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Testosterone concentrations, using different assays, in different types of ovarian insufficiency: a systematic review and meta-analysis

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BACKGROUND: Increasing age and post-menopausal status are associated with decreasing androgen concentrations in females. Women with premature loss of ovarian function, such as primary ovarian insufficiency (POI) or iatrogenic menopause may be at increased risk for diminished testosterone levels at a relatively young age. Differentiation between a hypoandrogenic or normoandrogenic state in women with premature loss of ovarian function is problematic due to trueness and precision problems using various testosterone assays. The current meta-analysis was conducted to evaluate current literature reporting serum total testosterone concentrations under these conditions, including stratification for various testosterone assays.

METHODS: A systematic review and meta-analysis of controlled observational studies were performed. The electronic databases of Pubmed, Embase and the Cochrane Library were systematically searched until October 2011 for comparative studies on total testosterone concentrations in women with spontaneous POI or iatrogenic menopause compared with controls. The literature search, data extraction and critical appraisal, using the Newcastle–Ottawa Scale, were performed by two independent investigators. The effect measure was the weighted mean difference (WMD) with 95% confidence interval (95% CI) in a random effects model.

RESULTS: A total of 206 articles for spontaneous POI and 1358 for iatrogenic menopause were reviewed, of which 9 and 17 papers, respectively, were selected for final analysis. Both groups demonstrated significantly lower total testosterone concentrations compared with controls [WMD (95% CI) −0.38 (−0.55 to −0.22) nmol/l, and −0.29 (−0.39 to −0.18) nmol/l, respectively], but with substantial

between-study heterogeneity. Subgroup analysis for assay type was statistically significant for spontaneous POI only. Sensitivity analyses of high-quality studies did not change the results, and resulted in a substantial decrease in heterogeneity in spontaneous POI studies.

CONCLUSIONS: The current meta-analysis demonstrates that total testosterone concentrations are decreased in women with spontaneous POI or iatrogenic menopause. The potential implications of hypoandrogenism in these women remain to be elucidated.

Key words: testosterone / androgen / primary ovarian insufficiency / iatrogenic menopause / surgical menopause

Introduction

While spontaneous menopause typically occurs around the age of 51 years (Treloar, 1981), ~1–2% of women experience menopause before the age of 40 years (Coulam *et al.*, 1986). Spontaneous premature cessation of ovarian function, currently known as primary ovarian insufficiency (POI) or premature ovarian failure (POF), is characterized by amenorrhea for at least 4 months along with repeatedly elevated FSH concentrations in the post-menopausal range and a hypo-estrogenic state (Cooper *et al.*, 2010; de Vos *et al.*, 2010). Spontaneous POI may be caused by steroidogenic cell autoimmunity (Bakalov *et al.*, 2005), numerical or structural chromosomal abnormalities, such as monosomy X (Mattei *et al.*, 1982), or monogenic causes, including the fragile X premutation (Wittenberger *et al.*, 2007) and other single gene mutations (Qin *et al.*, 2007; Rossetti *et al.*, 2009; Janse *et al.*, 2012). In the great majority of women diagnosed with POI, however, the mechanisms underlying premature exhaustion of the ovarian follicle pool remain unknown. Most probably, POI should be regarded as a complex genetic disease in which multiple genetic variants along with environmental factors may play important roles (Knauff *et al.*, 2009).

Another group of post-menopausal women, who do not fit the description of physiologic menopause, are women who experienced menopause due to gonadotoxic treatment (such as chemotherapeutic agents and irradiation), or extensive abdominal surgery and oophorectomy. Because the long-term survival in women treated for cancer has greatly improved during recent decades (Altekruse *et al.*, 2010), the incidence of women with iatrogenic menopause due to either benign or malignant disease has now increased to 3.4–4.5% (Graziottin, 2010).

Spontaneous POI and iatrogenic menopause are associated with infertility, and increased risk of osteoporosis (Yildiz *et al.*, 1996; Uygur *et al.*, 2005), cardiovascular disease (van der Schouw *et al.*, 1996; Kalantaridou *et al.*, 2004; Knauff *et al.*, 2008) and diminished emotional well-being (van der Stege *et al.*, 2008). Most known health risks in women with spontaneous POI and iatrogenic menopause are attributed to the low estrogen concentration in these women.

However, some studies suggest that these women may also be at risk for low testosterone concentrations (Bachmann *et al.*, 2002; Davis, 2002; Rivera-Woll *et al.*, 2004). Until now, it has been debated whether the post-menopausal ovary remains a significant source of androgen production. There is conflicting evidence indicating that hypoandrogenism in post-menopausal women may derive from a gradual decrease in both ovarian and adrenal production of androgens with increasing age (Davison *et al.*, 2005), while others suggest that only ovarian androgen production further decreases during the menopausal transition (Rannevik *et al.*, 1995; Couzinet *et al.*, 2001). Hypoandrogenemia is described in association with the female androgen insufficiency syndrome (FAIS). The Princeton Consensus Statement proposed the following clinical symptoms for the description of FAIS: (i) diminished sense of well-being or dysphoric mood, (ii) persistent, unexplained fatigue and (iii) sexual function changes (Bachmann *et al.*, 2002). Besides FAIS, decreased testosterone concentrations have been associated with multiple general health consequences in peri- and post-menopausal women. These include increased risks of dyslipidemia (Khatibi *et al.*, 2007) and coronary heart disease events (Patel *et al.*, 2009; Laughlin *et al.*, 2010). These associations remain controversial, however, because other studies have found no or even reverse effects on risks of metabolic syndrome (Bell *et al.*, 2006; Janssen *et al.*, 2010) or cardiovascular disease (Kalyani *et al.*, 2009). In contrast, from existing literature, it becomes clear that the lower total testosterone concentrations are associated with increased fracture risk (Lee *et al.*, 2008) and decreased bone density (Rariy *et al.*, 2011). Furthermore, associations between low testosterone concentrations and decreased verbal fluency (Drake *et al.*, 2000), and increased frailty in the elderly (Wu *et al.*, 2010) have been described.

Although some studies (Somboonporn *et al.*, 2005; Traish *et al.*, 2007) and guidelines (North American Menopause Society, 2005) have advocated a beneficial effect of androgen therapy in hypoandrogenic women, the evidence is controversial and the Endocrine Society Clinical Practice Guidelines do not recommend androgen therapy until the physiological role of androgens has become clear and the clinical syndrome of androgen deficiency is better defined (Wierman *et al.*, 2006).

The diagnoses of FAIS and other health risks possibly associated with hypoandrogenemia are complicated by the lack of reliable testosterone assays and the need for age-adjusted reference values (Rosner and Vesper, 2010; Haring *et al.*, 2011). Simple radioimmunoassay (RIA) and chemiluminescence immunoassay are performed directly in serum, but show more bias in the lower range encountered in women, due to increased interference and overestimation of steroid concentrations compared with other assays (Boots *et al.*, 1998; Stanczyk *et al.*, 2003). The addition of extraction and chromatography procedures before the use of RIA removes these interfering proteins and cross-reacting steroids, but extraction is labor intensive and time-consuming (Rosner and Vesper, 2010). A third type of testosterone assay is the liquid chromatography-tandem mass spectrometry (LC-MS/MS). This assay has the advantage of chromatographic separation and mass spectrometry analysis, leading to an equal or better precision compared with most other assays, and is much less time-consuming. However, like extraction/chromatography RIAs, standardization is still lacking for LC-MS/MS (Vesper *et al.*, 2008). Finally, most circulating testosterone is biologically inactive due to

binding to serum proteins, primarily sex hormone-binding protein (SHBG) and albumin (Dunn *et al.*, 1981). Free testosterone (FT) is the unbound component of total testosterone, while bioavailable testosterone is defined as the concentration of testosterone that is free or weakly-bound. While FT may correlate better with the patient's clinical androgenic state, the measurement of FT is even more complicated because FT is only a small proportion of total testosterone (Rosner *et al.*, 2007). Instead of measuring FT concentrations itself, the free androgen index (FAI; testosterone/SHBG) is often calculated. However, the FAI is also highly dependent on the quality of testosterone and SHBG assay measurements (Vermeulen *et al.*, 1999).

Multiple studies have investigated serum total testosterone concentrations in women with spontaneous POI or iatrogenic menopause. However, due to the low incidence of ovarian insufficiency and the heterogeneity of iatrogenic menopause, most studies included small sample sizes. Moreover, different total testosterone assays were applied and control groups were not uniform. The current systematic review and meta-analysis were aimed to investigate total testosterone concentrations in women with spontaneous POI and iatrogenic menopause, and to identify whether these women are at risk for hypoandrogenemia, while introducing subgroup analyses for the constitution of control group and testosterone assay that was used.

Methods

Search strategy

The electronic databases of MEDLINE, EMBASE and the Cochrane Library were consulted from inception until October 2011 for the identification of suitable papers. A search strategy was carried out based on synonyms of 'POI', 'iatrogenic menopause' and 'testosterone' in titles and abstracts. Synonyms were identified by selecting relevant items included in the indexed MeSH search terms and by reviewing relevant literature for new relevant synonyms not mentioned by MeSH. Two separate searches were carried out: one for testosterone concentrations in spontaneous POI, and one for testosterone concentrations in iatrogenic menopause.

For the first search, the following terms were included in the MEDLINE search: ('POF'[tiab] OR 'ovarian failure'[tiab] OR 'ovarian ageing'[tiab] OR 'ovarian aging'[tiab] OR 'POI'[tiab] OR 'POF'[tiab] OR 'premature menopause'[tiab] OR 'early menopause'[tiab] OR 'climacterium praecox'[tiab] OR 'gonadotrophin resistant ovary syndrome'[tiab] OR 'gonadotrophin-resistant ovary syndrome'[tiab] OR 'resistant ovary syndrome'[tiab] OR 'ovarian follicle depletion'[tiab]) AND [testosterone'[tiab] OR 'FT'[tiab] OR 'total testosterone'[tiab] OR 'androgen'[tiab] OR 'androgens'[tiab] OR 'circulating testosterone'[tiab]).

The search for testosterone concentrations in iatrogenic menopause consisted of the following search terms in MEDLINE: ('iatrogenic menopause'[tiab] OR 'surgical menopause'[tiab] OR 'bilateral oophorectomy'[tiab] OR 'oophorectomised'[tiab] OR 'oophorectomized'[tiab] OR 'ovariectomy'[tiab] OR 'chemotherapy-induced menopause'[tiab] OR 'chemically induced menopause'[tiab] OR 'post chemotherapy ovarian failure'[tiab] OR 'chemical ovarian failure'[tiab] OR 'chemical ovarian insufficiency'[tiab] OR 'chemotherapy-induced menopause'[tiab] OR ('cancer'[tiab] AND 'menopause'[tiab]) OR ('chemotherapy'[tiab] AND 'menopause'[tiab])) AND ('testosterone'[tiab] OR 'FT'[tiab] OR 'total testosterone'[tiab] OR 'androgen'[tiab] OR 'androgens'[tiab] OR 'circulating testosterone'[tiab]). The searches were modified for EMBASE and the Cochrane Library using their title/abstract headings. No limits were used in the advanced search.

In addition, a hand search of reference lists of relevant review articles and those of included studies was conducted to locate any other potentially eligible studies. When necessary, authors were contacted to gain additional information.

Selection criteria

All published studies in which serum total testosterone concentrations were described for women with spontaneous POI or surgical menopause and compared with healthy controls, were considered eligible for this systematic review and meta-analysis. The criteria for spontaneous POI had to be consistent with the definition of POI by the WHO III criteria: amenorrhea for at least 4 months, occurring before the age of 40 years, along with repeated elevated FSH to a menopausal level and decreased estradiol (E_2) concentrations (Coulam *et al.*, 1986; Cooper *et al.*, 2010). Iatrogenic menopause was defined as women who underwent bilateral salpingo-oophorectomy (BSO) or became post-menopausal due to gonadotoxic treatment (such as chemotherapy or irradiation) before natural menopause occurred (Wulf, 2004). Controls were required to be women without POI or iatrogenic menopause. Both similar-aged, cycling controls as well as naturally post-menopausal controls were considered eligible.

Exclusion criteria were hyperandrogenemia, BSO or gonadotoxic treatment performed after menopause had occurred, the use of hormone therapy, studies focusing on men or animals and studies without a control group. Studies focusing only on chromosomally abnormal POI patients, such as Turner syndrome, or women with galactosaemia, were excluded. Reviews, case-reports, letters to the editor, conference papers and studies published in languages other than English, Dutch or German were also excluded.

The process for study selection was conducted in two phases. First, titles and abstracts were screened to meet the inclusion criteria by two independent investigators (F.J. and S.J.T.) to avoid selection bias (Fig. 1). Final inclusion occurred after the examination of full text. Any disagreement was resolved by consensus or a third reviewer (B.C.J.M.F.).

Data extraction

From each study included for review, the following information was extracted by two investigators independently (F.J. and S.J.T.) using a standardized data extraction form: author, year of publication, study design, patient population characteristics (criteria, sample size, age, BMI and time since POI/iatrogenic menopause), constitution of controls and a description of the applied total testosterone assay (Tables I and II). Total testosterone concentrations were also extracted from relevant studies. When data were presented in subgroups within a study, pooled means and pooled mean standard deviations (SD) were calculated, using the following formula:

$$SD^2_{pooled} = \frac{[(n_1 - 1) \times SD_1^2 + (n_2 - 1) \times SD_2^2 + (m_1^2 + m_2^2 - 2 \times m_1 \times m_2) \times n_1 \times n_2/(n_1 + n_2)]}{(n_1 + n_2 - 1)},$$

where n is the sample size, SD the standard deviation and m the mean. Any testosterone concentrations reported as conventional units (ng/ml or ng/dl) were converted into SI units (nmol/l) by multiplication of the data by 3.467 or 0.03467, respectively. Reported SEM were converted to SD with the following formula:

$$SD = SEM \times \sqrt{n},$$

where SD is the standard deviation, SEM the standard error of the mean and n the sample size. Geometric means and 95% confidence

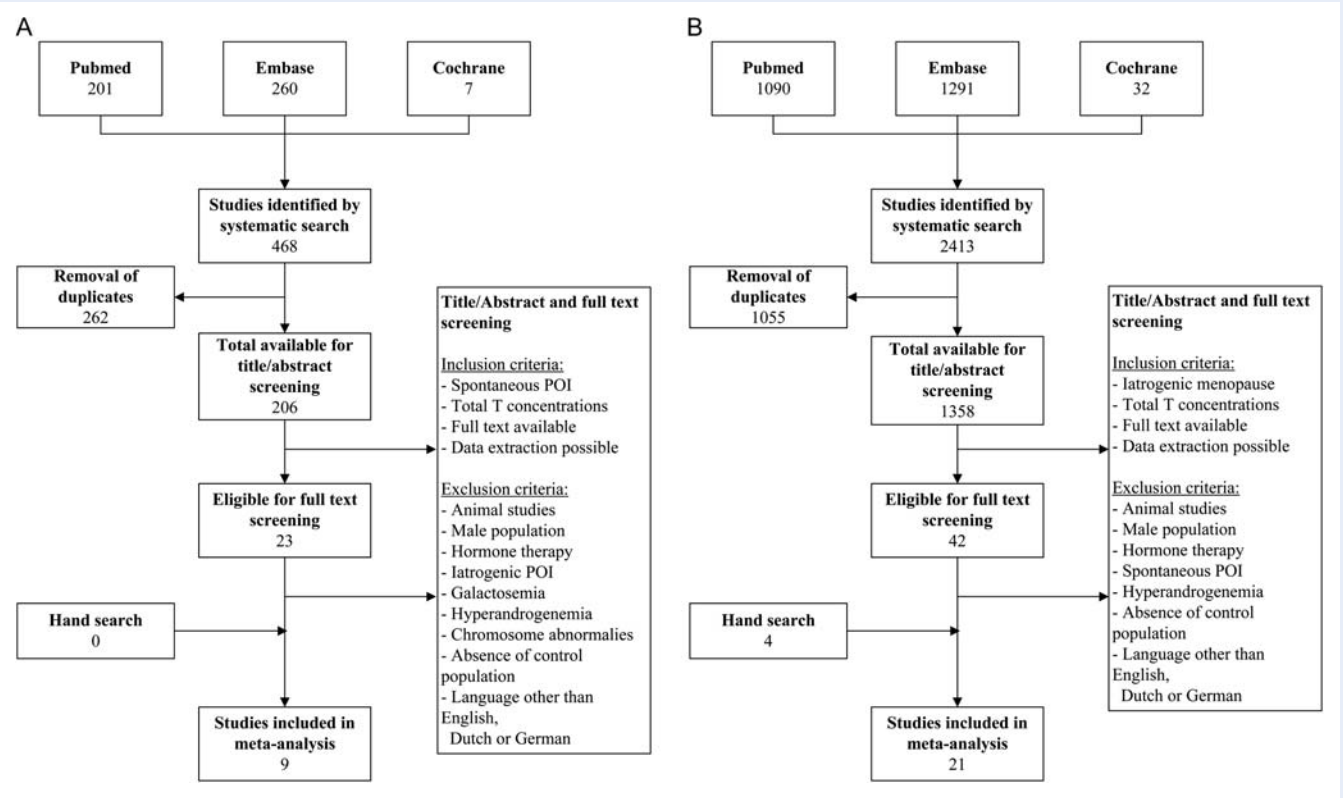

Figure 1 Search and selection for studies on total testosterone concentrations in women with spontaneous POI (**A**) and in women with iatrogenic menopause (**B**).

intervals (CIs) were converted into arithmetic means and SDs using the equivalence of the logarithm of a geometric mean and the log-normal distribution. When necessary, authors were contacted to gain additional information.

Critical appraisal

Included studies were critically appraised by two independent reviewers (F.J. and S.J.T.) according to the Newcastle–Ottawa Scale for meta-analysis of observational studies (Wells *et al.*, 2000) and following the Cochrane risk of bias assessment (Higgins and Green, 2011) (Table II and Supplementary data, Tables SI and SII). The following criteria were assessed: representativeness of cases (spontaneous POI or iatrogenic POI), selection of control groups and comparability between these groups. Studies in which control women were matched for important factors associated with testosterone concentration, such as age, BMI and duration of amenorrhea, were considered as higher quality studies. Furthermore, validity of the outcome measurement (testosterone concentration measurement) and non-response rates were assessed. Low methodological quality was not an exclusion criterion. Again, any disagreement among the investigators was dissolved by consensus.

Statistical analysis

Weighted mean differences (WMDs) and associated 95% CIs were calculated for the comparison of testosterone concentrations between spontaneous POI and controls, and between iatrogenic menopause and controls in a random effects model. The degree of heterogeneity between the results of the different studies was examined by inspection of funnel plots, the overlap in the CIs and the Higgins Index (I^2). Furthermore, subgroup analyses for testosterone assay and for constitution of controls were conducted to examine their contribution to testosterone concentrations. Finally, sensitivity analyses of high-quality studies were performed. The high-quality studies were defined as a high Newcastle–Ottawa score and studies in which confounding factors for testosterone concentration such as age, BMI and time since menopause did not differ between cases and controls. All analyses were performed with RevMan version 5.1 (2011).

Results

Characteristics of included studies

The systematic searches yielded 468 and 2413 studies for spontaneous POI and iatrogenic menopause, respectively (Fig. 1). Duplicates were removed using Reference Manager, which resulted in 206 and 1358 available studies for the review process, respectively. On the basis of *a priori* defined selection criteria, screening title and abstract resulted in the exclusion of 183 studies for spontaneous POI and 1316 studies for iatrogenic menopause. Full text papers were retrieved for both searches (23 and 42, respectively) and reviewed on the basis of the selection criteria.

For spontaneous POI, 14 full-text studies were excluded: 1 study (Bernardi *et al.*, 1998) was excluded due to identical results as a previously published study (Hartmann *et al.*, 1997), 2 studies were excluded because POI diagnosis did not meet the predefined criteria (Aloia *et al.*, 1985; Ludwig and Wolters, 2007), 3 studies did not

Table I Summary of studies assessing total testosterone concentrations in women with spontaneous POI and controls.

Author	Study design	POI	Control	Total testosterone assay
Bermudez *et al.* (1993)	Cross-sectional case control	$n = 7$, age range 20–34 years, normal BMI (20–25 kg/m^2)	$n = 6$, regular cycle, age range 27–29 years, normal BMI (20–25 kg/m^2)	Extraction/chromatography RIA; intra-assay CV 5%; inter-assay CV NR; LLOD NR
Hartmann *et al.* (1997)	Cross-sectional case control	$n = 33$, age 28.7 ± 4.9 years, BMI 22.5 ± 3.6 kg/m^2, duration of amenorrhea 2.5 ± 1.3 years	$n = 33$, regular cycle, age 28.3 ± 4.9 years, BMI 21.6 ± 2.6 kg/m^2 And $n = 32$, post-menopausal, age 53.1 ± 2.6 years, BMI 24.5 ± 2.6 kg/m^2, duration of amenorrhea 2.6 ± 1.4 years	Direct RIA; intra-assay CV 6.5%; inter-assay CV 11.2%; LLOD 0.14 nmol/l
Elias *et al.* (1997)	Cross-sectional case control	$n = 29$, age 34 ± 4 years. POI diagnosed when FSH 'elevated'	$n = 29$, regular cycle, age 34 ± 4 years	Extraction/chromatography RIA; intra-assay CV <11%; inter-assay CV <12%; LLOD <0.04 nmol/l
Doldi *et al.* (1998)	Cross-sectional case control	$n = 25$, age 30.2 years, BMI 22.4 kg/m^2. POI diagnosed when FSH >25.6 IU/l.	$n = 18$, regular cycle, age 29.4 years, BMI 21.1 kg/m^2	Direct RIA; intra-assay CV NR; inter-assay CV NR; LLOD NR
Falsetti (1999)	Cross-sectional case control	$n = 40$, age 32.6 ± 7.3 years, BMI 22.9 ± 3.8 kg/m^2	$n = 30$, regular cycle, age 35 ± 3.5 years, BMI 22.2 ± 2.2 kg/m^2	Direct RIA; intra-assay CV NR; inter-assay CV NR LLOD NR
Benetti-Pinto *et al.* (2005)	Cross-sectional case control	$n = 30$, age 34.4 ± 5.2 years, BMI 24.7 ± 5.0 kg/m^2, duration of amenorrhea 5.4 ± 3.8 years	$n = 30$, regular cycle, age 34.5 ± 5.5 years, BMI 24.4 ± 4.6 kg/m^2 And $n = 30$, naturally post-menopausal women, age 55.1 ± 3.9 years, duration of amenorrhea 5.4 ± 3.8 years	Direct RIA; intra-assay CV NR; inter-assay CV: 5.6%. LLOD NR
Kalantaridou *et al.* (2006)	Cross-sectional case control	$n = 130$, age 32.1 ± 5.5 years, BMI 23.2 ± 3.1 kg/m^2	$n = 65$, healthy, regular cycle, age 30.3 ± 7.1 years, BMI 23.0 ± 2.7 kg/m^2	Extraction/chromatography RIA; intra-assay CV 6.1%; inter-assay CV 15%; LLOD 0.10 nmol/l
van der Stege *et al.* (2008)	Cross-sectional case control	$n = 27$ (no HT use), age 35.8 ± 4.9 years, BMI 23.5 ± 3.4 kg/m^2	$n = 63$, regular cycle, age 35.0 ± 4.7 years, BMI 24.0 ± 4.6 kg/m^2	Direct RIA; intra-assay CV 4.9–7.1%; inter-assay CV 14–19%; LLOD NR
Janse *et al.* (2011)	Cross-sectional case control	$n = 208$, age 37.1 ± 7.6 years, BMI 24.4 ± 4.1 kg/m^2	$n = 45$, regular cycle, severe male infertility, FSH <12 IU/l, age 32.8 ± 3.5, BMI 24.7 ± 6.6 kg/m^2	Extraction/chromatography RIA; intra-assay CV 3.5–3.8%; inter-assay CV 6–10%; LLQ 0.10 nmol/l

Data shown as mean ± SD, unless stated otherwise. POI, primary ovarian insufficiency; BMI, body mass index; HT, hormone therapy; RIA, radioimmunoassay; CV, coefficient of variation; LLOD, lower limit of detection NR, not reported.

include female controls (Duignan *et al.*, 1978; Bachelot *et al.*, 2005; Knauff *et al.*, 2008), 1 study did not measure serum testosterone concentrations (Mason *et al.*, 2006), 1 study focused only on women with galactosaemia (Kaufman *et al.*, 1987), 1 study was a review paper (Arlt, 2003), 2 studies were conference abstracts (Grech and Panay, 2008; Woodman *et al.*, 2009) and 3 studies were excluded because of language other than English, Dutch or German (Stanosz *et al.*, 1995; Berlier *et al.*, 1999; Skalba *et al.*, 2006).

For iatrogenic menopause, 25 full-text studies were excluded: 1 study (Oriana *et al.*, 1982) was excluded due to identical results as a previously published study (Preda *et al.*, 1979), 3 studies were excluded because post-menopausal oophorectomy was performed (Lukanova *et al.*, 2004; Antoniucci *et al.*, 2005; Fogle *et al.*, 2007), in 1 study it was unclear whether the gonadotoxic treatment resulted in a post-menopausal status (Eby *et al.*, 1989), 12 studies did not include female controls (Horton *et al.*, 1966; Judd *et al.*, 1974; Chakravarti *et al.*, 1977; Dennerstein *et al.*, 1978; Dowsett *et al.*, 1988; Hughes, Jr. *et al.*, 1991; De Leo *et al.*, 1998; Azzena *et al.*, 2002; Nozaki *et al.*, 2004; Mason *et al.*, 2006; Nar *et al.*, 2009; Kalyan *et al.*, 2011), 2 studies did not measure total T (Forsling *et al.*, 1996; Acar *et al.*, 1998), in 1 study women used hormone therapy (Schmitt-Robe *et al.*, 1992), 1 study did not report post-oophorectomy testosterone-values (Preda *et al.*, 1979), 1 study was a conference record (Bucuras *et al.*, 2010) and 3 studies were excluded because of language other than English, Dutch or German (Bassalyk *et al.*, 1986; Halerz-Nowakowska, 1995; Kulak *et al.*, 2009). In addition, a hand search was performed which resulted in the identification of 4 new eligible studies for iatrogenic menopause, while no additional studies were found for spontaneous POI. Finally, 9 studies on spontaneous POI and 21 studies on iatrogenic menopause were included for critical appraisal and meta-analysis.

The characteristics of the included studies for spontaneous POI are reported in Table I. In most studies, POI diagnosis was reported as a post-menopausal FSH concentration >40 IU/l. Two studies did not adhere to this strict cut-off for FSH, due to describing FSH as 'elevated' (Elias *et al.*, 1997) or using a cut-off of only 25.6 IU/l (Doldi *et al.*, 1998), but these studies still met the selection criteria set by the investigators. All studies incorporated a control group with a regular menstrual cycle pattern, while two studies also included a post-menopausal control group (Hartmann *et al.*, 1997; Benetti-Pinto *et al.*, 2005). Direct RIA

Table II Summary of studies assessing total testosterone concentrations in women with iatrogenic menopause and controls.

Author	Study design	Iatrogenic menopause	Control	Testosterone assay
Lamb *et al.* (1964)	Cross-sectional case control	$n = 5$ BSO ($n = 3$ for pelvic inflammatory disease, $n = 2$ for breast cancer), age and BMI NR	$n = 20$, regular menstrual cycle, age 17–38 years. BMI NR	Extraction/chromatography RIA; intra-assay CV NR; inter-assay CV NR LOD NR
Abraham (1969)	Cross-sectional case control	$n = 6$ BSO and hysterectomy for cervical cancer ($n = 3$) or benign causes ($n = 3$), age 40–67 years. BMI NR	$n = 6$ cycling women, of whom at least 5 ovulatory, age 21–28 years. BMI NR	Extraction/chromatography RIA; intra-assay CV NR; inter-assay CV NR LOD NR
Vermeulen (1976)	Cross-sectional case control	$n = 8$ BSO, age 51–62 years, BMI and time since menopause not described	$n = 19$ natural menopause, age 51–65 years, time since menopause 4–10 years. BMI NR	Extraction/chromatography RIA; intra-assay CV NR; inter-assay CV NR; LOD NR
Studd *et al.* (1978)	Cross-sectional case control	$n = 100$ BSO and hysterectomy, 1–31 years after oophorectomy. Age and BMI NR	$n = 64$ natural menopause, 1–30 years after menopause. Age and BMI NR	Extraction/chromatography RIA; intra-assay CV NR; inter-assay CV NR; LOD NR
Beksac *et al.* (1983)	Prospective cohort case control	$n = 27$ women undergoing BSO and hysterectomy, measurement 7 days post-operatively. Mean age and BMI NR	$n = 25$ premenopausal women undergoing hysterectomy without oophorectomy, measurement 7 days post-operatively. Mean age and BMI NR	Method of RIA not specified; intra-assay CV NR; inter-assay CV NR; LOD NR
Sherwin (1985)	Prospective cohort case control	$n = 10$ women undergoing hysterectomy with BSO for benign causes, mean age 45.9 $\pm$ 3.3 years, measurement 8 months post-operatively (placebo for HT)	$n = 10$ women undergoing hysterectomy without BSO for benign causes, mean age 36.3 $\pm$ 2.2 years, measurement 8 months post-operatively	Direct RIA; intra-assay CV NR; inter-assay CV NR; LOD NR
Inskip *et al.* (1994)	Cross-sectional case control	$n = 21$ cervix carcinoma patients with BSO without irradiation, age at diagnosis 51 years (48% premenopausal), age at sampling 61 years (42–76) And $n = 79$ cervix carcinoma patients with irradiation ($\geq$38 Gy) without BSO, age at diagnosis 53 years (43% premenopausal), age at sampling 61 (26–86) years	$n = 32$ cervix carcinoma patients with hysterectomy or cervical amputation only, age at diagnosis 33 years (94% premenopausal, age at sampling 38 (25–66) years	Extraction/chromatography RIA; intra-assay CV 6%; inter-assay CV 10%; LOD 0.07 nmol/l
Wakatsuki (1995)	Cross-sectional case control	$n = 10$ BSO, age 43.67 $\pm$ 2.55 years, >2 years after BSO	$n = 10$ natural menopause, age 65.63 $\pm$ 5.58 years, >1 years after final menstrual period	Extraction/chromatography RIA; intra-assay CV NR; inter-assay CV NR; LOD NR
Laughlin *et al.* (2000)	Cross-sectional case control	$n = 123$ BSO, age 73 $\pm$ 7 years, BMI 24.5 $\pm$ 3.8 kg/m^2, age at BSO 46 $\pm$ 9 years	$n = 438$ natural menopause, age 74 $\pm$ 8 years, BMI 24.1 $\pm$ 3.7 kg/m^2, age at menopause 49 $\pm$ 5 years	Extraction/chromatography RIA; intra-assay CV 4.0%; inter-assay CV 4.9%; LOD 0.011 nmol/l
Sowers *et al.* (2001)	Prospective cohort case control	$n = 33$ BSO, age 38 years (27–47), latest measurement (1994–1995) used for review	$n = 509$ cycling women, age 38 years (27–47 years), latest measurement (1994–1995) used for review	Direct RIA; intra-assay CV 15.5%; inter-assay CV 15.5%; LOD NR
Couzinet *et al.* (2001)	Cross-sectional case control	$n = 15$ BSO, age 53 $\pm$ 3 years, time since BSO 15.3 $\pm$ 2.1 years	$n = 15$ natural menopause, age 57 $\pm$ 4 years, time since menopause 13.3 $\pm$ 2.0 years	Extraction/chromatography RIA; intra-assay CV 15%; inter-assay CV 17%; LOD 0.17 nmol/l;
García-Pérez (2004)	Cross-sectional case control	$n = 35$ BSO, age 49.31 $\pm$ 6.52 years, BMI 25.60 $\pm$ 3.64, time since BSO 4.31 $\pm$ 4.13 years	$n = 112$ natural menopause, age 54.23 $\pm$ 5.48, BMI 25.95 $\pm$ 4.14 kg/m^2, time since menopause 6.24 $\pm$ 5.29 years	Direct RIA; intra-assay CV 4%; inter-assay CV 9%; LOD NR
Davison *et al.* (2005)	Cross-sectional case control	$n = 27$ BSO, age 55–75 years	$n = 183$ natural menopause, age 55–75 years	Extraction/chromatography RIA; intra-assay CV 4.2–10.5%; inter-assay CV 7.1–12.8%; LOD 0.2 nmol/l
Hassa (2006)	Cross-sectional case control	$n = 35$ hysterectomy and BSO for benign causes, age 46.2 SEM 0.4 years, BMI 28.1 SEM 0.6 kg/m^2 . Data collected on post-operative day 7	$n = 57$ premenopausal hysterectomy for benign causes, age 41.7. SEM 0.5 years, BMI 26.3 SEM 0.6 kg/m^2. Data collected on post-operative day 7	Direct RIA; intra-assay CV 2.1%; inter-assay CV 2.5%; LOD NR

Continued

Table II *Continued*

Author	Study design	Iatrogenic menopause	Control	Testosterone assay
Cappola *et al.* (2007)	Cross-sectional case control	$n = 56$ BSO, mean age and BMI not stated	$n = 219$ natural menopause, mean age 74 (65–98) years, mean BMI 26,8 kg/m^2	LC-MS/MS; intra-assay CV 8.2%; inter-assay CV 13.2%; LOD 0.008 nmol/l
McTiernan (2008)	Cross-sectional case control	$n = 24$ BSO, mean age and time since menopause NR	$n = 241$ natural menopause, age at screening 64.6 ± 7.0 years, time since menopause 15.7 ± 8.9 years	Extraction/chromatography RIA; intra-assay CV 8.9% inter-assay CV 19.1%; LOD 0.10 nmol/l
Korse *et al.* (2009)	Cross-sectional case control	$n = 35$ BRCA1/2 carriers after prophylactic BSO, age 45.9 ± 6.1 years	$n = 40$ naturally post-menopausal BRCA1/2 carriers, age 56.5 ± 5.9 years	Direct RIA intra-assay CV NR inter-assay CV NR LOD 0.07 nmol/l
Danforth *et al.* (2010)	Cross-sectional case control	$n = 64$ hysterectomy and BSO, mean age and time since menopause NR	$n = 438$ natural menopause, median time since menopause 12 years, median BMI 25 kg/m^2	Extraction/chromatography RIA; intra-assay CV 6–13.6%; inter-assay CV NR; LOD 0.035 nmol/l
Bui (2010)	Retrospective cohort case control	$n = 8$ BRCA1/2 carriers after prophylactic BSO. Age at surgery 44.8 ± 6.6 years. Sample drawn <1 years post-operatively	$n = 16$ natural menopause, age at menopause 50.7 ± 2.5 years. Sample drawn <2 years after menopause date (12 mo after final menstrual period)	ID-LC-MS/MS; intra-assay CV NR; inter-assay CV 4–5%; LOD 0.027 nmol/l
Labrie *et al.* (2011)	Cross-sectional case control	$n = 71$ BSO, age 60.6 years. Time since menopause NR	$n = 442$ natural menopause, age 59.9 years And: $n = 47$ premenopausal normal cycling, age 33 years	GC–MS; inter-assay CV 2.9%; inter-assay CV 3.4%; LOD NR
Alarslan (2011)	Cross-sectional case control	$n = 35$ hysterectomy with BSO for benign causes. Age 51.7 ± 4.0 years, BMI 28.5 ± 4.1 kg/m^2. Sample drawn >1 years post-operatively	$n = 83$ natural menopause. Age 52.4 ± 4.6 years, BMI 28.4 ± 4.1 kg/m^2. Sample drawn >1 years after final menstrual period	Direct RIA; intra-assay CV NR; inter-assay CV NR; LOD NR

Data shown as mean ± SD, unless stated otherwise. POI, primary ovarian insufficiency; BMI, body mass index; BSO, bilateral salpingo-oophorectomy; HT, hormone therapy; ID-LC-MS/MS, isotope dilution-liquid chromatography- tandem mass spectrometry; GC–MS, gas chromatography-mass spectrometry; RIA, radioimmunoassay; CV, coefficient of variation; LOD, limit of detection; NR, not reported.

was applied in five studies, while four studies incorporated extraction/chromatography steps before RIA was applied.

Details for the included studies for iatrogenic menopause are described in Table II. In the included studies, iatrogenic menopause was mostly a result of BSO with ($n = 7$) or without hysterectomy ($n = 14$). One study in women with cervical cancer included two groups with possible iatrogenic menopause: one treated by BSO, and one by irradiation (Inskip *et al.*, 1994). Reasons for BSO were benign or prophylactic in five studies, while BSO was performed because of cancer in three studies. However, 13 studies did not describe reasons why BSO was carried out. Controls consisted of women with natural menopause ($n = 13$), women with hysterectomy only ($n = 4$) or were cycling women ($n = 3$). One study included both cycling controls and women with natural menopause (Labrie *et al.*, 2011). There were 11 studies which applied extraction/chromatography and RIA, two used (isotope dilution-) LC-MS/MS, one used gas chromatography-MS (GC–MS) and the remaining studies used direct RIA ($n = 6$). One study did not mention the type of testosterone assay that was used (Beksac *et al.*, 1983).

Methodological quality

The quality assessment for studies selected for the comparison of testosterone concentrations in spontaneous POI versus controls is reported in Table III (summary) and in Supplementary data, Table SI. All but two studies adjusted for the important confounders age and BMI, or duration of menopause and BMI. However, in five out of nine studies selection bias could not be excluded because recruitment procedures for women with spontaneous POI and controls were not described. Of the remaining four studies, two included all consecutive POI patients, while two invited these women with advertisements, internet or through recruitment letters to physicians. In these four studies, controls were selected from different population samples (general population or hospital population) than cases. Completeness of sample (non-response rate) was not reported in any of the studies, thus indicating a high risk for attrition bias. Blinding of laboratory personnel was not described in any study, thereby introducing a possible detection bias. However, performance bias was not suspected due to the fact that identical testosterone assays were employed in all case–control comparisons. The influence of reporting bias could not be assessed. In summary, three studies (Kalantaridou *et al.*, 2006; van der Stege *et al.*, 2008; Janse *et al.*, 2011) scored 7 points or higher on the Newcastle–Ottawa scale, indicating good methodological quality.

For the comparison of testosterone concentrations in iatrogenic menopause versus controls, the quality assessment for included studies are reported in Table III (summary) and in Supplementary data, Table SII. In most studies ($n = 12$), iatrogenic menopause was

Table III Summary of critical appraisal of included studies using the Newcastle–Ottawa Quality Assessment Scale for case–control studies.

Study ID	Selection (Max. four stars)	Comparability (Max. two stars)	Exposure (Max.three stars)
Spontaneous POI			
Bermudez *et al.* (1993)	**	—	*
Hartmann *et al.* (1997)	**	**	**
Elias *et al.* (1997)	*	*	*
Doldi *et al.* (1998)	**	**	**
Falsetti (1999)	**	**	*
Benetti-Pinto *et al.* (2005)	**	**	*
Kalantaridou *et al.* (2006)	****	**	**
van der Stege *et al.* (2008)	***	**	**
Janse *et al.* (2011)	***	**	**
Iatrogenic menopause			
Lamb (1964)	****	—	**
Abraham (1969)	*	*	*
Vermeulen (1976)	**	*	**
Studd *et al.* (1978)	**	—	**
Beksac *et al.* (1983)	**	—	**
Sherwin (1985)	***	*	**
Inskip *et al.* (1994)	**	**	**
Wakatsuki (1995)	*	*	*
Laughlin *et al.* (2000)	****	**	**
Sowers *et al.* (2001)	****	*	**
Couzinet *et al.* (2001)	**	**	*
García-Pérez (2004)	**	**	*
Davison *et al.* (2005)	***	*	*
Hassa (2006)	***	*	*
Cappola *et al.* (2007)	***	-	*
McTiernan (2008)	*	*	*
Korse *et al.* (2009)	****	**	***
Danforth *et al.* (2010)	*	**	*
Bui (2010)	**	—	*
Labrie *et al.* (2011)	**	*	*
Alarslan (2011)	**	*	**

Selection

1. Is the case definition adequate? (a) yes, with independent validation* (b) yes, e.g. record linkage or based on self-reports and (c) no description
2. Representativeness of cases (a) consecutive or obviously representative series of cases* (b) potential for selection biases or not stated
3. Selection of controls (a) community controls* (b) hospital controls and (c) no description
4. Definition of controls (a) no history of disease (end-point)* and (b) no description of source

Comparability

1. Comparability of cases and controls on the basis of the design or analysis (a) study controls for __ (select most important factor)* and (b) study controls for any additional factor* (this criteria could be modified to indicate specific control for a second important factor)

Exposure

1. Ascertainment of exposure (a) secure record (e.g. surgical records)* (b) structured interview where blind to case/control status* (c) interview not blinded to case/control status (d) written self-report or medical record only and (e) no description
2. Same method of ascertainment for cases and controls (a) yes* (b) no
3. Non-response rate (a) same rate for both groups* (b) non-respondents described and (c) rate different and no designation

confirmed by medical records, however, four studies relied on self-report only and three studies did not mention any procedure for how the diagnosis was confirmed. Cases and controls were matched on or adjusted for age and BMI, or time since menopause and BMI, in six studies. There are indications for selection bias in most studies, because 10 papers only matched on or adjusted for one of these confounders, while in five studies no matching or adjustment was performed at all. Moreover, recruitment procedures of cases or controls were not described in nine and six studies, respectively. There were eight studies which used identical populations for the recruitment of controls. All studies were at risk for attrition bias, because response rate for both cases and controls was not described for any of the studies. Again, blinding of laboratory personnel was not described in any study (introduction of possible detection bias), but performance bias was not suspected because of the use of identical testosterone assays in all case–control comparisons. Assessment of reporting bias was not possible. Summarizing the Newcastle–Ottawa scale assessment, three studies (Sowers *et al.*, 2001; Laughlin *et al.*, 2000; Korse *et al.*, 2009) scored 7 points or higher, and were therefore considered as the best available evidence.

Testosterone concentrations in spontaneous POI

The nine studies included in the meta-analysis reported total testosterone concentrations in 529 women with spontaneous POI and in 319 controls. Women with spontaneous POI demonstrated significantly lower total testosterone concentrations compared with controls (nine studies); WMD (95% CI) −0.38 (−0.55 to −0.22) nmol/l. However, substantial between-study heterogeneity was identified ($I^2 = 81\%$) (Fig. 2). Subgroup analyses for comparison between the associations with cycling controls and post-menopausal controls identified that total testosterone concentrations in spontaneous POI are significantly lower compared with the first control group (cycling women) [WMD (95% CI): −0.38 (−0.55 to −0.22) nmol/l], but not compared with the latter control group (post-menopausal women) [WMD (95% CI): 0.07 (−0.17 to 0.32) nmol/l]. This between-subgroup difference reached statistical significance ($P = 0.002$) (Supplementary data, Fig. S1). In a subgroup analysis for assay type, comparing between the associations with direct RIA [WMD (95% CI): −0.54 (−0.79 to −0.29) nmol/l] and extraction/chromatography RIA [WMD (95% CI): −0.19 (−0.35 to −0.04) nmol/l], the difference between assay subgroups was statistically significant ($P = 0.02$) (Supplementary data, Fig. S2). In the sensitivity analysis, the difference in total testosterone concentrations between women with spontaneous POI and controls remained robust [three studies, WMD (95% CI): −0.31 (−0.46 to −0.15) nmol/l], and resulted in a substantial decrease in heterogeneity ($I^2 = 51\%$) (Fig. 3).

Testosterone concentrations in iatrogenic menopause

Due to incomplete data and non-responsive authors, the following studies could not be included in the meta-analysis ($n = 4$): in one study only mean ratios without original data were reported (Inskip *et al.*, 1994), two studies reported geometric means without 95% CIs and data could therefore not be converted into means and SDs (Studd *et al.*, 1978; Danforth *et al.*, 2010) and one study did not mention SD for cases (Lanb *et al.*, 1964). The 17 studies included in the meta-analysis reported data on 558 women with iatrogenic menopause and 2425 controls. In women with iatrogenic menopause, total testosterone concentrations were significantly lower than in controls (17 studies); WMD (95% CI): −0.29 (−0.39 to −0.18) nmol/l. For this comparison, major heterogeneity was identified ($I^2 = 97\%$) (Fig. 4). Subgroup analysis for constitution of controls, i.e. cycling or premenopausally hysterectomized women [WMD (95% CI): −0.49 (−0.83 to −0.14) nmol/l] versus post-menopausal or post-menopausally hysterectomized women [WMD (95% CI): −0.18 (−0.27 to −0.10) nmol/l], identified that differences between the associations according to control type did

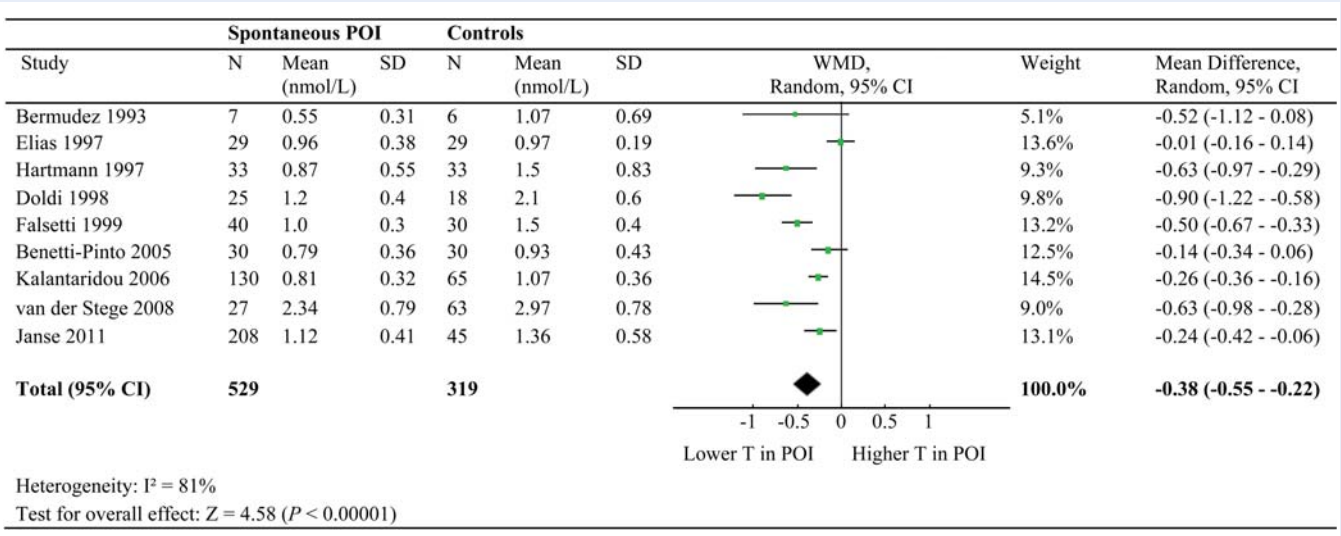

Figure 2 Meta-analysis of nine comparative studies on total testosterone concentrations in women with spontaneous POI compared with controls.

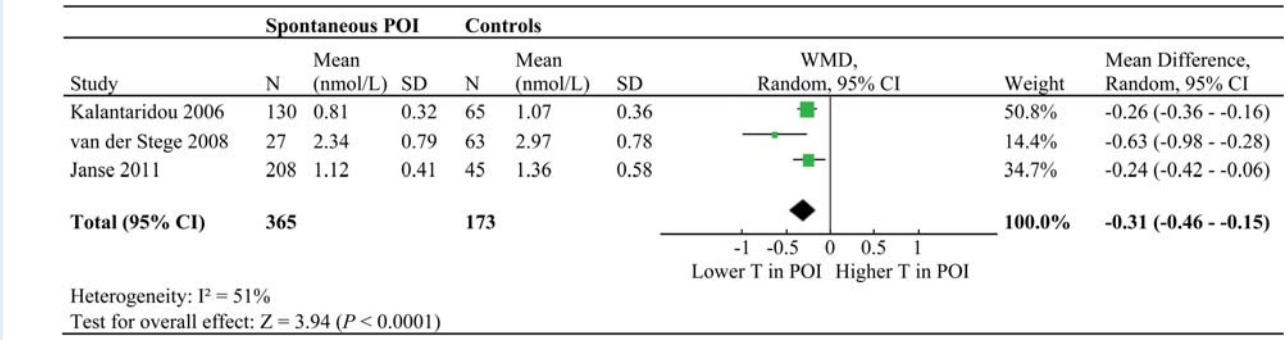

Figure 3 Sensitivity analysis of the three best-quality studies included in the meta-analysis on total testosterone in women with spontaneous POI.

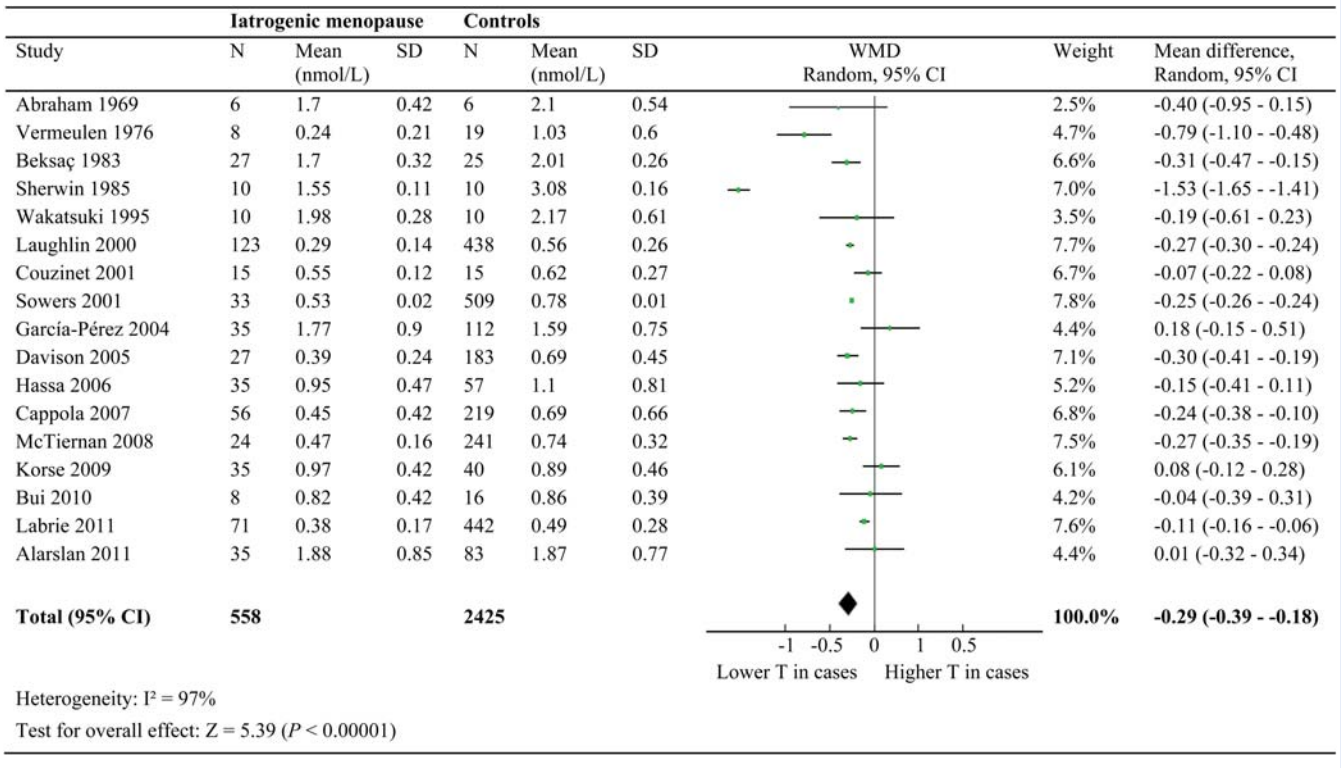

Figure 4 Meta-analysis of 17 comparative studies on total testosterone concentrations in women with iatrogenic menopause compared with controls.

not reach statistical significance ($P = 0.09$) (Supplementary data, Fig. S3). Subgroup analysis for assay type did not identify statistical differences between testosterone measurement by direct RIA [WMD (95% CI): −0.29 (−0.74 to 0.16) nmol/l] versus extraction/chromatography RIA or LC-MS/MS or GC–MS [WMD (95% CI): −0.24 (−0.32 to −0.16) nmol/l] in this population ($P = 0.09$) (Supplementary data, Fig. S4). Sensitivity analyses performed for the best-quality studies confirmed the overall findings [three studies, WMD (95% CI): −0.24 (−0.29 to −0.18) nmol/l], but between-study heterogeneity remained substantial ($I^2 = 83\%$) (Fig. 5).

Discussion

The current systematic review of the literature and subsequent meta-analysis was set up to assess for the first time whether serum total testosterone concentrations in women with spontaneous POI or iatrogenic menopause are different from women who experience natural menopause at a regular age. For spontaneous POI, pooled total testosterone concentrations were significantly lower compared with controls: WMD (95% CI) −0.38 (−0.55 to −0.22) nmol/l. The difference between pooled total testosterone concentrations in

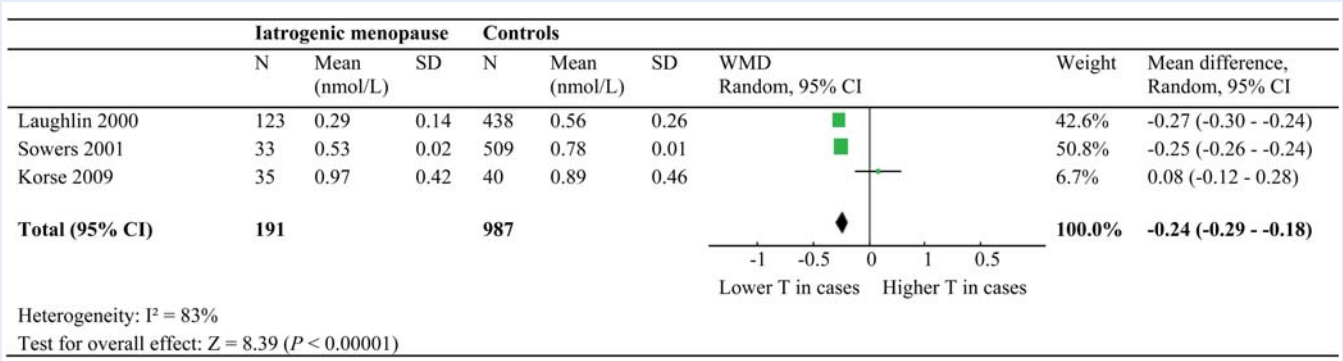

Figure 5 Sensitivity analysis of the three best-quality studies included in the meta-analysis on total testosterone in women with iatrogenic menopause.

women with iatrogenic menopause compared with controls was also statistically significant: WMD (95% CI): −0.29 (−0.39 to −0.18) nmol/l. Sensitivity analyses identified that these differences remained robust; heterogeneity decreased significantly in the analysis for spontaneous POI, but remained substantial in the analysis for iatrogenic menopause.

It has been hypothesized that women with iatrogenic menopause or spontaneous POI may be at increased risk for hypoandrogenism. Because hypoandrogenism is related to decreased estrogen concentrations (decreased peripheral conversion of testosterone to E_2 by fatty tissue), and possibly to diminished well-being and sexual health (Bachmann *et al.*, 2002), and increased risk for cardiovascular disease (Laughlin *et al.*, 2010), it is relevant to evaluate the testosterone concentrations in women with POI or iatrogenic menopause. For spontaneous POI, it is hypothesized that the premature cessation of ovarian function may result in a hypoandrogenic state, which is supported by the findings of the current meta-analysis. However, there is also increasing evidence in the general female population that advancing age *per se* is associated with decreasing total testosterone concentrations (Labrie *et al.*, 1997; Parker, Jr. *et al.*, 2000; Haring *et al.*, 2011). The decline in circulating testosterone may occur as a result of atrophy of the ageing adrenal zona reticularis (diminished production of testosterone precursors), which may lead to decreased peripheral conversion of dehydroepiandrosterone sulphate (DHEA-S) (through DHEA) to testosterone (Davison *et al.*, 2005; Panzer and Guay, 2009). Furthermore, it is debated whether menopause as such is associated with serum testosterone alterations. It has been shown that testosterone levels do not fall abruptly in women undergoing natural menopause due to the preservation of androgen producing theca cells along with elevated LH concentrations (Braunstein, 2002; Fogle *et al.*, 2007), although the ovarian contribution to circulating testosterone in post-menopausal women remains the topic of extensive debate (Couzinet *et al.*, 2001; Labrie *et al.*, 2011). Clearly, any ovarian contribution of androgen production is completely stopped in iatrogenic menopause, which is supported by the current findings. The current results for POI may indicate that ovarian production of testosterone is decreased in these women as well, but the underlying mechanisms remain to be clarified.

To control or adjust for the two possible confounders age and BMI (Santoro *et al.*, 2005; Cappola *et al.*, 2007) was an important part of the quality assessment in the current review of total testosterone concentrations in women with spontaneous POI or iatrogenic menopause. Especially in the latter group, these confounders were often not adjusted for, which may have lead to significant between-study heterogeneity. Another explanation for the significant between-study heterogeneity may be the heterogeneous constitution of the control groups in both meta-analyses. While subgroup analyses for type of controls (pre- versus post-menopausal women) also showed high between-study heterogeneity, different age ranges and unclear recruitment procedures for controls may still have been present. Moreover, in the analysis for iatrogenic menopause, heterogeneity in the patient groups existed: BSO was performed for different indications (benign or cancer) and at different ages. A third reason for the encountered between-study heterogeneity may be that none of the studies applied identical testosterone assays. The measurement of testosterone in women is challenging due to lack of trueness, precision and sensitivity of various available testosterone assays (Rosner and Vesper, 2010), and between-study comparability of results is therefore problematic. Subgroup analyses for direct RIA versus extraction/chromatography RIA or LC-MS/MS identified that heterogeneity between studies became less important when the latter assay types were applied, while a significant difference in the results was identified between the use of these assays in spontaneous POI (χ^2 5.23, $P = 0.02$, $I^2 = 80.9\%$). This is consistent with previously published studies on the performance of testosterone assays (Herold and Fitzgerald, 2003; Stanczyk *et al.*, 2003; Rosner and Vesper, 2010). Finally, most applied testosterone assays were reported to have high intra- and inter-assay coefficients of variation. The identified differences between cases and controls in this meta-analysis were mostly larger than the analytical variance, and therefore we consider that we may still interpret these results as statistically significant. However, this once again demonstrates that the trueness and precision of testosterone assays need to be improved before testosterone measurements could be used in a clinical setting to distinguish women with hypoandrogenism from normoandrogenic women.

Only a limited number of studies have directly investigated the consequences of decreased testosterone concentrations in spontaneous POI and iatrogenic menopause. One study, also included in the current meta-analysis, identified that women with spontaneous POI have diminished general and sexual well-being compared with

controls. Although significantly lower testosterone concentrations were identified in these women with POI, an independent role for testosterone concentration could not be identified (van der Stege *et al.*, 2008). However, in agreement with findings in normal menopause, multiple studies have identified that women with iatrogenic menopause are at increased risk for hypoactive sexual desire disorder (Castelo-Branco *et al.*, 2009; Nappi *et al.*, 2009), diminished health-related quality of life (Bhattacharya, 2009), osteoporosis (Yildiz *et al.*, 1996) and fracture risk (Melton *et al.*, 2003), parkinsonism (Rocca *et al.*, 2008) or cognitive impairment (Rocca *et al.*, 2007). These findings are suggestive of a common pathological pathway, but the exact association with decreased testosterone concentrations remains to be determined. Moreover, up until now there is limited evidence for the subscription of androgen therapy in these groups of women. All available studies have investigated androgen therapy in co-treatment with estrogen therapy (Shifren *et al.*, 2000; Buster *et al.*, 2005), and effects of higher serum testosterone concentrations on the remission of symptoms, such as memory function, are not clear (Moller *et al.*, 2010).

The current meta-analysis has several limitations. Because only a limited number of studies identified by the literature search met the inclusion criteria, all selected studies were included for review and meta-analysis. This has led to the inclusion of lower-quality studies with small samples sizes, and significant between-study heterogeneity. We have further addressed the issue of low quality androgen assays by additional sensitivity analyses of the best quality studies only (Figs. 3 and 5). Furthermore, the search was restricted to total testosterone concentrations only. While SHBG, bioavailable testosterone and FT have also been shown to correlate with clinical states such as the metabolic syndrome (Santoro *et al.*, 2005), it is unclear which of these measurements best reflects the availability of testosterone at tissue level (Matsumoto and Bremner, 2004).

In conclusion, this literature review and meta-analysis demonstrate that women with spontaneous POI or iatrogenic menopause are at risk for decreased testosterone concentrations. Differentiation between hypoandrogenic and normoandrogenic states within groups of women with premature loss of ovarian function is problematic due to trueness and precision problems in various testosterone assays. The possible effects of hypoandrogenism, and the possible role for androgen therapy in these women remain to be determined.

Supplementary data

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

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Authors' roles

Every author has contributed in a substantial way to the work described in the manuscript and its preparation. F.J. and S.J.T. were responsible for the conception and design, data interpretation and writing of the manuscript. M.J.C.E. was responsible for the interpretation of the data, statistical analysis, writing and final approval of the manuscript. B.C.J.M.F. significantly contributed to the conception of the study, interpretation of data, revising the manuscript and final approval of the version to be submitted.

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Conflict of interest

We have no other conflict of interest to declare.

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