsistent findings on the influence of sex hormones on key health outcomes in aging men. In fully adjusted analyses, only men with very low total testosterone concentrations had higher risks for all-cause and CVD mortality. A key finding was that men with a testosterone concentration below 7.4 nmol/L (<213 ng/dL) had higher risk for all-cause mortality, regardless of LH concentration. In addition, a higher concentration of circulating SHBG was associated with risk for all-cause mortality and was marginally associated with CVD deaths. Furthermore, the estimated relative risks for all-cause and CVD mortality were elevated in men with low or very high baseline concentrations of DHT. However, only men with very low DHT concentrations had higher risk for incident CVD events. Men with very high LH or very low estradiol concentrations had higher risk for all-cause mortality.

We found that men with low testosterone concentrations (<7.4 nmol/L [<213 ng/dL]) had higher all-cause mortality, and this was consistent in exploratory analyses whether LH was categorized as low, normal, or high. This suggests that the association is with low

Figure 1. Summary curves and forest plots for the association of baseline testosterone concentration with relative risk for all-cause mortality (*top*), CVD death (*middle*), and incident CVD events (*bottom*): model 2.

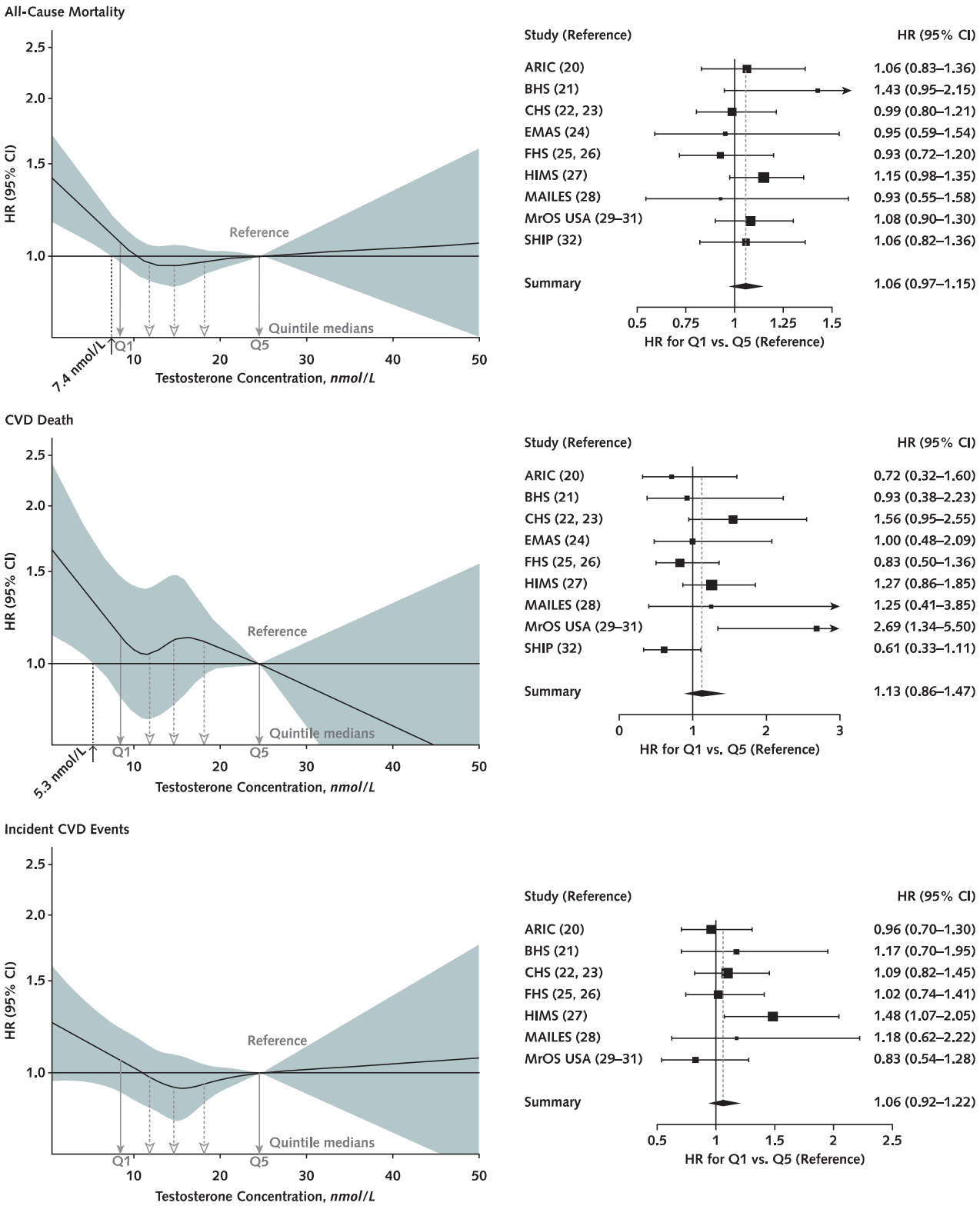

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Figure 1–Continued.

Estimates are controlled for baseline age, body mass index, marital status, alcohol consumption, smoking, physical activity, ratio of total to high-density lipoprotein cholesterol, blood creatinine concentration, lipid medication use, hypertension, and diabetes. Estimates for all-cause mortality are also controlled for history of cancer at baseline. Men with prevalent CVD were excluded from the analysis of incident CVD. Dashed lines and arrowheads indicate testosterone concentrations below which the HR and the 95% CI for all-cause mortality (*top*) and CVD death (*middle*) are >1.0 (7.4 nmol/L [213 ng/dL] and 5.3 nmol/L [153 ng/dL], respectively). To convert testosterone values from nanomoles per liter to nanograms per deciliter, divide by 0.0347. ARIC = Atherosclerosis Risk In Communities; BHS = Busselton Health Study; CHS = Cardiovascular Health Study; CVD = cardiovascular disease; EMAS = European Male Ageing Study; FHS = Framingham Heart Study; HIMS = Health In Men Study; HR = hazard ratio; MAILES = Men Androgen Inflammation Lifestyle Environment and Stress; MrOS = Osteoporotic Fractures in Men; Q = quintile; SHIP = Study of Health in Pomerania.

testosterone itself, whether due to an underlying testicular cause or centrally mediated. Men with even lower testosterone concentrations (<5.3 nmol/L [<153 ng/dL]) had higher risk for CVD death. In an exploratory analysis, lower testosterone concentration was associated with higher risk for stroke, but not MI or HF. Lower testosterone concentrations may predispose men to poorer outcomes via associations with lower muscle mass and greater adiposity and with other adverse cardiovascular risk factors (2, 10). Very low testosterone concentrations might also be associated with other medical comorbidities and hence higher mortality risk (2, 41). By contrast, in other exploratory analyses, men with both low testosterone and normal or high SHBG concentrations had higher all-cause mortality risk, whereas men with low testosterone and low SHBG concentrations had lower risk. Therefore, the combination of low testosterone and low SHBG concentrations seems to be benign, which is consistent with the concept that men with low SHBG may also have low circulating testosterone in the absence of androgen deficiency (5, 7). Our IPDMA resolves previous inconsistent findings from studies using immunoassay (11, 42–51) or mass spectrometry (23, 52–57), which reported either null or inverse associations of testosterone concentration with mortality in men. Similarly, previous studies reported neutral (11, 23, 42–45, 54, 56, 57) or inverse (46, 48, 50) associations of testosterone with CVD mortality in men. By using a large, combined data set of cohorts with testosterone measured using mass spectrometry to obtain more precise estimates, we demonstrate that lower testosterone concentrations are associated with higher all-cause and CVD mortality in a nonlinear fashion.

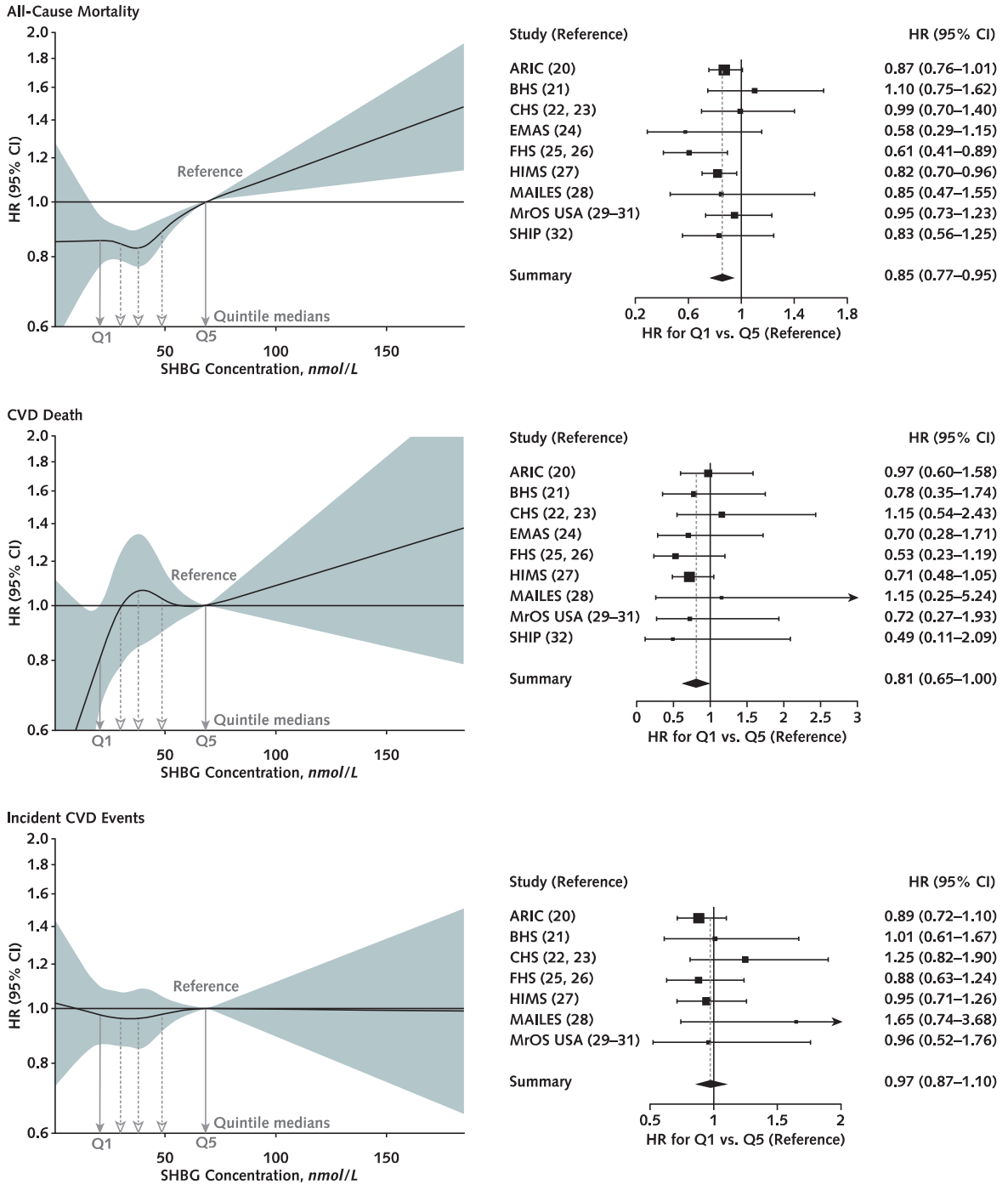
In our IPDMA, men with higher SHBG concentrations had higher risk for all-cause mortality; this association was linear for SHBG values above the median concentration. There was a marginal association of lower SHBG with lower risk for CVD death. It is possible that higher SHBG concentrations could modulate the bioavailability of testosterone to tissues (6). This concept is supported by our exploratory analysis that found an association between lower testosterone concentrations and higher all-cause mortality when SHBG concentration was normal or high but not when it was low. A direct role for SHBG signaling via receptors on cell surfaces has also been postulated (58, 59). Alternatively, SHBG might influence risk for all-cause and CVD mortality in a manner distinct from its relationship with total testosterone, but further investigation of potential underlying nutritional or metabolic pathways would be needed (60). This IPDMA clarifies results for SHBG where previous studies reported inconsistent findings for all-cause or CVD mortality risk in men (11, 23, 44, 46, 51, 54, 55, 57, 61–63).

Only men with high LH concentrations (as might be consistent with primary hypogonadism) or very low estradiol concentrations (at the margins of the distributions where data were relatively scant) had higher all-cause mortality risk. There was no clear association of LH or estradiol with risk for CVD death. This resolves inconsistent findings from previous studies that reported no association of LH with all-cause mortality (45), higher risk among men with LH in the highest quartile (46), or higher risk among older men with relatively high LH concentrations (61) and from estradiol studies using mass spectrometry that found no association with all-cause mortality (54, 56) or found associations of estradiol in the lowest quartile (55) or decreasing estradiol concentrations with higher all-cause mortality risk (57).

We found that the relationship between DHT and all-cause and CVD mortality risk was consistent with a U-shaped curve. There was increased all-cause mortality risk for men with DHT concentrations in the lowest and second-lowest quintiles and increased CVD mortality risk for the lowest quintile compared with the highest quintile median (2.45 nmol/L) as well as higher risk for both outcomes in men with DHT concentrations above 2.45 nmol/L, although data at these high concentrations were sparse. This clarifies previous inconsistent results from other studies (23, 42, 54, 56, 57). Of note, men with very low DHT had higher risk for incident CVD events; none of the other exposures was related to this outcome. DHT activates androgen receptor signaling, and downstream metabolites of DHT can also activate signaling through other receptors (8), which are potential pathways to modulation of outcomes. Thus, although lower circulating DHT level is a biomarker for poorer health outcomes in men, higher DHT concentrations are not beneficial.

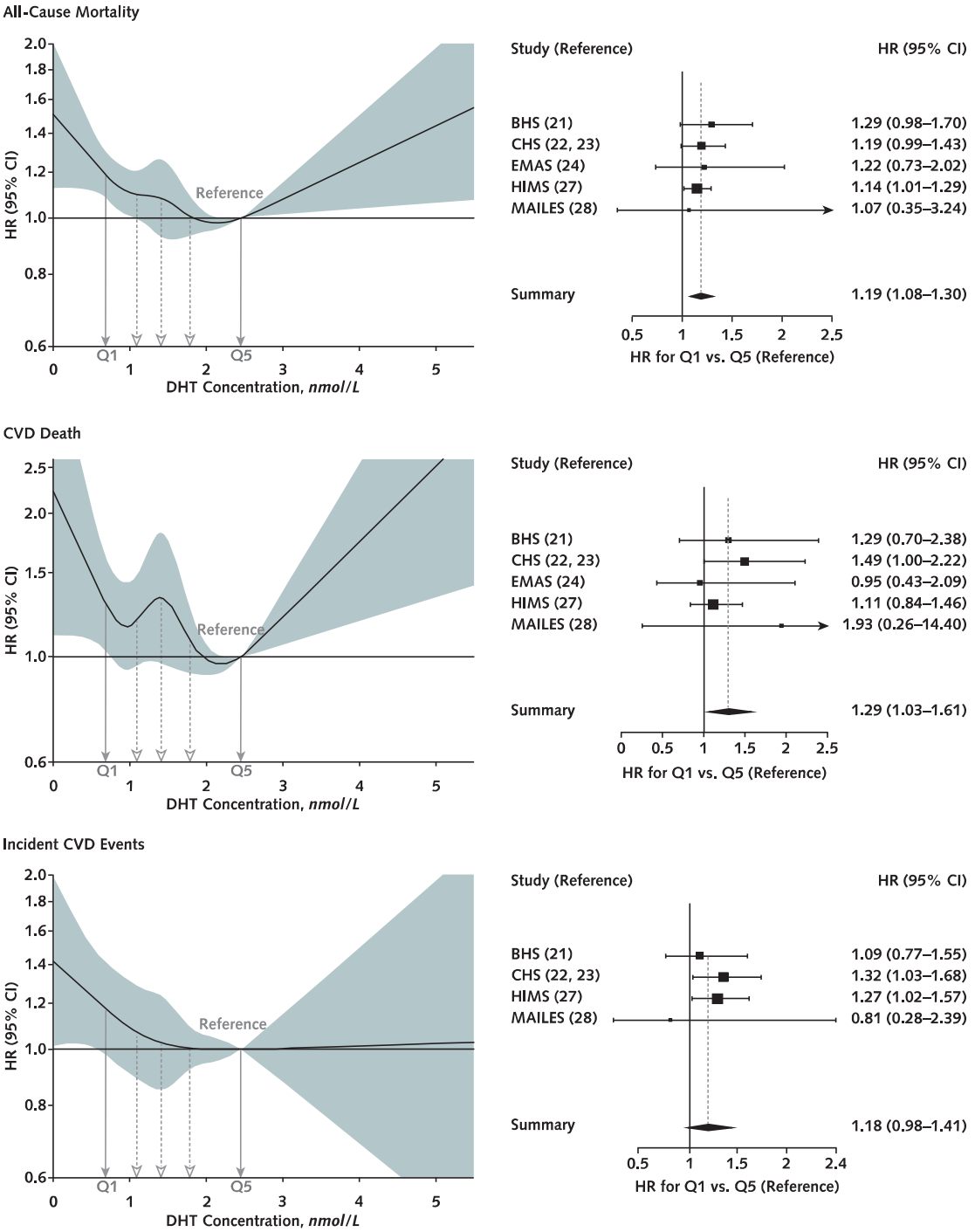
Thus, when examining 3 major sex steroids that activate nuclear transcription factors, we found that only men with low testosterone, low DHT, or very low estradiol concentrations have higher risk for all-cause mortality, and only men with very low testosterone or low DHT concentrations have increased CVD mortality. On the other hand, men with higher concentrations of SHBG,

Figure 2. Summary curves and forest plots for the association of baseline SHBG concentration with relative risk for all-cause mortality (*top*), CVD death (*middle*), and incident CVD events (*bottom*): model 2.



Estimates are controlled for baseline age, body mass index, marital status, alcohol consumption, smoking, physical activity, ratio of total to high-density lipoprotein cholesterol, blood creatinine concentration, lipid medication use, hypertension, and diabetes. Estimates for all-cause mortality are also controlled for history of cancer at baseline. Men with prevalent CVD were excluded from the analysis of incident CVD. ARIC = Atherosclerosis Risk In Communities; BHS = Busselton Health Study; CHS = Cardiovascular Health Study; CVD = cardiovascular disease; EMAS = European Male Ageing Study; FHS = Framingham Heart Study; HIMS = Health In Men Study; HR = hazard ratio; MAILES = Men Androgen Inflammation Lifestyle Environment and Stress; MrOS = Osteoporotic Fractures in Men; Q = quintile; SHBG = sex hormone–binding globulin; SHIP = Study of Health in Pomerania.

Figure 3. Summary curves and forest plots for the association of baseline DHT concentration with relative risk for all-cause mortality (*top*), CVD death (*middle*), and incident CVD events (*bottom*): model 2.



Estimates are controlled for baseline age, body mass index, marital status, alcohol consumption, smoking, physical activity, ratio of total to high-density lipoprotein cholesterol, blood creatinine concentration, lipid medication use, hypertension, and diabetes. Estimates for all-cause mortality are also controlled for history of cancer at baseline. Men with prevalent CVD were excluded from the analysis of incident CVD. BHS = Busselton Health Study; CHS = Cardiovascular Health Study; CVD = cardiovascular disease; DHT = dihydrotestosterone; EMAS = European Male Ageing Study; HIMS = Health In Men Study; HR = hazard ratio; MAILES = Men Androgen Inflammation Lifestyle Environment and Stress; Q = quintile.

which binds all 3 sex steroids, have higher risks for all-cause and CVD mortality. Higher LH concentration, which might be found in men with primary hypogonadism (5, 7), is also associated with higher all-cause mortality.

Strengths of this study include the availability of IPD from 9 prospective cohort studies and AD from 2 other studies, representing prospective data for 24 109 men from 11 major cohort studies (median ages, 49 to 76 years) from Australia, Europe, and North America (20–34). Mass spectrometry was used to measure circulating testosterone, DHT, and estradiol concentrations, providing a more accurate and precise estimate of sex hormone concentrations (13, 14). We conducted prespecified, comprehensive analyses examining 3 key outcomes (all-cause mortality, CVD deaths, and incident CVD events); performed multiple sensitivity analyses; and assessed for publication bias. The size of the combined data set and the statistical approach facilitated robust analyses to resolve different findings from individual studies and to account for potential confounding from cardiovascular risk factors. Limitations of the study include its observational nature, which precluded determination of causation. In bridge searches covering 2019 to 2024, 2 other similarly sized studies were identified that may be eligible for inclusion in future meta-analyses. Sex hormones were assayed in different laboratories at different times. Testosterone is affected by circadian rhythm, and although most studies collected blood samples in the morning, 3 studies did not (2). Validated measures of unbound ("free") testosterone were not available; thus, we presented exploratory analyses of testosterone including SHBG in the model, which provided some insight into outcomes when testosterone concentration was low and SHBG concentration was low, normal, or high. Some variables were recorded differently across studies; these were categorized to enable data to be harmonized. Some cohorts used internal or adjudicated definitions of CVD-related outcomes, and when these were not available, ICD codes were used. We performed analyses to evaluate sensitivity to the inclusion of other variables not featured in the main models, but there may be residual confounding from unmeasured variables, including other chronic diseases that could affect sex hormones or mortality risk, such as depression or thyroid disease. The analyses and conclusions apply to men in the contributing cohorts, who had baseline testosterone concentrations measured using mass spectrometry and were predominantly White and from Australia, Europe, and North America; therefore, our results should be confirmed in studies involving men of different ethnicities from other geographic regions. Nevertheless, findings from this IPDMA represent a broader population than individual studies and provide greater statistical power. Relative heterogeneity in the IPDMAs ranged from negligible to moderate, with 95% CIs for the I^2 values of 0% to 41.1% for analyses of testosterone and SHBG, 0% to 48.1% for DHT, and 0% to 62.2% for LH and estradiol.

In conclusion, we found that the testosterone concentration below which men had higher risk for all-cause mortality was 7.4 nmol/L (213 ng/dL). This adds to information on reference ranges based on distributions of testosterone in selected samples of healthy men (5, 7). Higher SHBG concentrations were associated with higher all-cause mortality, which may be related to its role as the major binding protein for sex steroids in the circulation. We found a U-shaped association of DHT with all-cause and CVD-related mortality risks, which were higher at lower and very high DHT concentrations. Men with very low DHT concentrations also had increased risk for incident CVD events. Further investigation into potential underlying mechanisms for these associations is warranted.

From Medical School, University of Western Australia, and Department of Endocrinology and Diabetes, Fiona Stanley Hospital, Perth, Western Australia, Australia (B.B.Y.); School of Population and Global Health, University of Western Australia, Perth, Western Australia, Australia (R.J.M., K.M.); Medical School, University of Western Australia; Harry Perkins Institute of Medical Research; and Department of Cardiology, Fiona Stanley Hospital, Perth, Western Australia, Australia (G.D.); Adelaide Institute for Sleep Health, Flinders University, Bedford Park, South Australia, Australia (R.J.A.); Laboratory of Clinical and Experimental Endocrinology, KU Leuven, Leuven, Belgium (L.A., D.V.); Internal Medicine, Baylor College of Medicine, Houston, Texas (C.M.B.); General Internal Medicine, University of California, San Francisco, San Francisco, California (D.C.B.); Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts (S.B.); Department of Biostatistics, School of Public Health, University of Washington, Seattle, Washington (M.L.B.); San Francisco Coordinating Center, California Pacific Medical Center Research Institute, San Francisco, California (P.M.C.); Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (D.J.C.); School of Medicine, Division of Endocrinology, Diabetes and Metabolism, Johns Hopkins University, Baltimore, Maryland (A.S.D.); Medical School, University of Western Australia, and Western Australian Centre for Healthy Ageing, University of Western Australia, Perth, Western Australia, Australia (L.F.); ANZAC Research Institute, University of Sydney, Sydney, New South Wales, Australia (D.J.H.); Medical School, University of Western Australia, and Perron Institute for Neurological and Translational Science, Perth, Western Australia, Australia (G.J.H.); Institute of Clinical Chemistry and Laboratory Medicine, University Medicine Greifswald, and German Centre for Cardiovascular Research, Partner Site Greifswald, Greifswald, Germany (A.H.); School of Public Health and Preventive Medicine, Monash University, Melbourne, Victoria, Australia, and European University of Applied Sciences, Faculty of Applied Public Health, Rostock, Germany (R.H.); Centre for Big Data Research in Health, University of New South Wales, Sydney, New South Wales, Australia (B.H.); Australian Institute of Family Studies, Southbank, Victoria, Australia (S.A.M.); Department of Medicine, University of Washington School of Medicine, and Geriatric Research, Education and Clinical Center, VA Puget Sound Health Care System, Seattle, Washington (A.M.M.);

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Reproducible Research Statement: *Study protocol:* The study protocol and systematic review have been published (18, 19). *Statistical code:* Computer code used to generate the results is available on reasonable request (e-mail, bu.yeap@uwa.edu.au). *Data set:* Availability of IPD and AD is governed by ethical approvals of the original cohort studies.

Corresponding Author: Bu B. Yeap, MBBS, PhD, Medical School, M582, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia; e-mail, bu.yeap@uwa.edu.au.

Author contributions are available at Annals.org.

References

1. **Yeap BB, Alfonso H, Chubb SA, et al.** Reference ranges and determinants of testosterone, dihydrotestosterone, and estradiol levels measured using liquid chromatography-tandem mass spectrometry in a population-based cohort of older men. J Clin Endocrinol Metab. 2012;97:4030-4039. [PMID: 22977273] doi:10.1210/jc.2012-2265
2. **Marriott RJ, Murray K, Adams RJ, et al.** Factors associated with circulating sex hormones in men: individual participant data meta-analyses. Ann Intern Med. 2023;176:1221-1234. [PMID: 37639720] doi:10.7326/M23-0342
3. **Shi Z, Araujo AB, Martin S, et al.** Longitudinal changes in testosterone over five years in community-dwelling men. J Clin Endocrinol Metab. 2013;98:3289-3297. [PMID: 23775354] doi:10.1210/jc.2012-3842
4. **Camacho EM, Huhtaniemi IT, O'Neill TW, et al; EMAS Group.** Age-associated changes in hypothalamic-pituitary-testicular function in middle-aged and older men are modified by weight change and lifestyle factors: longitudinal results from the European Male Ageing Study. Eur J Endocrinol. 2013;168:445-455. [PMID: 23425925] doi:10.1530/EJE-12-0890
5. **Yeap BB, Grossmann M, McLachlan RI, et al.** Endocrine Society of Australia position statement on male hypogonadism (part 1): assessment and indications for testosterone therapy. Med J Aust. 2016;205:173-178. [PMID: 27510348] doi:10.5694/mja16.00393
6. **Handelsman DJ.** Androgen physiology, pharmacology, use and misuse. In: Feingold KR, Anawalt B, Boyce A, et al, eds. Endotext. MDText.com, Inc.; 2000.
7. **Bhasin S, Brito JP, Cunningham GR, et al.** Testosterone therapy in men with hypogonadism: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2018;103:1715-1744. [PMID: 29562364] doi:10.1210/jc.2018-00229
8. **Bhasin S, Valderrabano RJ, Gagliano-Juca T.** Age-related changes in the male reproductive system. In: Feingold KR, Anawalt B, Boyce A, et al, eds. Endotext. MDText.com, Inc.; 2000.
9. **Russell N, Grossmann M.** Mechanisms in endocrinology: estradiol as a male hormone. Eur J Endocrinol. 2019;181:R23-R43. [PMID: 31096185] doi:10.1530/EJE-18-1000
10. **Yeap BB, Dwivedi G.** Androgens and cardiovascular disease in men. In: Feingold KR, Anawalt B, Boyce A, et al, eds. Endotext. MDText.com, Inc.; 2000.

11. **Yeap BB, Marriott RJ, Antonio L, et al.** Serum testosterone is inversely and sex hormone-binding globulin is directly associated with all-cause mortality in men. J Clin Endocrinol Metab. 2021;106: e625-e637. [PMID: 33059368] doi:10.1210/clinem/dgaa743
12. **Yeap BB, Marriott RJ, Antonio L, et al.** Associations of serum testosterone and sex hormone-binding globulin with incident cardiovascular events in middle-aged to older men. Ann Intern Med. 2022; 175:159-170. [PMID: 34958606] doi:10.7326/M21-0551
13. **Demers LM.** Testosterone and estradiol assays: current and future trends. Steroids. 2008;73:1333-1338. [PMID: 18565562] doi:10.1016/j.steroids.2008.05.002
14. **French D, Drees J, Stone JA, et al.** Comparison of four clinically validated testosterone LC-MS/MS assays: harmonization is an attainable goal. Clin Mass Spectrom. 2018;11:12-20. [PMID: 34841068] doi:10.1016/j.clinms.2018.11.005
15. **Ohlsson C, Barrett-Connor E, Bhasin S, et al.** High serum testosterone is associated with reduced risk of cardiovascular events in elderly men. The MrOS (Osteoporotic Fractures in Men) Study in Sweden. J Am Coll Cardiol. 2011;58:1674-1681. [PMID: 21982312] doi:10.1016/j.jacc.2011.07.019
16. **Yeap BB, Alfonso H, Chubb SAP, et al.** In older men, higher plasma testosterone or dihydrotestosterone is an independent predictor for reduced incidence of stroke but not myocardial infarction. J Clin Endocrinol Metab. 2014;99:4565-4573. [PMID: 25268392] doi:10.1210/jc.2014-2664
17. **Lincoff AM, Bhasin S, Flevaris P, et al; TRAVERSE Study Investigators.** Cardiovascular safety of testosterone-replacement therapy. N Engl J Med. 2023;389:107-117. [PMID: 37326322] doi:10.1056/NEJMoa2215025
18. **Yeap BB, Marriott RJ, Adams RJ, et al.** Androgens In Men Study (AIMS): protocol for meta-analyses of individual participant data investigating associations of androgens with health outcomes in men. BMJ Open. 2020;10:e034777. [PMID: 32398333] doi:10.1136/bmjopen-2019-034777
19. **Marriott RJ, Harse J, Murray K, et al.** Systematic review and meta-analyses on associations of endogenous testosterone concentration with health outcomes in community-dwelling men. BMJ Open. 2021;11:e048013. [PMID: 34728442] doi:10.1136/bmjopen-2020-048013
20. **Wright JD, Folsom AR, Coresh J, et al.** The ARIC (Atherosclerosis Risk In Communities) Study: JACC Focus Seminar 3/8. J Am Coll Cardiol. 2021;77:2939-2959. [PMID: 34112321] doi:10.1016/j.jacc.2021.04.035
21. **Knuiman MW, Jamrozik K, Welborn TA, et al.** Age and secular trends in risk factors for cardiovascular disease in Busselton. Aust J Public Health. 1995;19:375-382. [PMID: 7578538] doi:10.1111/j.1753-6405.1995.tb00389.x
22. **Fried LP, Borhani NO, Enright P, et al.** The Cardiovascular Health Study: design and rationale. Ann Epidemiol. 1991;1:263-276. [PMID: 1669507] doi:10.1016/1047-2797(91)90005-w
23. **Shores MM, Biggs ML, Arnold AM, et al.** Testosterone, dihydrotestosterone, and incident cardiovascular disease and mortality in the Cardiovascular Health Study. J Clin Endocrinol Metab. 2014;99: 2061-2068. [PMID: 24628549] doi:10.1210/jc.2013-3576
24. **Lee DM, O'Neill TW, Pye SR, et al; EMAS study group.** The European Male Ageing Study (EMAS): design, methods and recruitment. Int J Androl. 2009;32:11-24. [PMID: 18328041] doi:10.1111/j.1365-2605.2008.00879.x
25. **Kannel WB, Feinleib M, McNamara PM, et al.** An investigation of coronary heart disease in families. The Framingham Offspring Study. Am J Epidemiol. 1979;110:281-290. [PMID: 474565] doi:10.1093/oxfordjournals.aje.a112813
26. **Splansky GL, Corey D, Yang Q, et al.** The Third Generation Cohort of the National Heart, Lung, and Blood Institute's Framingham Heart Study: design, recruitment, and initial examination. Am J Epidemiol. 2007;165:1328-1335. [PMID: 17372189] doi:10.1093/aje/kwm021
27. **Norman PE, Flicker L, Almeida OP, et al.** Cohort profile: the Health In Men Study (HIMS). Int J Epidemiol. 2009;38:48-52. [PMID: 18316347] doi:10.1093/ije/dyn041
28. **Grant JF, Martin SA, Taylor AW, et al.** Cohort profile: the Men Androgen Inflammation Lifestyle Environment and Stress (MAILES) study. Int J Epidemiol. 2014;43:1040-1053. [PMID: 23785097] doi:10.1093/ije/dyt064
29. **Orwoll E, Blank JB, Barrett-Connor E, et al.** Design and baseline characteristics of the Osteoporotic Fractures in Men (MrOS) Study–a large observational study of the determinants of fracture in older men. Contemp Clin Trials. 2005;26:569-585. [PMID: 16084776] doi:10.1016/j.cct.2005.05.006
30. **Blank JB, Cawthon PM, Carrion-Petersen ML, et al.** Overview of recruitment for the Osteoporotic Fractures in Men Study (MrOS). Contemp Clin Trials. 2005;26:557-568. [PMID: 16085466] doi:10.1016/j.cct.2005.05.005
31. **Blackwell T, Yaffe K, Ancoli-Israel S, et al; Osteoporotic Fractures in Men (MrOS) Study Group.** Association of sleep characteristics and cognition in older community-dwelling men: the MrOS Sleep Study. Sleep. 2011;34:1347-1356. [PMID: 21966066] doi:10.5665/SLEEP.1276
32. **Völzke H, Alte D, Schmidt CO, et al.** Cohort profile: the Study of Health in Pomerania. Int J Epidemiol. 2011;40:294-307. [PMID: 20167617] doi:10.1093/ije/dyp394
33. **Cumming RG, Handelsman D, Seibel MJ, et al.** Cohort profile: the Concord Health and Ageing in Men Project (CHAMP). Int J Epidemiol. 2009;38:374-378. [PMID: 18480109] doi:10.1093/ije/dyn071
34. **Mellström D, Johnell O, Ljunggren Ö, et al.** Free testosterone is an independent predictor of BMD and prevalent fractures in elderly men: MrOS Sweden. J Bone Miner Res. 2006;21:529-535. [PMID: 16598372] doi:10.1359/jbmr.060110
35. **Borenstein M, Hedges LV, Higgins JPT, et al.** Introduction to Meta-analysis. J Wiley; 2009.
36. **Riley RD, Tierney JF, Steward LA.** Individual Participant Meta-Analysis: A Handbook for Healthcare Research. J Wiley; 2021.
37. **Lewington S, Whitlock G, Clarke R, et al; Prospective Studies Collaboration.** Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. Lancet. 2007;370:1829-1839. [PMID: 18061058] doi:10.1016/S0140-6736(07)61778-4
38. **Bartlett J, Keogh R, Bonneville EF, et al.** smcfcs: Multiple Imputation of Covariates by Substantive Model Compatible Fully Conditional Specification. R package version 1.6.1. 2022. Accessed at https://CRAN.R-project.org/package=smcfcs on 2 April 2024.
39. **Rubin DB.** Multiple Imputation for Nonresponse in Surveys. Wiley; 1987.
40. **Higgins JPT, Thompson SG.** Quantifying heterogeneity in a meta-analysis. Stat Med. 2002;21:1539-1558. [PMID: 12111919] doi:10.1002/sim.1186
41. **Ahern T, Swiecicka A, Eendebak RJAH, et al; EMAS study group.** Natural history, risk factors and clinical features of primary hypogonadism in ageing men: Longitudinal Data from the European Male Ageing Study. Clin Endocrinol (Oxf). 2016;85:891-901. [PMID: 27374987] doi:10.1111/cen.13152
42. **Araujo AB, Kupelian V, Page ST, et al.** Sex steroids and all-cause and cause-specific mortality in men. Arch Intern Med. 2007;167:1252-1260. [PMID: 17592098] doi:10.1001/archinte.167.12.1252
43. **Vikan T, Schirmer H, Njølstad I, et al.** Endogenous sex hormones and the prospective association with cardiovascular disease and mortality in men: the Tromsø Study. Eur J Endocrinol. 2009;161:435-442. [PMID: 19542243] doi:10.1530/EJE-09-0284
44. **Menke A, Guallar E, Rohrmann S, et al.** Sex steroid hormone concentrations and risk of death in US men. Am J Epidemiol. 2010;171:583-592. [PMID: 20083549] doi:10.1093/aje/kwp415
45. **Haring R, Teng Z, Xanthakis V, et al.** Association of sex steroids, gonadotrophins, and their trajectories with clinical cardiovascular disease and all-cause mortality in elderly men from the Framingham

Heart Study. Clin Endocrinol (Oxf). 2013;78:629-634. [PMID: 22901104] doi:10.1111/cen.12013
46. **Holmboe SA, Vradi E, Jensen TK, et al.** The association of reproductive hormone levels and all-cause, cancer, and cardiovascular disease mortality in men. J Clin Endocrinol Metab. 2015;100:4472-4480. [PMID: 26488309] doi:10.1210/jc.2015-2460
47. **Shores MM, Matsumoto AM, Sloan KL, et al.** Low serum testosterone and mortality in male veterans. Arch Intern Med. 2006;166:1660-1665. [PMID: 16908801] doi:10.1001/archinte.166.15.1660
48. **Khaw K-T, Dowsett M, Folkerd E, et al.** Endogenous testosterone and mortality due to all causes, cardiovascular disease, and cancer in men: European prospective investigation into cancer in Norfolk (EPIC-Norfolk) Prospective Population Study. Circulation. 2007;116:2694-2701. [PMID: 18040028] doi:10.1161/CIRCULATIONAHA.107.719005
49. **Laughlin GA, Barrett-Connor E, Bergstrom J.** Low serum testosterone and mortality in older men. J Clin Endocrinol Metab. 2008;93:68-75. [PMID: 17911176] doi:10.1210/jc.2007-1792
50. **Haring R, Völzke H, Steveling A, et al.** Low serum testosterone levels are associated with increased risk of mortality in a population-based cohort of men aged 20–79. Eur Heart J. 2010;31:1494-1501. [PMID: 20164245] doi:10.1093/eurheartj/ehq009
51. **Schederecker F, Cecil A, Prehn C, et al.** Sex hormone-binding globulin, androgens and mortality: the KORA-F4 cohort study. Endocr Connect. 2020;9:326-336. [PMID: 32168474] doi:10.1530/EC-20-0080
52. **Pye SR, Huhtaniemi IT, Finn JD, et al; EMAS Study Group.** Late-onset hypogonadism and mortality in aging men. J Clin Endocrinol Metab. 2014;99:1357-1366. [PMID: 24423283] doi:10.1210/jc.2013-2052
53. **Srinath R, Golden SH, Carson KA, et al.** Endogenous testosterone and its relationship to preclinical and clinical measures of cardiovascular disease in the Atherosclerosis Risk In Communities Study. J Clin Endocrinol Metab. 2015;100:1602-1608. [PMID: 25584720] doi:10.1210/jc.2014-3934
54. **Chan YX, Knuiman MW, Hung J, et al.** Neutral associations of testosterone, dihydrotestosterone and estradiol with fatal and non-fatal cardiovascular events, and mortality in men aged 17–97 years. Clin Endocrinol (Oxf). 2016;85:575-582. [PMID: 27106765] doi:10.1111/cen.13089
55. **Tivesten A, Vandenput L, Labrie F, et al.** Low serum testosterone and estradiol predict mortality in elderly men. J Clin Endocrinol Metab. 2009;94:2482-2488. [PMID: 19401373] doi:10.1210/jc.2008-2650
56. **Yeap BB, Alfonso H, Chubb SAP, et al.** In older men an optimal plasma testosterone is associated with reduced all-cause mortality and higher dihydrotestosterone with reduced ischemic heart disease mortality, while estradiol levels do not predict mortality. J Clin Endocrinol Metab. 2014;99:E9-18. [PMID: 24257908] doi:10.1210/jc.2013-3272
57. **Hsu B, Cumming RG, Naganathan V, et al.** Temporal changes in androgens and estrogens are associated with all-cause and cause-specific mortality in older men. J Clin Endocrinol Metab. 2016;101:2201-2210. [PMID: 26963953] doi:10.1210/jc.2016-1025
58. **Rosner W, Hryb DJ, Khan MS, et al.** Sex hormone-binding globulin mediates steroid hormone signal transduction at the plasma membrane. J Steroid Biochem Mol Biol. 1999;69:481-485. [PMID: 10419028] doi:10.1016/s0960-0760(99)00070-9
59. **Rosner W, Hryb DJ, Kahn SM, et al.** Interactions of sex hormone-binding globulin with target cells. Mol Cell Endocrinol. 2010;316:79-85. [PMID: 19698759] doi:10.1016/j.mce.2009.08.009
60. **Simó R, Sáez-López C, Barbosa-Desongles A, et al.** Novel insights in SHBG regulation and clinical implications. Trends Endocrinol Metab. 2015;26:376-383. [PMID: 26044465] doi:10.1016/j.tem.2015.05.001
61. **Hyde Z, Norman PE, Flicker L, et al.** Low free testosterone predicts mortality from cardiovascular disease but not other causes: the Health In Men Study. J Clin Endocrinol Metab. 2012;97:179-189. [PMID: 22013106] doi:10.1210/jc.2011-1617
62. **Kalme T, Seppälä M, Qiao Q, et al.** Sex hormone-binding globulin and insulin-like growth factor-binding protein-1 as indicators of metabolic syndrome, cardiovascular risk, and mortality in elderly men. J Clin Endocrinol Metab. 2005;90:1550-1556. [PMID: 15613437] doi:10.1210/jc.2004-0762
63. **Wang A, Arver S, Boman K, et al.** Testosterone, sex hormone-binding globulin and risk of cardiovascular events: a report from the Outcome Reduction with an Initial Glargine Intervention trial. Eur J Prev Cardiol. 2019;26:847-854. [PMID: 30567457] doi:10.1177/2047487318819142

Author Contributions: Conception and design: B.B. Yeap, R.J. Marriott, G. Dwivedi, A.S. Dobs, L. Flicker, G.J. Hankey, A.M. Matsumoto, C. Ohlsson, E.S. Orwoll, G.A. Wittert, K. Murray.
Analysis and interpretation of the data: B.B. Yeap, R.J. Marriott, G. Dwivedi, R.J. Adams, L. Antonio, A.S. Dobs, S.A. Martin, A.M. Matsumoto, D. Mellström, C. Ohlsson, M. Quartagno, T.G. Travison, D. Vanderschueren, G.A. Wittert, K. Murray.
Drafting of the article: B.B. Yeap, R.J. Marriott, G. Dwivedi, R.J. Adams, A.S. Dobs, D.J. Handelsman, B. Hsu, A.M. Matsumoto.
Critical revision for important intellectual content: B.B. Yeap, R. J. Marriott, G. Dwivedi, R.J. Adams, D.C. Bauer, S. Bhasin, M.L. Biggs, P.M. Cawthon, D.J. Couper, L. Flicker, D.J. Handelsman, G.J. Hankey, A. Hannemann, R. Haring, S.A. Martin, A.M. Matsumoto, D. Mellström, C. Ohlsson, T.W. O'Neill, E.S. Orwoll, M. Quartagno, A. Steveling, Å. Tivesten, D. Vanderschueren, G. A. Wittert, F.C.W. Wu, K. Murray.
Final approval of the article: B.B. Yeap, R.J. Marriott, G. Dwivedi, R.J. Adams, L. Antonio, C.M. Ballantyne, D.C. Bauer, S. Bhasin, M.L. Biggs, P.M. Cawthon, D.J. Couper, A.S. Dobs, L. Flicker, D. J. Handelsman, G.J. Hankey, A. Hannemann, R. Haring, B. Hsu, S.A. Martin, A.M. Matsumoto, D. Mellström, C. Ohlsson, T.W. O'Neill, E.S. Orwoll, M. Quartagno, M.M. Shores, A. Steveling, Å. Tivesten, T.G. Travison, D. Vanderschueren, G.A. Wittert, F.C. W. Wu, K. Murray.
Provision of study materials or patients: P.M. Cawthon, D.J. Handelsman, R. Haring, B. Hsu, A.M. Matsumoto, T.W. O'Neill, E.S. Orwoll, D. Vanderschueren, F.C.W. Wu.
Statistical expertise: R.J. Marriott, M. Quartagno, T.G. Travison, K. Murray.
Obtaining of funding: B.B. Yeap, P.M. Cawthon, G.J. Hankey.
Administrative, technical, or logistic support: R.J. Marriott, M.L. Biggs, P.M. Cawthon, D.J. Handelsman.
Collection and assembly of data: B.B. Yeap, R.J. Marriott, R.J. Adams, L. Antonio, M.L. Biggs, P.M. Cawthon, D.J. Couper, L. Flicker, A. Hannemann, B. Hsu, S.A. Martin, A.M. Matsumoto, D. Mellström, M.M. Shores, Å. Tivesten, G.A. Wittert.

Appendix: Additional Details on Analyses

Methods

Data Extraction

Individual participant data were requested from each individual study after approvals had been granted by the respective institutional review boards (18, 19). Once prepared, IPD were securely sent, stored in a central repository, and checked, and baseline variables from different data sets were harmonized by being converted into a consistent format (categorical variables) or unit of measurement (continuous variables). Primary health outcomes of interest were all-cause mortality, death caused by CVD, and incident CVD events. Deaths caused by CVD were defined as deaths from MI, cardiac arrest, HF, or stroke, and incident CVD events were defined as occurrence of fatal or nonfatal MI, HF, or stroke. Whenever a study routinely used existing definitions for an outcome (such as for adjudicated events), those definitions were used; otherwise, they were derived from supplied IPD using ICD codes (Supplement Table 2).

Quality Assessment and Risk of Bias

A description of the literature search and selection of studies, which was conducted by 2 independent investigators with discussion to resolve disagreements, has been published (19). The Newcastle-Ottawa Quality Assessment Scale for Cohort Studies was used to assess selected studies for risk of bias (see Supplementary Table 8 in the previously published systematic review [19]). For the criterion of participant selection (representativeness of the exposed cohort, selection of the nonexposed cohort, ascertainment of exposure, and demonstration that the outcome of interest was not present at the start of the study), the quality of the selected studies ranged from 3 to 4 stars out of a maximum of 4. For the criterion of comparability (based on the study design or analysis), all of the selected studies rated 2 stars (the maximum score). For the criterion of outcome (method of assessment, adequate period of follow-up, adequacy of follow-up), the selected studies rated 2 or 3 stars out of a maximum of 3. Overall, the studies were generally of high quality and had low risk of bias. This reflected the nature of the studies, which were population-based, used an accurate method for measuring testosterone, adjusted for participant age and other risk factors, and had at least 5 years of follow-up for relevant health outcomes (19).

Exclusions

Men with a history of orchidectomy were excluded because we were unable to distinguish those who had unilateral surgery (such as those who had a single testis removed due to testicular cancer or trauma and may or may not have had preserved Leydig cell function from the remaining testis) versus bilateral surgery (such as those who might have had surgical castration for treatment of prostate cancer). Men with a known diagnosis of severe hypogonadism due to hypothalamus–pituitary–gonadal axis pathology would likely have been treated with testosterone, and men reporting use of androgens were excluded. We excluded men using androgens or antiandrogens, as these might have affected circulating testosterone concentrations or interfered with effects of testosterone on the outcomes of interest.

Missing Data

Within each study, the percentage of incomplete cases ranged from 0.5% to 47.2%, and within variables (across all IPD), the percentage ranged from 0% to 6.9% (Supplement Table 3). The total number lost to follow-up or with undetermined event status was 419 for mortality analyses and 16 for CVD analyses, representing 2.0% and 0.1% of IPD, respectively, after all other exclusions (Supplement Tables 3A to 3C).

Additional Analyses

Additional analyses explored sensitivity to inclusion of additional predictors not available in all IPD-level data sets, sensitivity to inclusion of AD from the 2 studies that did not supply IPD, sensitivity to extended follow-up of vital status for most but not all EMAS (European Male Ageing Study) participants, associations with specific types of CVD events (HF, MI, and stroke), and whether

associations of testosterone with all-cause mortality risk were influenced by SHBG or LH concentrations (Supplement Methods).

Results

Analysis Population

Nine studies provided IPD with follow-up on all-cause and/or CVD deaths (deaths due to MI, cardiac arrest, HF, or stroke) (20–32), and 2 other studies provided AD statistics (33, 34) (Appendix Table). After exclusion of men with missing testosterone measurements ($n = 6547$), those with prior orchidectomy ($n = 61$), those using androgens or antiandrogens ($n = 244$), those who were lost to follow-up ($n = 394$), and those with undetermined event status ($n = 25$), there were IPD for 20 654 men for mortality analyses (Appendix Figure 1), comprising 255 830 participant-years of risk, with 7241 deaths and 1351 CVD deaths. Aggregate data statistics were provided for 3455 men, with 1099 deaths and 326 CVD deaths. Seven studies provided IPD with follow-up on incident CVD events (occurrence of fatal or nonfatal HF, MI, or stroke). After the same exclusions were applied and men with prior CVD were excluded, there were IPD for 12 829 men for CVD events analyses (Appendix Figure 1), comprising 158 445 participant-years of risk and 2803 incident CVD events, and AD for another 1956 men, with 326 CVD events.

Other Analyses

Supplementary analyses controlled for additional covariates that were not available in all of the data sets, with results largely consistent with those of the main analyses (Supplement Results and Supplement Figures 7 to 15). The estimated risk for HF was higher in men with very low baseline testosterone concentrations (<3.2 nmol/L). The estimated risk for stroke was higher in men with Q1 median testosterone concentrations (8.46 nmol/L) relative to the Q5 median (fully adjusted HR, 1.49 [CI, 1.04 to 2.15]). There was no association of baseline testosterone with risk for MI and no association of SHBG with these outcomes (Supplement Results and Supplement Figures 16 and 17).

Appendix Table. Summary Attributes: Cross-Sectional Data at Baseline, Numbers of Outcome Events, and Length of Follow-up, by Study

Study (Reference)*	Location	Participants, *n*	Median Age (IQR), *y*	Median Testosterone Concentration (IQR), *nmol/L*	Outcome	Events, *n*	Median Follow-up (IQR), *y*
Mortality analyses†							
ARIC (20)	United States	1556	63.0 (58.0–68.0)	13.1 (10.0–16.6)	All-cause	696	20.7 (19.9–21.4)
					Cardiovascular	70	19.7 (14.9–20.9)
BHS (21)	Australia	2020	49.9 (37.6–64.8)	13.0 (10.1–16.6)	All-cause	476	19.6 (18.9–19.6)
					Cardiovascular	110	19.6 (18.6–19.6)
CHS (22, 23)	United States	1123	76.0 (73.3–79.9)	12.7 (9.5–16.5)	All-cause	997	20.7 (20.5–20.9)
					Cardiovascular	214	12.4 (7.2–18.4)
EMAS (24)	Europe	2445	59.1 (50.3–68.7)	16.1 (12.6–20.3)	All-cause	169	4.3 (4.1–4.6)
					Cardiovascular	69	4.3 (4.1–4.6)
EMAS subset‡	Europe	1742	59.1 (50.4–68.7)	16.5 (12.9–20.7)	All-cause	411	14.0 (13.5–14.2)
FHS (25, 26)	United States	3329	49.0 (39.0–59.0)	20.4 (15.7–26.2)	All-cause	645	16.9 (15.8–19.6)
					Cardiovascular	163	16.6 (15.4–19.2)
HIMS (27)	Australia	4121	76.0 (74.0–79.0)	12.4 (9.5–15.6)	All-cause	2368	13.3 (12.7–14.1)
					Cardiovascular	425	12.5 (8.1–13.3)
MAILES (28)	Australia	1975	55.0 (46.0–64.0)	16.5 (12.8–20.6)	All-cause	138	9.8 (9.4–11.1)
					Cardiovascular	42	9.7 (9.3–11)
MrOS USA (29–31)	United States	1976	73.0 (69.0–77.0)	13.4 (10.3–17.0)	All-cause	1162	17.7 (17.2–18.3)
					Cardiovascular	149	14.7 (8.8–17.5)
SHIP (32)§	Germany	2109	51.0 (37.0–64.0)	15.4 (11.9–19.6)	All-cause	590	19.5 (18.2–20.5)
					Cardiovascular	109	19.0 (9.9–20.1)
CHAMP (33)	Australia	1526	76.0 (72.0–80.0)	14.3 (11.1–18.4)	All-cause	774	11.8 (11.3–12.4)
					Cardiovascular	202	9.3 (8.5–10.1)
MrOS Sweden (34)	Sweden	1929	75.3 (72.8–78.3)	15.4 (11.9–19.3)	All-cause	325	6.5 (5.9–7.1)
					Cardiovascular	124	6.4 (5.8–7.0)
Incident CVD events‖							
ARIC (20)	United States	1502	63.0 (58.0–68.0)	13.1 (10.0–16.7)	CVD	450	19.9 (16.6–21.1)
BHS (21)	Australia	1908	48.7 (36.8–63.2)	13.0 (10.2–16.6)	CVD	303	19.6 (18.7–19.6)
CHS (22, 23)	United States	1123	76.0 (73.3–79.9)	12.7 (9.5–16.5)	CVD	568	14.0 (8.4–20.2)
FHS (25, 26)	United States	3016	47.0 (38.0–57.0)	20.6 (15.9–26.6)	CVD	413	15.9 (13.9–17.4)
HIMS (27)	Australia	2718	76.0 (74.0–78.0)	12.6 (9.7–15.8)	CVD	734	12.7 (9.9–13.5)
MAILES (28)	Australia	1770	54.0 (45.0–63.0)	16.6 (13.0–20.8)	CVD	115	9.8 (9.4–11.0)
MrOS USA (29–31)	United States	792	75.0 (71.0–79.0)	13.6 (10.4–17.3)	CVD	220	10.4 (9.6–10.6)¶
CHAMP (33)	Australia	488	76.0 (72.0–79.0)	15.6 (11.6–19.7)	CVD	114	11.3 (9.4–12.2)
MrOS Sweden (34)	Sweden	1468	75.3 (72.7–78.1)	15.8 (12.4–19.7)	CVD	212	5.5 (4.8–6.0)

ARIC = Atherosclerosis Risk in Communities Study; BHS = Busselton Health Study; CHAMP = Concord Health and Ageing in Men Project; CHS = Cardiovascular Health Study; CVD = cardiovascular disease; EMAS = European Male Ageing Study; FHS = Framingham Heart Study; HIMS = Health In Men Study; IPD = individual participant data; MAILES = Men Androgen Inflammation Lifestyle Environment and Stress study; MrOS = Osteoporotic Fractures in Men; SHIP = Study of Health in Pomerania.

* All studies supplied IPD-level data except CHAMP and MrOS Sweden, which supplied aggregate-level statistics.

† Among men for whom mass spectrometry measurements of testosterone were available after exclusions and losses to follow-up.

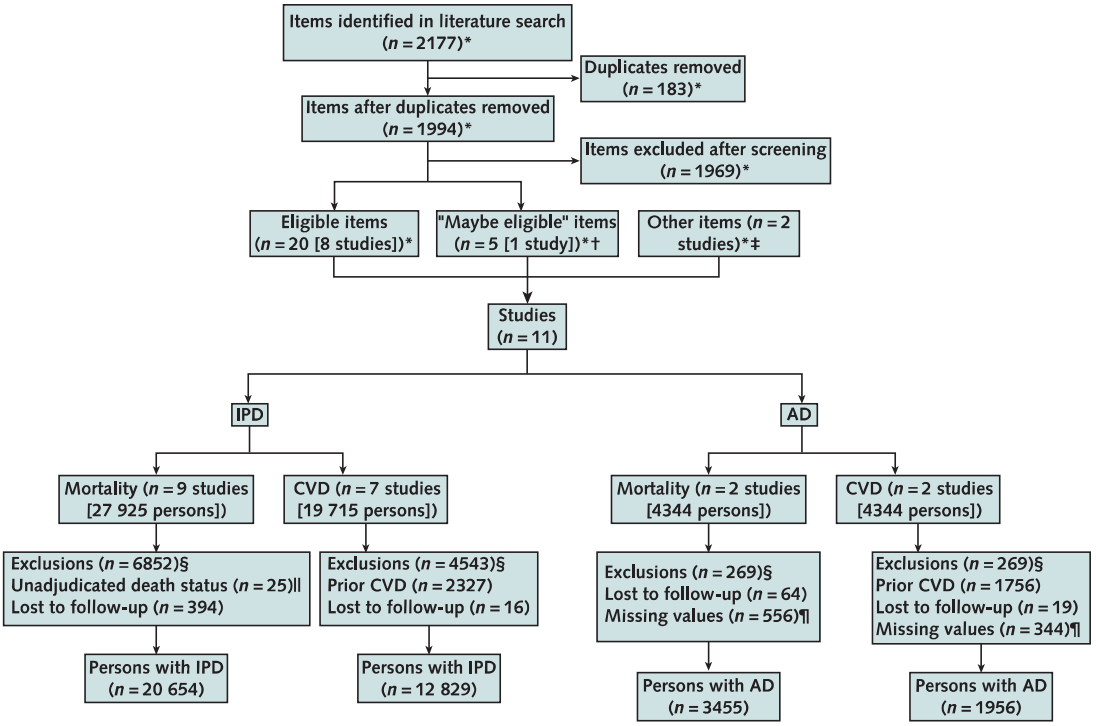
‡ Extended follow-up of deaths from any cause was available for a subset of EMAS participants, excluding those from the Manchester and Szeged cohorts.

§ Data are from the SHIP-0 and SHIP-TREND cohorts.

‖ Participants with a history of CVD were excluded from analyses of incident CVD events.

¶ Follow-up of adjudicated cardiovascular events was from the MrOS Sleep Study visit (December 2003 to March 2005) until 28 February 2015, whereas follow-up of deaths in the MrOS USA study was from the initial baseline (March 2000 to April 2002) until August 2019. A small number of participants ($n = 25$) were excluded from mortality analyses due to unadjudicated death status.

Appendix Figure 1. Flowchart identifying studies selected from the systematic review and the IPD and AD obtained from those studies.



AD = aggregate data; CVD = cardiovascular disease; IPD = individual participant data; MrOS = Osteoporotic Fractures in Men.

* Further details on systematic literature searches and screening and selection of items (journal articles, reports, theses, webpage articles) and a PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) flowchart for the systematic review are provided in the article by Marriott and colleagues (19).

† Items identified as "maybe eligible" at completion of systematic screening were further investigated using information external to the systematic review, resulting in identification of 1 additional eligible study with IPD-level data.

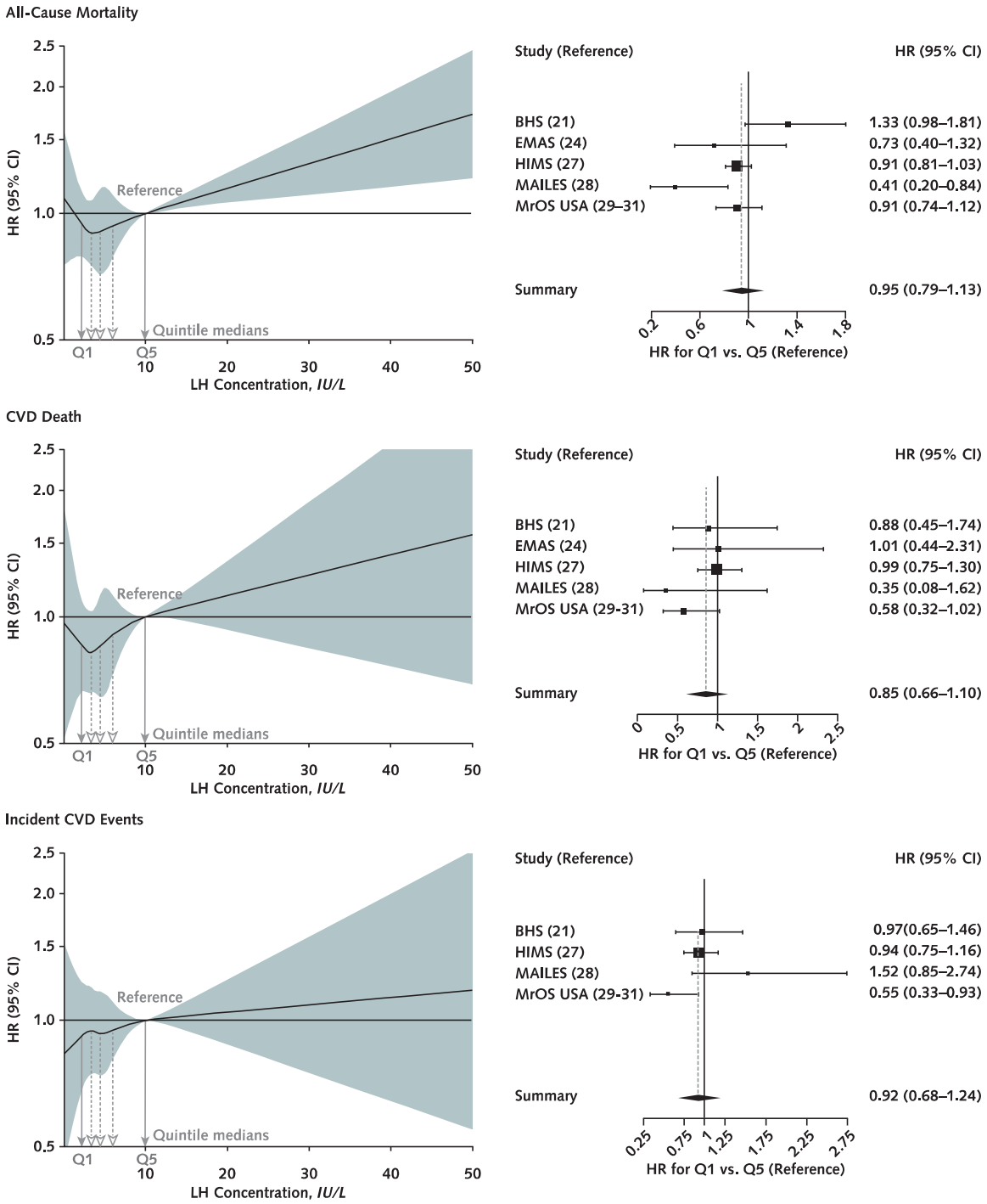
‡ Additional studies were identified through known contacts of Marriott and colleagues (18).

§ For analyses of testosterone associations, data were excluded for participants who were missing measurements of total testosterone made using mass spectrometry at baseline, had a history of orchidectomy, were using androgen or antiandrogen medications, or were lost to follow-up.

‖ Participants with unadjudicated death status were excluded from the longer follow-up of mortality outcomes, as at the latest date of data provision by the MrOS USA study.

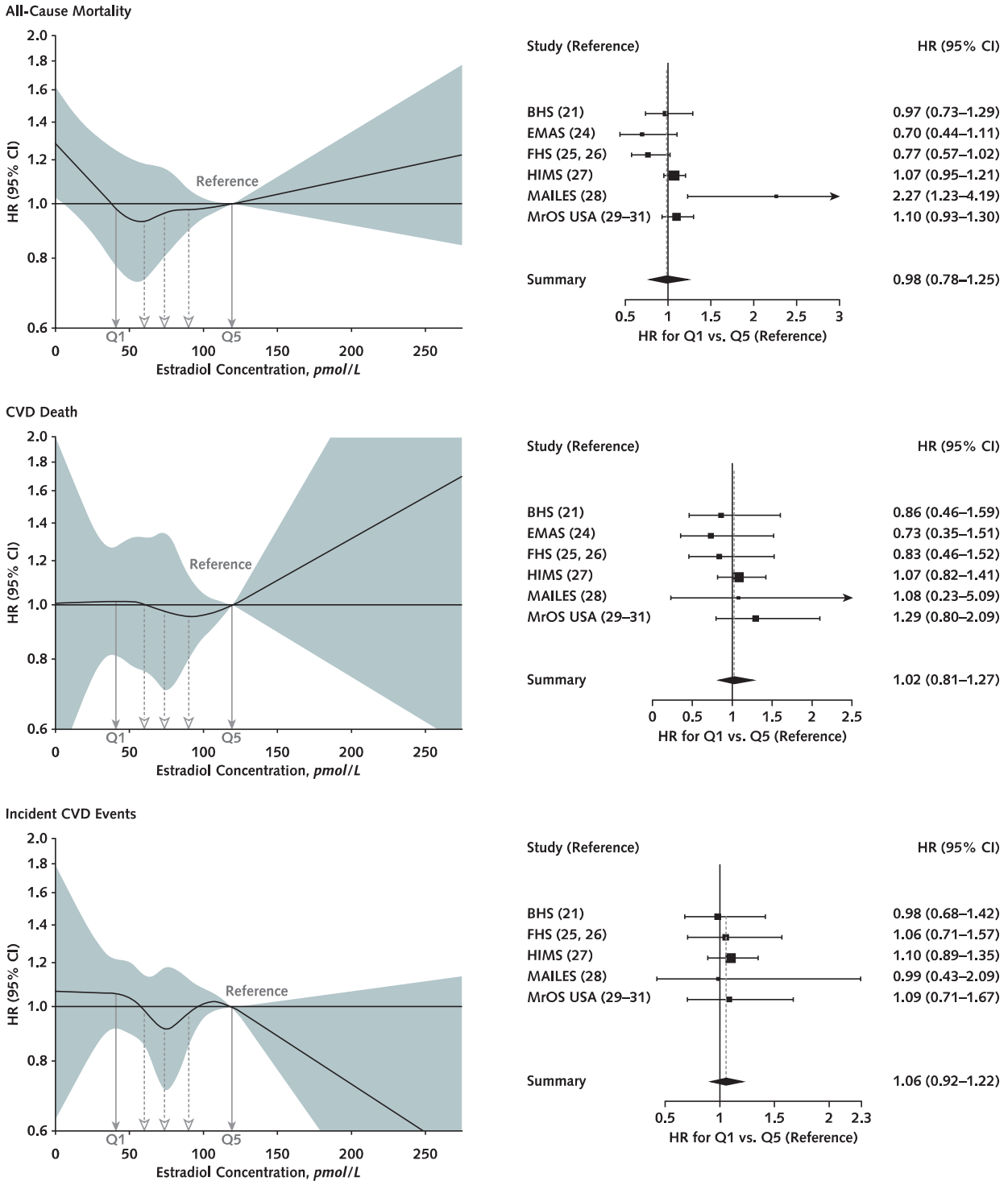
¶ AD were included from analyses of complete-cases data (missing values excluded).

Appendix Figure 2. Summary curves and forest plots for the association of baseline LH concentration with relative risk for all-cause mortality (*top*), CVD death (*middle*), and incident CVD events (*bottom*): model 2.



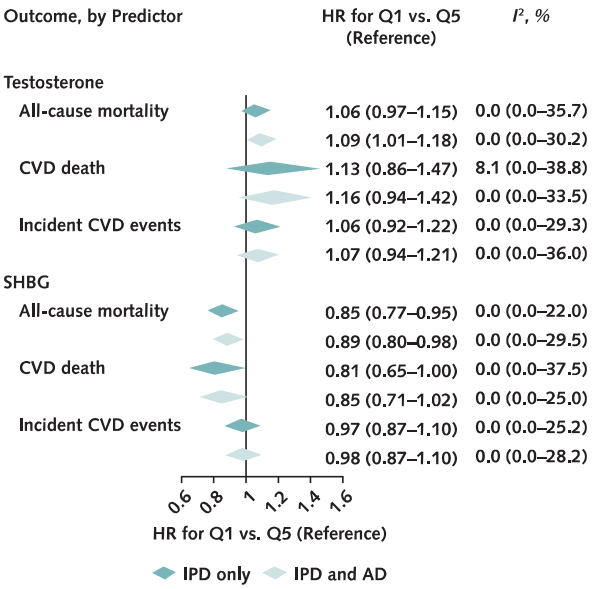
Estimates are controlled for baseline age, body mass index, marital status, alcohol consumption, smoking, physical activity, ratio of total to high-density lipoprotein cholesterol, blood creatinine concentration, lipid medication use, hypertension, and diabetes. Estimates for all-cause mortality are also controlled for history of cancer at baseline. BHS = Busselton Health Study; CVD = cardiovascular disease; EMAS = European Male Ageing Study; HIMS = Health In Men Study; HR = hazard ratio; LH = luteinizing hormone; MAILES = Men Androgen Inflammation Lifestyle Environment and Stress; MrOS = Osteoporotic Fractures in Men; Q = quintile.

Appendix Figure 3. Summary curves and forest plots for the association of baseline estradiol concentration with relative risk for all-cause mortality (*top*), CVD death (*middle*), and incident CVD events (*bottom*): model 2.



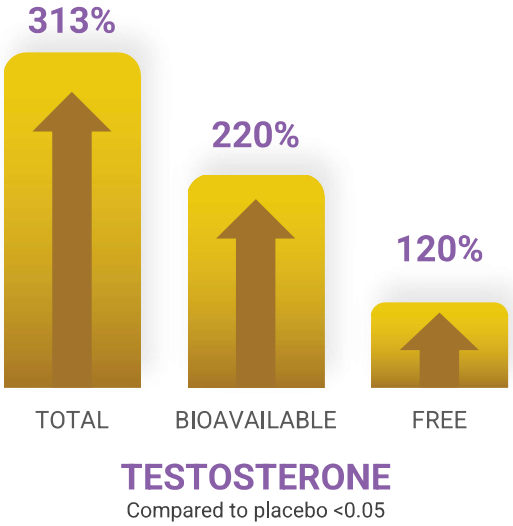
Estimates are controlled for baseline age, body mass index, marital status, alcohol consumption, smoking, physical activity, ratio of total to high-density lipoprotein cholesterol, blood creatinine concentration, lipid medication use, hypertension, and diabetes. Estimates for all-cause mortality are also controlled for history of cancer at baseline. BHS = Busselton Health Study; CVD = cardiovascular disease; EMAS = European Male Ageing Study; FHS = Framingham Heart Study; HIMS = Health In Men Study; HR = hazard ratio; MAILES = Men Androgen Inflammation Lifestyle Environment and Stress; MrOS = Osteoporotic Fractures in Men; Q = quintile.

Appendix Figure 4. Sensitivity of summary estimates (IPD only) to the inclusion of aggregate-level data (IPD and AD) provided by 2 additional studies (CHAMP and MrOS Sweden).



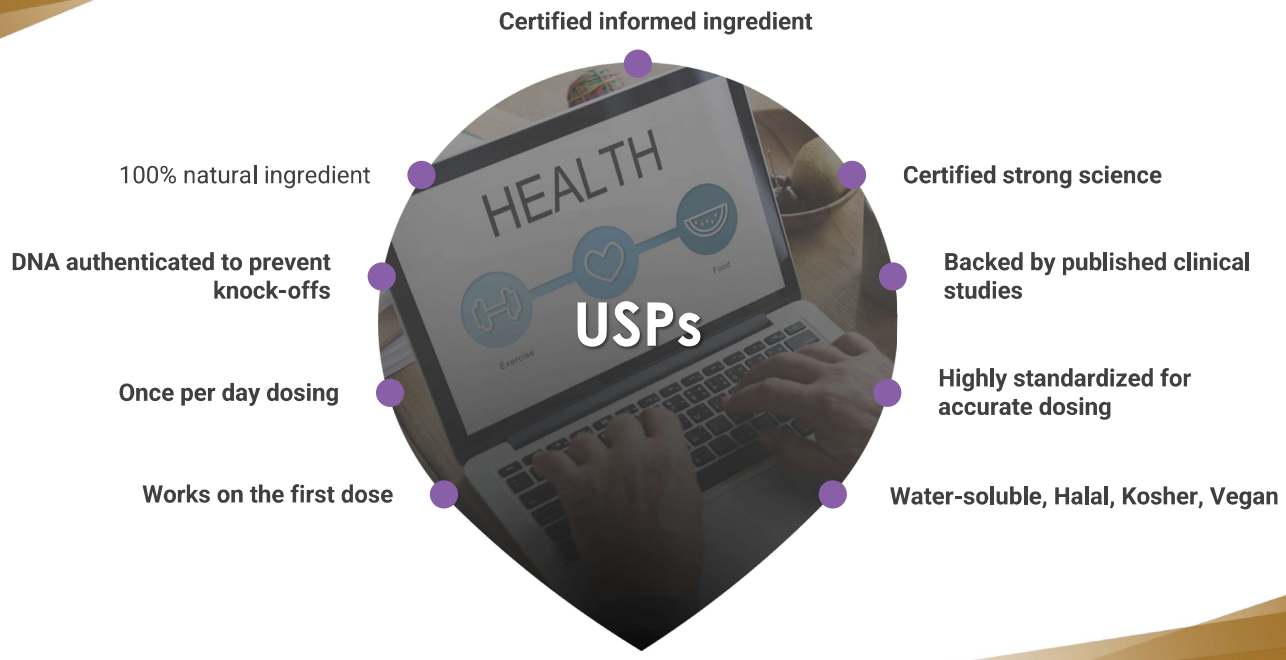
AD = aggregate data; CHAMP = Concord Health and Ageing in Men Project; CVD = cardiovascular disease; HR = hazard ratio; IPD = individual participant data; MrOS = Osteoporotic Fractures in Men; Q = quintile; SHBG = sex hormone–binding globulin.

Increases Testosterone After 10 Hours
TESTOSTERONE VS PLACEBO



Effects of Single dose TESTOSURGE on serum testosterone levels of healthy sedentary male subjects

Outcome: *Single dose of TESTOSURGE significantly increased Free Testosterone, Bioavailable Testosterone, and Total Testosterone Levels after 10 hours of administered*



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