Primary Ovarian Insufficiency: Genes, hormones, and beyond

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Primary Ovarian Insufficiency: Genes, hormones, and beyond

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(met een samenvatting in het Nederlands)

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Voor mijn ouders

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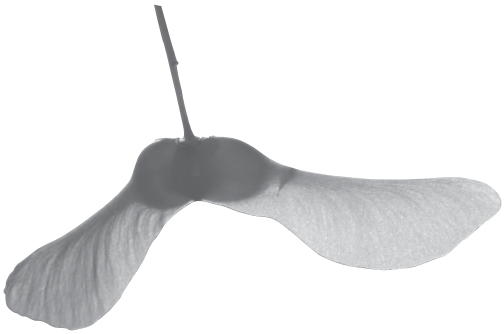
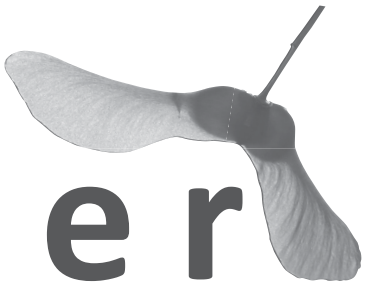




Chapter 1

General introduction

Primary ovarian insufficiency greatly influences a woman's fertility potential and her overall health. This condition, due to accelerated follicle loss or dysfunction of the ovarian follicles, is extensively studied in the current thesis. This general introduction provides a background against which the studies in this thesis were initiated.

Ovarian follicle pool exhaustion

In human fetal ovaries the maximum number of resting follicles is reached by the gestational age of approximately 20 weeks (1-3). The maximum number of 6 to 7 million oocytes decreases to an estimated 1 to 2 million oocytes at birth, due to massive apoptosis of the fetal primordial follicles (4). After birth, atresia continues although in a slower pace resulting in the survival of around 300,000 to 500,000 follicles at the time when a girl experiences her menarche (1). After menarche, the ovarian follicle pool further declines with a rate of approximately 1,000 follicles each month, until ultimately only 100 to 1,000 follicles remain (3,5,6). This situation is marked by the occurrence of the final menstrual cycle: menopause. In a relatively fixed time relation to menopause, fertility is compromised due to a decreasing amount of ovarian follicles together with decreasing quality of the oocytes (7). This process is referred to as ovarian aging.
Menopause typically occurs at around 51 years of age (8). However, there is substantial variability in menopausal age and related fertility decline among women of similar age. Approximately 10% of women become postmenopausal before the age of 45 years, and 1% to 2% of women reach menopause before 40 years of age (9). This condition is referred to as primary ovarian insufficiency (POI).

Definitions

Historically, various terms have been used to describe the clinical entity of what is nowadays called primary ovarian insufficiency. In 1942, Albright was the first to describe the phenomenon of amenorrhea and estradiol deficiency in association with menopausal follicle-stimulating hormone (FSH) levels in young women as primary ovarian insufficiency (10). The term 'primary' relates to the fact that the defect resides in the ovary itself, and is not due to other, secondary causes such as hypothalamic-pituitary disorders, or adrenal dysfunction. Other terms for POI include premature ovarian failure, premature menopause, climacterium praecox, and hypergonadotropic amenorrhea. However, POI is now regarded as the more accurate term (11,12), because it also suggests that ovarian follicular activity might intermittently recover (even years after the diagnosis) (13).

Etiology

The exact cause of primary ovarian insufficiency remains to be elucidated in most women. POI arises from either the premature depletion of the ovarian follicles or ovarian follicle dysfunction. Premature depletion of the ovarian follicle pool may be due to destruction of primordial follicles by toxic agents, autoimmune response, activation of proapoptotic pathways, or accelerated follicular recruitment (14). Direct evidence of depletion of the resting pool of follicles can only reliably be assessed through assessment of the total number of follicles in whole ovaries after oophorectomy (15). In case of follicular dysfunction, sufficient ovarian follicles are present upon ultrasonography or ovarian biopsy, but they do not function properly (16,17). *Figure 1* presents the distribution of the incidence of etiologic factors in our own data.

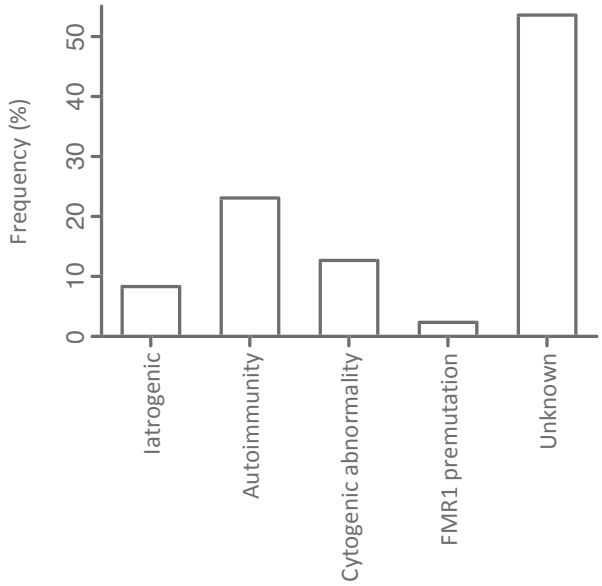


Figure 1. Incidence of etiologic factors in women with POI included in the Dutch primary ovarian insufficiency consortium (n = 528). (Own data, unpublished)

Iatrogenic POI

Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly increased the life expectancy of young women with cancer. Approximately 82% of patients diagnosed with cancer before the age of 19 years survive the disease for at least five years (18). Among the long-term effects of treatment strategies applied for childhood cancer, especially the high incidence of

POI and associated infertility have become apparent (12,19,20). Alkylating agents, such as cyclophosphamide and procarbazine, and irradiation are particularly toxic to the ovaries (21,22). Other iatrogenic causes of POI include extensive abdominal surgery or oophorectomy. Iatrogenic causes explain approximately 5% of all POI cases (9,23,24).

Autoimmunity

It is estimated that approximately 20% of women with POI have a history of autoimmune diseases, most frequently thyroid disease (23). Autoimmune oophoritis may occur as part of the autoimmune polyendocrine syndrome (APS). APS type I, also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), has its onset in childhood. First, mucocutaneous candidiasis (75%) and/or hypoparathyroidism (89%) appear, later adrenal insufficiency (60% to 79%) and POI (60%) may also occur (25). APS type II usually develops in adulthood, and is manifested by autoimmune Addison's disease and thyroid autoimmunity, or type I diabetes. In APS type II, 10% of women develop POI (26). Moreover, POI may also arise from an inflammatory autoimmune response against ovarian-specific antigens or regulatory factors, and circulating steroidogenic cell antibodies have been recorded (27,28).

Genetics

Studies in mother-daughter pairs and in twins have identified that age at menopause is highly heritable: an estimated 40% to 70% (29-31). Moreover, POI tends to run in families and it is estimated that familial POI accounts for 12.5% to 50% of all cases (32-37). Varying definitions and different standards for family history and cytogenetic and molecular diagnosis may explain the wide interval in reported incidences (*Table 1*). The identification of the high heritability of POI suggests that genetic factors play an important role in the pathogenesis of POI.

Table 1. Incidence of familial POI according to varying definitions

Incidence	Definition	Inheritance	Reference
37.5%	≥ 1 OI (EMP or POI)	X-linked inheritance Female Sex preponderance	(32)
31%	≥ 1 POI	Dominant X-linked or autosomal inheritance with variable penetrance	(34)
50%	≥ 1 OI (EMP or POI)	Dominant X-linked or autosomal inheritance	(35)
12.7%	≥ 2 POI	X-linked or autosomal-dominant sex-limited inheritance	(36)
28.5%	≥ 1 POI	Autosomal or X-linked dominant sex-limited inheritance, with incomplete penetrance	(38)

POI: primary ovarian insufficiency; OI: ovarian insufficiency; EMP: early menopause (between age 40 and 45 years).

Structural / numerical chromosomal abnormalities

The X-chromosome plays an important role in the development and maintenance of ovarian function. Complete absence of the X-chromosome (Turner syndrome), whether or not occurring in a mosaic fashion, is a strong risk factor for the development of POI (39). Moreover, cytogenetic studies in women with POI identified at least two so-called "critical regions" on the X-chromosome; POF 1: Xq26-27 (40), and POF 2: Xq13.3-q21 (41). Deletions and/or translocations in these regions are highly associated with POI (42). Overall, numerical and structural chromosomal aberrations account for approximately 1% of all POI cases (43).

Monogenic causes

The most common single gene mutation to cause POI is the premutation of the fragile-X mental retardation gene 1 (*FMR1*), located on Xq27.3 (44). CGG repeat size exceeding 200 repeats is considered a full mutation, and causes the fragile-X syndrome which is characterized by mental retardation, characteristic facial features, and behavioral problems (OMIM ID number 300624). The *FMR1* premutation is an amplification of the CGG repeat length to 55-200 repeats (45) above the normal range (< 45 repeats) (46). Approximately 21% of *FMR1* premutation carriers will develop POI (FXPOI) (47). Moreover, the *FMR1* premutation accounts for 0.8% to 13% of all women with POI (44).

Many other candidate genes have been suggested in association with POI etiology. In some of these genes, POI presents as part of a broader syndrome. Examples of syndromal POI include APS1 caused by an *AIRE* gene mutation (48); the blepharophimosis, ptosis, epicanthus inversus syndrome (BPES) caused by *FOXL2* gene mutations (49); and galactosemia due to homozygous *GALT* gene mutations (50,51). Some of the nonsyndromal candidate genes for POI are *BMP15* (52), *NR5A1* (53), LH-receptor (*LHR*) and FSH-receptor (*FSHR*) genes (16,54), and *NOBOX* (55). Unfortunately, most of these mutations are rare with frequencies < 1%, and, to date, most studies were performed in limited-sample size populations or in which POI phenotype was poorly defined.

POI as a complex genetic trait

The high heritability of age at menopause and the tendency for POI to run in families, imply a strong genetic component underlying POI. However, environmental factors, such as smoking, have also been shown to contribute to the variation in age at menopause (56,57). Finally, in most POI cases, no cause can be established using current methodologies. Together, this has lead to the understanding that POI should be regarded a complex genetic disease. Typically, the phenotype of complex genetic diseases is controlled by many genes and their variants, in interaction with environmental factors (58). Complex genetic disorders are often characterized by familial clustering without an obvious

Mendelian pattern of inheritance because several genes, their mutations and environmental factors contribute (59). The identification of the multitude of genes and gene variants involved in the multifactorial forms of POI has now begun, using techniques to study large numbers of identified polymorphic gene markers, such as single nucleotide polymorphisms or copy number variations in localized chromosomal areas. Such genome-wide association studies initiated by our group and others recently delivered their first, promising results (60-63).

Health effects

The premature hypo-estrogenic status in women with POI is associated with decreased fertility and quality of life, while general health may also be affected negatively. A summary of POI-related health effects is displayed in *Table 2*.

Short-term health effects

Fertility

Notwithstanding the premature exhaustion of the follicle pool, waxing and waning of ovarian function may occur for several years (64,65). Ovulation has been documented in a substantial number of POI patients (64). However, the reported lifetime-incidence of spontaneous pregnancy in POI patients is estimated to be only approximately 4% (13), and assisted reproduction techniques relying on the woman's own oocytes do not significantly improve the chance to conceive (66,67). In an IVF setting, markers of ovarian reserve, such as anti-müllerian hormone (AMH), have been proven valuable for predicting a woman's fertility potential (68). However, women with POI typically have AMH levels below the assay's lower limit of detection (69) and thus the usefulness of these markers for discriminating women who are more likely to conceive versus those who are not within the POI group is questionable. For the vast majority of women with POI to start a family, oocyte donation or adoption are the only options. In contrast, several options for fertility preservation may be offered to women about to undergo gonadotoxic treatment. These include relatively new techniques, such as oocyte cryopreservation and ovarian tissue cryopreservation, although varying success rates on pregnancy chances have been published and both methods are still considered experimental (70,71).

Climacteric symptoms and quality of life

Despite the clearly negative impact on fertility, effects of POI on perceived quality of life are hardly substantiated due to lack of good quality studies. Women with POI may experience typical climacteric symptoms such as night sweats, hot flushes, and suffer from urogenital atrophy leading to dyspareunia, vaginitis and micturition complaints similarly to women with menopause at a normal age (72).

Table 2. Consequences of POI

Organ/Function	Effect of POI	Reference
Ovary	*Ovarian follicle pool depleted or follicle dysfunction:*	
	- Decreased estradiol concentrations	(10,13)
	- Waxing and waning of ovarian function may possibly occur	(74,83,84)
	- Possible decrease of total testosterone	
Fertility	- Lifetime chance to conceive after diagnosis POI: 5-10 %	(13)
	- Assisted reproduction techniques do not improve pregnancy rate	(66)
	- oocyte donation or adoption only options	
Well-being	*Climacteric symptoms:*	(72)
	- Similar to menopause at regular age	
	Emotional well-being:	(73,74)
	- Anxiety, depression, somatization, sensitivity, hostility and psychological distress	
	Sexual function:	(74)
	- Less satisfaction, fewer sexual fantasies, less sexual arousal, increased incidence of genital pain and reduced lubrication.	
	- Association with possibly decreased androgens unclear	
Bone	- Increased risk for osteoporosis	(86)
	- Increased fracture risk	(87)
Cardiovascular	- Epidemiologic studies: increased incidence of cardiovascular disease and increased cardiovascular mortality	(92,90)
	- Slightly unfavorable serum lipid profile	(95)
	- Impaired endothelial function	(96)
	- Exact mechanism of cardiovascular disease development in POI remains unclear	
Brain	- Higher incidence of cognitive impairment, dementia and parkinsonism in women with previous oophorectomy.	(97)
	- A possible association between cognitive function with time since natural menopause or age at natural menopause in non-oophorectomized women is not well-established.	(100)

POI: primary ovarian insufficiency.

Feelings of depression and sadness, as well as severe emotional stress at time of diagnosis have been reported (73). In a Dutch study it was concluded that POI patients were less satisfied with their sexual life, had fewer sexual fantasies and less sexual arousal, as well as increased incidence of genital pain and reduced lubrication compared to controls (74). *Figure 2* provides an overview of the incidence of climacteric symptoms in our own cohort of women with POI.

Most known health risks in women with POI are attributed to the low estrogen concentration. However, some studies suggest that these women may also be sensitive to low testosterone concentrations (75-77). It is under debate if

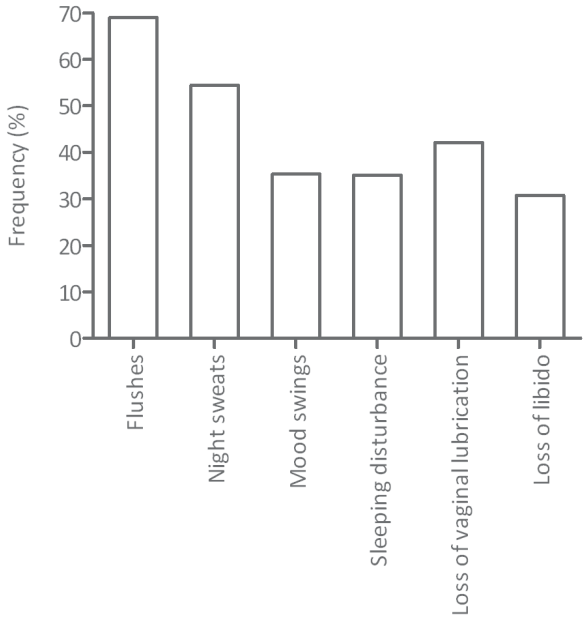


Figure 2. Incidence of climacteric symptoms in women with POI included in the Dutch primary ovarian insufficiency consortium (n = 436). (Own data, unpublished).

androgens, which partly originate from the ovaries, decrease in concordance with estrogens. Initially it was shown that testosterone and androstenedione levels in POI patients and controls did not differ (78,79), however more recent studies demonstrated significantly lower testosterone levels in POI compared to healthy, age-matched, regularly cycling controls (74,80-84). The major problem encountered when evaluating androgen levels in women is that the available testosterone assays have difficulty to reliably determine the low androgen levels in women, let alone in specific female patient groups, such as POI (85).

Long-term health effects

Bone health

Observational studies have identified lower bone mineral density and increased risk for osteopenia in women with POI compared with controls (86). Moreover, women with earlier age at menopause are at increased risk for fractures (relative risk 1.5) (87). A possible protective role for the often prescribed hormone therapy has not been substantiated until now (88). Risk factors within the entire group of women with POI are age at diagnosis and time since diagnosis, while smoking is probably not related (89).

Cardiovascular risk

Epidemiological data have shown earlier age at menopause to be associated with an increased incidence of cardiovascular disease (90,91). Moreover, a calculated 2% decrease of cardiovascular mortality for each year that menopause is delayed was identified (92). It is under debate whether early age at menopause induces cardiovascular disease (for example due to estrogen deficiency), or whether the opposite, early menopause as a consequence of cardiovascular damage, holds true (90,93,94). A previous study by our group demonstrated that women recently diagnosed with POI have a slightly unfavorable serum lipid profile compared to healthy cycling controls, irrespective of the time of estrogen deprivation (95). Another small study identified impaired endothelial function in POI compared to controls, which improved with use of hormone therapy (96).

Neurologic health

Higher incidences of cognitive impairment, dementia and parkinsonism have been documented in women who underwent bilateral oophorectomy before the occurrence of natural menopause (97,98). The risk for cognitive impairment increases with younger age at oophorectomy ($P < 0.0001$) (97). In contrast to the studies in oophorectomized women, other studies did not find an association of cognitive function and time since natural menopause and age at natural menopause (99,100). To date, no studies have been performed on cognitive function in POI patients.

Aims and outline of the thesis

In 2004, the Dutch primary ovarian insufficiency consortium was established. This national collaboration is aimed at unraveling the genetic components of POI and to further describe the phenotype of women affected by POI. Up until time of writing of this introduction, approximately 500 women with POI were included, and therefore the Dutch cohort is one of the largest worldwide and offers great research opportunities.
The studies present in this thesis focus on the assessment of the contribution of genetic factors to (the phenotype of) POI. Also, POI phenotype was examined by extensive assessment of the circulating androgens in women with POI. Finally, the value of ovarian reserve tests was investigated in women with iatrogenic POI, who had undergone ovarian transplantation.

Outline

Chapter 2 describes the phenotypes of women affected by familial POI compared with those with sporadic POI to assess possible different genetic makeups and environmental backgrounds.
Chapter 3 studies the contribution of mutations in the *NR5A1* gene in women with POI.
Chapter 4 studies the relevance of normal- and intermediate *FMR1* CGG repeat size in association with age at POI diagnosis, as a measure of POI severity.
Chapter 5 gives a systematic review and meta-analysis of the available literature on serum total testosterone concentrations in women with spontaneous or iatrogenic POI.
In *Chapter 6*, serum androgen concentrations of women with POI, polycystic ovary syndrome (PCOS) and controls are investigated, and the accuracy and precision of two androgen assays in these groups of women are evaluated.
Chapter 7 describes the value of ovarian reserve tests in women who have undergone ovarian transplantation after cryopreservation following gonadotoxic treatment.
Chapter 8 summarizes the results of the conducted studies and discusses the implications for clinical practice and future research.

References

1. Block E. Quantitative morphological investigations of the follicular system in women; variations at different ages. Acta Anat (Basel) 1952;14:108-123.
2. Baker TG. A quantitative and cytological study of germ cells in human ovaries. Proc R Soc Lond B Biol Sci 1963;158:417-433.
3. Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause. PLoS One 2010;5:e8772.
4. Block E. A quantitative morphological investigation of the follicular system in newborn female infants. Acta Anat (Basel) 1953;17:201-206.
5. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod 1992;7:1342-1346.
6. Gougeon A, Ecochard R, Thalabard JC. Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women. Biology of Reproduction 1994;50:653-663.
7. Broekmans FJ, Soules MR, Fauser BC. Ovarian aging: mechanisms and clinical consequences. Endocr Rev 2009;30:465-493.
8. Treloar AE. Menstrual cyclicity and the pre-menopause. Maturitas 1981;3:249-264.
9. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986;67:604-606.
10. Albright F, Smith PH, Fraser R. A syndrome characterized by primary ovarian insufficiency an decreased stature. The American Journal of the Medical Sciences 1942;204:625-648.
11. Cooper AR, Baker VL, Sterling EW, Ryan ME, Woodruff TK, Nelson LM. The time is now for a new approach to primary ovarian insufficiency. Fertil Steril 2010;95:1890-1897.
12. de Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. Lancet 2010;376:911-921.
13. Bidet M, Bachelot A, Bissauge E, Golmard JL, Gricourt S, Dulon J, Coussieu C, Badachi Y, Touraine P. Resumption of ovarian function and pregnancies in 358 patients with premature ovarian failure. J Clin Endocrinol Metab 2011;96:3864-3872.
14. Anasti JN. Premature ovarian failure: an update. Fertil Steril 1998;70:1-15.
15. Russell P, Bannatyne P, Shearman RP, Fraser IS, Corbett P. Premature hypergonadotropic ovarian failure: clinicopathological study of 19 cases. Int J Gynecol Pathol 1982;1:185-201.
16. Aittomaki K, Herva R, Stenman UH, Juntunen K, Ylostalo P, Hovatta O, de la Chapelle A. Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. J Clin Endocrinol Metab 1996;81:3722-3726.
17. Hubayter ZR, Popat V, Vanderhoof VH, Ndubizu O, Johnson D, Mao E, Calis KA, Troendle JF, Nelson LM. A prospective evaluation of antral follicle function in women with 46,XX spontaneous primary ovarian insufficiency. Fertil Steril 2010;94:1769-1774.

18. Altekruse SF, Kosary CL, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlader N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK. SEER Cancer Statistics Review, 1975-2007. Available at seer.cancer.gov/csr/1975_2007/ 2010.
19. Meirow D. Reproduction post-chemotherapy in young cancer patients. Mol Cell Endocrinol 2000;169:123-131.
20. Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Oncol 2005;6:209-218.
21. Wallace WH, Thomson AB, Saran F, Kelsey TW. Predicting age of ovarian failure after radiation to a field that includes the ovaries. Int J Radiat Oncol Biol Phys 2005;62:738-744.
22. Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. Cancer 2007;110:2222-2229.
23. Hoek A, Schoemaker J, Drexhage HA. Premature ovarian failure and ovarian autoimmunity. Endocr Rev 1997;18:107-134.
24. Rebar RW. Premature ovarian "failure" in the adolescent. Ann NY Acad Sci 2008;1135:138-145.
25. Perheentupa J. APS-I/APECED: the clinical disease and therapy. Endocrinol Metab Clin North Am 2002;31:295-320.
26. Eisenbarth GS, Gottlieb PA. Autoimmune polyendocrine syndromes. N Engl J Med 2004;350:2068-2079.
27. Bakalov VK, Anasti JN, Calis KA, Vanderhoof VH, Premkumar A, Chen S, Furmaniak J, Smith BR, Merino MJ, Nelson LM. Autoimmune oophoritis as a mechanism of follicular dysfunction in women with 46,XX spontaneous premature ovarian failure. Fertil Steril 2005;84:958-965.
28. Welt CK. Autoimmune oophoritis in the adolescent. Ann N Y Acad Sci 2008;1135:118-122.
29. Snieder H, MacGregor AJ, Spector TD. Genes control the cessation of a woman's reproductive life: a twin study of hysterectomy and age at menopause. J Clin Endocrinol Metab 1998;83:1875-1880.
30. Treloar SA, Do KA, Martin NG. Genetic influences on the age at menopause. Lancet 1998;352:1084-1085.
31. van Asselt KM, Kok HS, Pearson PL, Dubas JS, Peeters PH, Te Velde ER, van Noord PA. Heritability of menopausal age in mothers and daughters. Fertil Steril 2004;82:1348-1351.
32. Cramer DW, Xu H, Harlow BL. Family history as a predictor of early menopause. Fertil Steril 1995;64:740-745.
33. Torgerson DJ, Thomas RE, Reid DM. Mothers and daughters menopausal ages: is there a link? Eur J Obstet Gynecol Reprod Biol 1997;74:63-66.
34. Vegetti W, Grazia Tibiletti M, Testa G, de Lauretis Yankowski L, Alagna F, Castoldi E, Taborelli M, Motta T, Bolis PF, Dalpra L, Crosignani PG. Inheritance in idiopathic premature ovarian failure: analysis of 71 cases. Hum Reprod 1998;13:1796-1800.

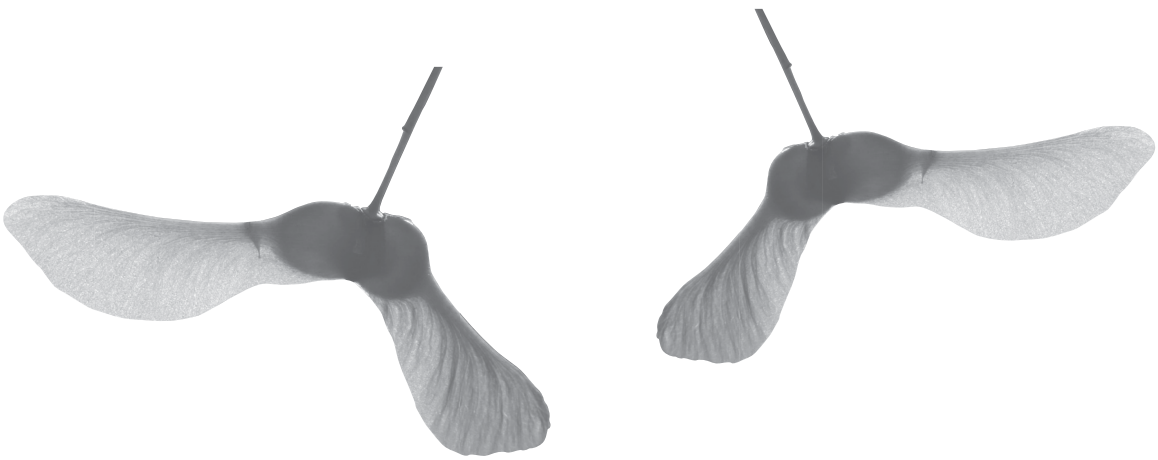
35. Tibiletti MG, Testa G, Vegetti W, Alagna F, Taborelli M, Dalpra L, Bolis PF, Crosignani PG. The idiopathic forms of premature menopause and early menopause show the same genetic pattern. Hum Reprod 1999;14:2731-2734.
36. van Kasteren YM, Hundscheid RD, Smits AP, Cremers FP, van Zonneveld P, Braat DD. Familial idiopathic premature ovarian failure: an overrated and underestimated genetic disease? Hum Reprod 1999;14:2455-2459.
37. Davis CJ, Davison RM, Payne NN, Rodeck CH, Conway GS. Female sex preponderance for idiopathic familial premature ovarian failure suggests an X chromosome defect: opinion. Hum Reprod 2000;15:2418-2422.
38. Vegetti W, Marozzi A, Manfredini E, Testa G, Alagna F, Nicolosi A, Caliari I, Taborelli M, Tibiletti MG, Dalpra L, Crosignani PG. Premature ovarian failure. Mol Cell Endocrinol 2000;161:53-57.
39. Ford CE, Jones KW, Polani PE, De Almeida JC, Briggs JH. A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome). Lancet 1959;1:711-713.
40. Krauss CM, Turksoy RN, Atkins L, McLaughlin C, Brown LG, Page DC. Familial premature ovarian failure due to an interstitial deletion of the long arm of the X chromosome. N Engl J Med 1987;317:125-131.
41. Powell CM, Taggart RT, Drumheller TC, Wangsa D, Qian C, Nelson LM, White BJ. Molecular and cytogenetic studies of an X;autosome translocation in a patient with premature ovarian failure and review of the literature. Am J Med Genet 1994;52:19-26.
42. Marozzi A, Manfredini E, Tibiletti MG, Furlan D, Villa N, Vegetti W, Crosignani PG, Ginelli E, Meneveri R, Dalpra L. Molecular definition of Xq common-deleted region in patients affected by premature ovarian failure. Hum Genet 2000;107:304-311.
43. Schlessinger D, Herrera L, Crisponi L, Mumm S, Percesepe A, Pellegrini M, Pilia G, Forabosco A. Genes and translocations involved in POF. Am J Med Genet 2002;111:328-333.
44. Wittenberger MD, Hagerman RJ, Sherman SL, Conkie-Rosell A, Welt CK, Rebar RW, Corrigan EC, Simpson JL, Nelson LM. The *FMR1* premutation and reproduction. Fertil Steril 2007;87:456-465.
45. Sherman S, Pletcher BA, Driscoll DA. Fragile-X syndrome: diagnostic and carrier testing. Genet Med 2005;7:584-587.
46. Kronquist KE, Sherman SL, Spector EB. Clinical significance of tri-nucleotide repeats in Fragile-X testing: a clarification of American College of Medical Genetics guidelines. Genet Med 2008;10:845-847.
47. Sherman SL. Premature ovarian failure in the fragile-X syndrome. Am J Med Genet 2000;97:189-194.
48. Aaltonen J, Bjorses P, Sandkuijl L, Perheentupa J, Peltonen L. An autosomal locus causing autoimmune disease: autoimmune polyglandular disease type I assigned to chromosome 21. Nat Genet 1994;8:83-87.

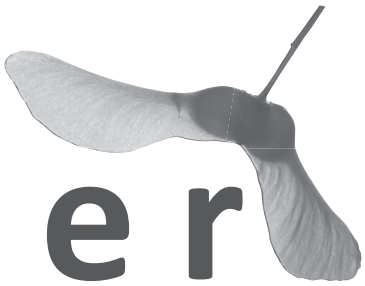
49. Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G. The putative forkhead transcription factor *FOXL2* is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 2001;27:159-166.
50. Waggoner DD, Buist NR, Donnell GN. Long-term prognosis in galactosaemia: results of a survey of 350 cases. J Inherit Metab Dis 1990;13:802-818.
51. Knauff EA, Richardus R, Eijkemans MJ, Broekmans FJ, de Jong FJ, Fauser BC, Bosch AM. Heterozygosity for the classical galactosemia mutation does not affect ovarian reserve and menopausal age. Reprod Sci 2007;14:780-785.
52. Rossetti R, Di Pasquale E, Marozzi A, Bione S, Toniolo D, Grammatico P, Nelson LM, Beck-Peccoz P, Persani L. *BMP15* mutations associated with primary ovarian insufficiency cause a defective production of bioactive protein. Hum Mutat 2009;30:804-810.
53. Lourenço D, Brauner R, Lin L, De Perdigo A., Weryha G, Muresan M, Boudjenah R, Guerra-Junior G, Maciel-Guerra AT, Achermann JC, McElreavey K, Bashamboo A. Mutations in *NR5A1* associated with ovarian insufficiency. N Engl J Med 2009;360:1200-1210.
54. Latronico AC, Chai Y, Arnhold IJ, Liu X, Mendonca BB, Segaloff DL. A homozygous microdeletion in helix 7 of the luteinizing hormone receptor associated with familial testicular and ovarian resistance is due to both decreased cell surface expression and imp*AIRE*d effector activation by the cell surface receptor. Mol Endocrinol 1998;12:442-450.
55. Qin Y, Choi Y, Zhao H, Simpson JL, Chen ZJ, Rajkovic A. *NOBOX* homeobox mutation causes premature ovarian failure. Am J Hum Genet 2007;81:576-581.
56. Bromberger JT, Matthews KA, Kuller LH, Wing RR, Meilahn EN, Plantinga P. Prospective study of the determinants of age at menopause. Am J Epidemiol 1997;145:124-133.
57. van Asselt KM, Kok HS, van der Schouw YT, Grobbee DE, te Velde ER, Pearson PL, Peeters PH. Current smoking at menopause rather than duration determines the onset of natural menopause. Epidemiology 2004;15:634-639.
58. Lamberts SW, Uitterlinden AG. Genetic Testing in Clinical Practice. Annu Rev Med 2008;60:431-442.
59. Manolio TA. Cohort studies and the genetics of complex disease. Nat Genet 2009;41:5-6.
60. Oldenburg RA, van Dooren MF, de Graaf B, Simons E, Govaerts L, Swagemakers S, Verkerk JM, Oostra BA, Bertoli-Avella AM. A genome-wide linkage scan in a Dutch family identifies a premature ovarian failure susceptibility locus. Hum Reprod 2008;23:2835-2841.
61. Knauff EA, Franke L, van Es MA, van den Berg LH, van der Schouw YT, Laven JS, Lambalk CB, Hoek A, Goverde AJ, Christin-Maitre S, Hsueh AJ, Wijmenga C, Fauser BC. Genome-wide association study in premature ovarian failure patients suggests *ADAMTS19* as a possible candidate gene. Hum Reprod 2009;24:2372-2378.

62. van Dooren MF, Bertoli-Avella AM, Oldenburg RA. Premature ovarian failure and gene polymorphisms. Curr Opin Obstet Gynecol 2009;21:313-317.
63. Knauff EA, Blauw HM, Pearson PL, Kok K, Wijmenga C, Veldink JH, van den Berg LH, Bouchard P, Fauser BC, Franke L. Copy number variants on the X chromosome in women with primary ovarian insufficiency. Fertil Steril 2011;95:1584-1588.
64. Nelson LM, Anasti JN, Kimzey LM, Defensor RA, Lipetz KJ, White BJ, Shawker TH, Merino MJ. Development of luteinized graafian follicles in patients with karyotypically normal spontaneous premature ovarian failure. J Clin Endocrinol Metab 1994;79:1470-1475.
65. Conway GS, Kaltsas G, Patel A, Davies MC, Jacobs HS. Characterization of idiopathic premature ovarian failure. Fertil Steril 1996;65:337-341.
66. van Kasteren YM, Schoemaker J. Premature ovarian failure: a systematic review on therapeutic interventions to restore ovarian function and achieve pregnancy. Hum Reprod Update 1999;5:483-492.
67. Bidet M, Bachelot A, Touraine P. Premature ovarian failure: predictability of intermittent ovarian function and response to ovulation induction agents. Curr Opin Obstet Gynecol 2008;20:416-420.
68. Broer SL, Mol BW, Hendriks D, Broekmans FJM. The role of antimullerian hormone in prediction of outcome after IVF: comparison with the antral follicle count. Fertil Steril 2009;91:705-714.
69. Knauff EAH, Eijkemans MJC, Lambalk CB, ten Kate-Booij MJ, Hoek A, Beerendonk CCM, Laven JSE, Goverde AJ, Broekmans FJM, Themmen APN, de Jong FH, Fauser BCJM, on behalf of the Dutch Premature Ovarian Failure Consortium. Anti-mullerian hormone, inhibin B, and antral follicle count in young women with ovarian failure. J Clin Endocrinol Metab 2009;94:786-792.
70. Silber S, Kagawa N, Kuwayama M, Gosden R. Duration of fertility after fresh and frozen ovary transplantation. Fertil Steril 2010;94:2191-2196.
71. Baker V. Life plans and family-building options for women with primary ovarian insufficiency. Semin Reprod Med 2011;29:362-372.
72. Schover LR. Premature ovarian failure and its consequences: vasomotor symptoms, sexuality, and fertility. J Clin Oncol 2008;26:753-758.
73. Groff AA, Covington SN, Halverson LR, Fitzgerald OR, Vanderhoof V, Calis K, Nelson LM. Assessing the emotional needs of women with spontaneous premature ovarian failure. Fertil Steril 2005;83:1734-1741.
74. van der Stege JG, Groen H, van Zadelhoff SJ, Lambalk CB, Braat DD, van Kasteren YM, van Santbrink EJ, Apperloo MJ, Weijmar Schultz WC, Hoek A. Decreased androgen concentrations and diminished general and sexual well-being in women with premature ovarian failure. Menopause 2008;15:23-31.
75. Bachmann G, Bancroft J, Braunstein G, Burger H, Davis S, Dennerstein L, Goldstein I, Guay A, Leiblum S, Lobo R, Notelovitz M, Rosen R, Sarrel P, Sherwin B, Simon J, Simpson E, Shifren J, Spark R, Traish A. Female androgen insufficiency: the princeton consensus statement on definition, classification, and assessment. Fertil Steril 2002;77:660-665.

76. Davis SR. When to suspect androgen deficiency other than at menopause. Fertil Steril 2002;77 Suppl 4:S68-S71.
77. Rivera-Woll LM, Papalia M, Davis SR, Burger HG. Androgen insufficiency in women: diagnostic and therapeutic implications. Hum Reprod Update 2004;10:421-432.
78. Duignan NM, Shaw RW, Glass MR, Butt WR, Edwards RL. Sex hormone levels and gonadotrophin release in premature ovarian failure. Br J Obstet Gynaecol 1978;85:862-867.
79. Falsetti L, Scalchi S, Villani MT, Bugari G. Premature ovarian failure. Gynecol Endocrinol 1999;13:189-195.
80. Bernardi F, Hartmann B, Casarosa E, Luisi S, Stomati M, Fadalti M, Florio P, Santuz M, Luisi M, Petraglia F, Genazzani AR. High levels of serum allopregnanolone in women with premature ovarian failure. Gynecol Endocrinol 1998;12:339-345.
81. Hartmann BW, Kirchengast S, Albrecht A, Laml T, Soregi G, Huber JC. Androgen serum levels in women with premature ovarian failure compared to fertile and menopausal controls. Gynecol Obstet Invest 1997;44:127-131.
82. Doldi N, Belvisi L, Bassan M, Fusi FM, Ferrari A. Premature ovarian failure: steroid synthesis and autoimmunity. Gynecol Endocrinol 1998;12:23-28.
83. Benetti-Pinto CL, Bedone AJ, Magna LA. Evaluation of serum androgen levels in women with premature ovarian failure. Fertil Steril 2005;83:508-510.
84. Kalantaridou SN, Calis KA, Vanderhoof VH, Bakalov VK, Corrigan EC, Troendle JF, Nelson LM. Testosterone deficiency in young women with 46,XX spontaneous premature ovarian failure. Fertil Steril 2006;86:1475-1482.
85. Rosner W, Vesper H. Toward excellence in testosterone testing: a consensus statement. J Clin Endocrinol Metab 2010;95:4542-4548.
86. Uygur D, Sengul O, Bayar D, Erdinc S, Batioglu S, Mollamahmutoglu L. Bone loss in young women with premature ovarian failure. Arch Gynecol Obstet 2005;273:17-19.
87. van der Voort DJ, van Der Weijer PH, Barentsen R. Early menopause: increased fracture risk at older age. Osteoporos Int 2003;14:525-530.
88. Leite-Silva P, Bedone A, Pinto-Neto AM, Costa JV, Costa-Paiva L. Factors associated with bone density in young women with karyotypically normal spontaneous premature ovarian failure. Arch Gynecol Obstet 2009;280:177-181.
89. Bachelot A, Rouxel A, Massin N, Dulon J, Courtillot C, Matuchansky C, Badachi Y, Fortin A, Paniel B, Lecuru F, Lefrere-Belda MA, Constancis E, Thibaud E, Meduri G, Mantel A, Misrahi M, Kuttenn F, Touraine P. Phenotyping and genetic studies of 357 consecutive patients presenting Premature Ovarian Failure. Eur J Endocrinol 2009;161:179-187.
90. Atsma F, Bartelink ML, Grobbee DE, van der Schouw YT. Postmenopausal status and early menopause as independent risk factors for cardiovascular disease: a meta-analysis. Menopause 2006;13:265-279.

91. Hu FB, Grodstein F, Hennekens CH, Colditz GA, Johnson M, Manson JE, Rosner B, Stampfer MJ. Age at natural menopause and risk of cardiovascular disease. Arch Intern Med 1999;159:1061-1066.
92. van der Schouw YT, van der Graaf Y, Steyerberg EW, Eijkemans JC, Banga JD. Age at menopause as a risk factor for cardiovascular mortality. Lancet 1996;347:714-718.
93. Snowdon DA, Kane RL, Beeson WL, Burke GL, Sprafka JM, Potter J, Iso H, Jacobs DR, Jr., Phillips RL. Is early natural menopause a biologic marker of health and aging? Am J Public Health 1989;79:709-714.
94. Kok HS, van Asselt KM, van der Schouw YT, van dT, I, Peeters PH, Wilson PW, Pearson PL, Grobbee DE. Heart disease risk determines menopausal age rather than the reverse. J Am Coll Cardiol 2006;47:1976-1983.
95. Knauff EA, Westerveld HE, Goverde AJ, Eijkemans MJ, Valkenburg O, van Santbrink EJ, Fauser BC, van der Schouw YT. Lipid profile of women with premature ovarian failure. Menopause 2008;15:919-923.
96. Kalantaridou SN, Naka KK, Papanikolaou E, Kazakos N, Kravariti M, Calis KA, Paraskevaidis EA, Sideris DA, Tsatsoulis A, Chrousos GP, Michalis LK. Imp*AIRE*d endothelial function in young women with premature ovarian failure: normalization with hormone therapy. J Clin Endocrinol Metab 2004;89:3907-3913.
97. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, Melton LJ, III. Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. Neurology 2007;69:1074-1083.
98. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, Melton LJ, III. Increased risk of parkinsonism in women who underwent oophorectomy before menopause. Neurology 2008;70:200-209.
99. Whalley LJ, Fox HC, Starr JM, Deary IJ. Age at natural menopause and cognition. Maturitas 2004;49:148-156.
100. Lebrun CE, van der Schouw YT, de Jong FH, Pols HA, Grobbee DE, Lamberts SW. Endogenous oestrogens are related to cognition in healthy elderly women. Clin Endocrinol (Oxf) 2005;63:50-55.





Chapter 2

Similar phenotype characteristics comparing familial and sporadic premature ovarian failure (POF)

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Abstract

Objective

Premature ovarian failure (POF) is characterized by secondary amenorrhea prior to age 40 years, along with repeated increased follicle-stimulating hormone and low estrogen concentrations. POF is considered a complex genetic disease with a familial presentation in 12% to 50% of cases. POF may originate from different genes and various gene-environment interactions. The aim of this study was to identify possible differences in phenotype comparing women with familial and sporadic POF.

Methods

A multicenter study was initiated in the Netherlands using standardized phenotyping. For each woman, medical history, menstrual cycle, and fertility and smoking status were assessed and standardized examination was performed. Based on a detailed 3-generation family history, women were identified as having either familial (defined as having at least one relative with POF) or sporadic POF.

Results

A total of 58 familial cases and 142 sporadic cases of POF were identified. Maternal age at menopause was significantly lower in the women with familial compared to the women with sporadic POF (41.0 ± 7.5 and 49.7 ± 2.6 years, respectively; $P < 0.001$). Sex hormone-binding globulin concentration was significantly higher in the women with familial than in the women with sporadic POF (73.6 ± 37.1 and 55.2 ± 26.9 nmol/L, respectively; $P = 0.002$). All other characteristics, such as parity, bone mineral density, and serum follicle-stimulating hormone and lipid levels were similar, as was the incidence of autoimmunity and cytogenetic abnormalities.

Conclusions

Familial and sporadic POF do not differ in phenotype except for maternal menopause age and sex hormone-binding globulin concentration. Future studies are needed to unravel the genotype- phenotype interactions in POF.

Introduction

The mean age at menopause for women in Western industrialized countries is 51 years (1). However, 10% of women become postmenopausal before the age of 45 years and 1% to 2% of women reach menopause before 40 years of age (2,3). The phenomenon of secondary amenorrhea occurring prior to age of 40 years, along with repeated elevated follicle-stimulating hormone (FSH) and low estrogen concentrations is referred to as premature ovarian failure (POF) (2).

Besides its implications for a woman's fertility potential, POF is also associated with a variety of health problems ascribed to deprivation from estrogen from an early age. Studies have shown bone mineral density to be significantly lower in women with POF within 2 years after the diagnosis (4,5). Early menopause is also related to a higher incidence of cardiovascular disease, neurological disease (including stroke), impaired cognition and a reduced life expectancy (6-11). Moreover, well-being is greatly affected by POF, with high incidences of depression and low self-esteem being reported (12).

POF is considered to be a heterogeneous, multicausal disorder. Iatrogenic causes (e.g., extensive pelvic surgery or oophorectomy, radiation, and chemotherapy) explain only 5% of POF cases (13-15). POF is also associated with autoimmunity, such as adrenal disease, oophoritis, or thyroid dysfunction (14,16). However, the role of endocrine autoimmune diseases in developing POF remains unclear, and reported incidences vary between 3% to 20% (14,17). Well-known genetic causes for POF include chromosomal aberrations, such as 45,X mosaicism and other X-chromosomal numerical and structural defects (prevalence of 1%) (18). Up to now, the only common single gene mutation on the X chromosome shown to have a significant prevalence in POF (3% to 15%) is the carrier status of the fragile-X premutation (*FMR1*) (19-22). Recently, mutations in the X-linked ovarian follicle organizer bone morphogenic protein 15 were observed in 2% in a series of 300 patients, and bone morphogenic protein 15 mutation screening in women with POF was suggested (23). Many other candidate genes involving follicle function and oogenesis, such as *FOXL2*, *GDF9*, *NR5A1*, *NOBOX*, *LHR*, and *FSHR*, have been suggested as other less frequent causes of monogenic POF (24-27). Nonetheless, only a small minority of POF cases can be explained by mutations in these candidate genes, and their exact mechanism in the development of POF remains speculative.

In most POF cases, no cause can be established using current methodologies. Therefore, the concept of POF as a complex genetic disease arises. Typically, the phenotype of complex genetic diseases is controlled by many genes and their variants, in interaction with environmental factors (28). Complex genetic disorders are often characterized by familial clustering without an obvious Mendelian pattern of inheritance because, often, several genes, their mutations

and environmental factors contribute (29). Identifying the multitude of genes and gene variants involved in the multifactorial forms of POF has now begun, using techniques to study large numbers of identified polymorphic gene markers, such as single nucleotide polymorphisms or copy number variations in localized chromosomal areas. Such genome-wide association studies initiated by our group and others recently delivered their first results (30,31). Several loci containing interesting candidate genes were identified for POF (30-32).

POF presents in both sporadic and familial forms, and it is estimated that familial POF accounts for 12.5% to 50% of all cases (3,33-38). Varying definitions and different standards for family history and cytogenetic and molecular diagnosis may explain the wide interval in reported incidences. Different genetic makeups and phenotype expressions comparing isolated versus familial POF may be proposed.

Carefully defining familial POF as compared with sporadic POF becomes more relevant as it may influence the informational content of clinical cohorts, because differences in phenotype could indicate that different genes play a role in the development of familial and sporadic POF. The aim of the present study was to identify possible differences in phenotype characteristics and background between familial and sporadic cases of POF.

Materials and methods

Participants

In 2005, we initiated a large nationwide, multicenter study of hypergonadotropic oligomenorrhea/amenorrhea (World Health Organization group 3) (39). Women presenting with cycle abnormalities, for example, oligomenorrhea or amenorrhea, and suspected of a World Health Organization class 3 status were invited to the outpatient clinic for detailed standardized evaluation. All women with POF, defined as spontaneous cessation of menses for at least 4 months in women less than 40 years, and FSH concentrations exceeding 40 IU/L were identified from the databases of participating hospitals. This study was approved by the local ethics committees of all participating hospitals, and all women included gave written informed consent.

We defined POF as familial when the index woman had at least one family member also affected by POF. POF was defined as sporadic when the index woman was the only family member affected by POF and did not have any other family member affected by early menopause (menopause before the age of 45 years). The remaining POF women who met neither of our definitions of familial or POF were excluded from the current analysis.

Women were asked about their medical history, particularly relating to age at menarche, menstrual cycle irregularities, menopausal age, obstetric history, symptoms of hypothyroidism and hyperthyroidism, as well as alcohol consump-

tion and smoking status (current, former or never smokers). Ethnicity and socioeconomic status were assessed, the latter being based on educational level and profession. Women filled out standardized questionnaires concerning family history, with a special focus on menopause age, menstrual cycle irregularity, parity, fertility problems, and cardiovascular disease. Family history covered three generations, including paternal and maternal grandparents, paternal and maternal aunts and uncles, and the proband's parents and siblings.

Physical examination was performed, including height and weight measurement. Blood pressure was measured twice in sitting position after 10 minutes of rest with calibrated IntelliSense M5-I (Omron)/HEM 757A-E (Omron Healthcare Europe, Hoofddorp, Netherlands). Transvaginal ultrasound was performed to assess the antral follicle count (AFC), using the 7.5 MHz transvaginal probe on a Voluson 530D (Kretz Technik, Zipf, Austria). AFC is calculated by adding up the follicles with a diameter of 2 to 5 millimeters from both ovaries.

Serum samples were drawn in a fasting state for measurement of serum FSH, luteinizing hormone, estradiol, progesterone, testosterone, 17-hydroxyprogesterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, sex hormone-binding globulin (SHBG), androstenedione, cortisol, anti-müllerian hormone, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total cholesterol, triglycerides, glucose, insulin and thyrotropin levels. Free Androgen Index (FAI) was calculated as follows: testosterone/SHBG x 100. To obtain valid laboratory results, none of the women had used any hormonal medication for at least two weeks. Details on laboratory testing, for example interassay and intra-assay coefficients of variation and detection limits, have been described in previously published papers from our group (40,41). In addition, serum was screened for autoimmunity (antithyroid peroxidase, antiadrenal, antiparietal, antiovarian, antithyrotropin receptor autoantibodies). Karyotyping and DNA testing for fragile-X premutation (FRAXA) were done. Finally, bone densitometry was performed using Hologic Discovery A densitometer (serial no. 80675, Tromp Medical, Castricum, Netherlands) if at least six months of amenorrhea had passed.

Statistical analysis

Descriptive and reproductive parameters and endocrine and lipid measurements are reported as means ± SD. Categorical data are expressed as percentages. The differences between the two groups for mean age at screening, menarche, childbirth and secondary amenorrhea, as well as for levels of lipids, gonadotropins and steroids; blood pressure; body mass index; and bone densitometry, were statistically tested using the Student's *t* test. Because high-density lipoprotein cholesterol and triglyceride values, as well as most of the gonadotropins did not show a normal distribution, logarithmic transformation was performed before executing the Student's *t* test. We used χ^2 tests to assess statistical differences between groups for the difference in education, ethnicity, smoking

status, alcohol consumption, reproductive characteristics, vaginal ultrasound, and the occurrence of autoimmunity, karyotypical abnormalities, and FRAXA premutation. Correction for age at screening was done by analysis of covariance for all variables. Logistic regression with a backward stepwise selection method was performed for parameters with a $P < 0.05$, and age at screening forced in as covariate to determine independent predictive variables for familial versus sporadic POF. Statistical analysis was performed using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL).

Results

A total of 256 consecutive women were diagnosed with POF. Women were mostly referred to the outpatient clinic for investigation of menstrual cycle irregularities or infertility or for counseling after a previous diagnosis or positive family history of POF. A total of 53 women with familial early menopause but without relatives affected by POF were identified. This group was excluded from further analysis. There were 61 women with familial POF and 142 women with sporadic POF identified. Within the familial cases, three pairs of index patients from the same families were identified. To avoid biased results based on the assumption that two family members might share genetic and environmental factors and, thus, may have a similar phenotypical presentation, we included from each pair only the patient who presented herself first at the outpatient clinic (*Figure 1*).

The descriptive characteristics of both patient groups are shown in *Table 1*. Mean age at screening was statistically significantly different between the two groups (women with familial POF, 38.8 ± 6.4 years; women with sporadic POF, 35.4 ± 7.3 years; $P = 0.002$). Educational level, smoking status, and ethnicity did not differ significantly between groups. Alcohol consumption, however, was significantly higher in women with familial POF compared to women with sporadic POF ($P = 0.048$). For physical examination parameters, no significant differences were identified.

Table 2 lists the reproductive characteristics of the two groups. Mean age of secondary amenorrhea related to menopause was similar in both groups, as were parity, age at first and last childbirth, and the incidence of miscarriages. Previous fertility treatment had been sought by 27% and 29% of the familial and sporadic groups, respectively, and the incidence of familial fertility problems was similar in both groups. However, age at menopause of the mothers of the index women was significantly lower for familial cases than for sporadic cases (41.0 ± 7.5 and 49.7 ± 2.6 years, respectively; $P < 0.001$). The parity of mothers of index women was not significantly different. The use of oral contraceptives or hormone therapy was equal in both groups. No differences were found for antral follicle count on transvaginal ultrasound.

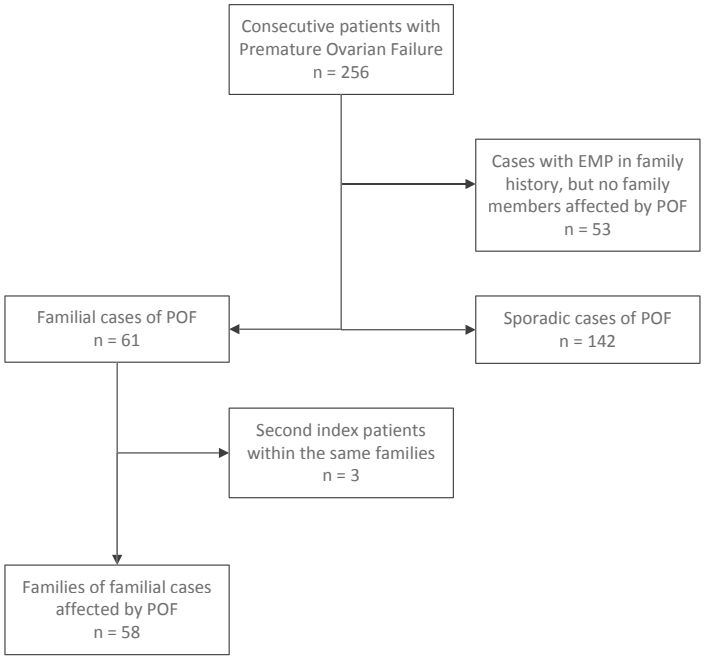

Figure 1. Flowchart for the selection of women.
Legend: EMP, early menopause; POF, premature ovarian failure.

Laboratory findings are reported in *Table 3*. Gonadotropin and sex hormone levels did not differ significantly between the two groups, except for SHBG (73.6 ± 37.1 nmol/L in women with familial POF vs. 55.2 ± 26.9 nmol/L in women with sporadic POF; P = 0.002) and free androgen index (1.89 ± 1.29 in women with familial POF vs. 2.45 ± 1.47 in women with sporadic POF, P = 0.008). The lipid profile, also when adjusted for age at screening, was similar for both POF groups, and all lipid levels were within the normal physiological range.

Table 4 displays the relations between possible etiological factors and the occurrence of POF. No differences for autoimmunity were found between the two groups. The incidence of numeric cytogenetic abnormalities was 13% in women with familial POF and 6% in women with sporadic POF (P = 0.23). The numeric cytogenetic abnormalities found were mostly 46,XX/45,XO mosaicism (familial POF, n = 4; sporadic POF, n = 4) and four cases of 46,XX/47,XXX mosaicism (two in each POF group). The incidences of fragile-X premutation were similar in the familial and sporadic POF groups.

Correction for age at screening did not create significant differences in any of the variables. Logistic regression shows that maternal age at menopause and SHBG are both independently associated with familial versus sporadic POF (P < 0.001 for both parameters).

Table 1. Descriptive characteristics of 200 women with POF

	Familial POF (n = 58)	Sporadic POF (n = 142)	P	P adjusted for age at screening
Age at screening, years	38.8 ± 6.4	35.4 ± 7.3	0.002	
Educational level			0.67	0.68
Primary school	1 (2)	7 (5)		
Secondary school	9 (15)	13 (10)		
Vocational education	22 (39)	48 (36)		
College	17 (30)	40 (30)		
University	8 (14)	26 (19)		
Smoking status			0.13	0.10
Never	24 (44)	79 (56)		
Quit	15 (27)	22 (16)		
Current	16 (29)	40 (28)		
Smoking status, pack year				
Quit	6.3 ± 4.2	8.1 ± 5.6	0.30	0.31
Current	10.8 ± 8.5	9.3 ± 8.8	0.56	0.77
Alcohol consumption			0.51	0.85
Yes	34 (59)	76 (54)		
No	24 (41)	66 (47)		
Alcohol consumption, U/wk	5.9 ± 4.9	4.0 ± 3.1	0.048	0.040
Incidence of cardiovascular disease in family	10 (17)	19 (13)	0.48	0.23
Ethnicity			0.42	0.44
Caucasian	52 (96)	113 (80)		
Black	2 (3)	10 (7)		
Mediterranean	1 (2)	11 (8)		
Asian	1 (2)	3 (2)		
Other	2 (3)	5 (3)		
BMI, kg/m^2	23.3 ± 7.6	23.6 ± 7.2	0.81	0.89
Systolic blood pressure, mmHg	128.2 ± 17.0	126.5 ± 17.7	0.57	0.71
Diastolic blood pressure, mmHg	83.0 ± 11.2	81.1 ± 10.4	0.31	0.60
Bone densitometry, Z score				
Lumbar spine	-0.57 ± 1.29	-0.54 ± 1.14	0.91	0.79
Left hip	-0.21 ± 0.91	-0.19 ± 0.97	0.92	0.75
Right hip	-0.34 ± 0.97	-0.15 ± 0.98	0.39	0.31

Data are shown as mean ± SD or n (%).
POF, premature ovarian failure; BMI, body mass index.

Table 2. Reproductive characteristics of 200 women with POF

	Familial POF (n = 58)	Sporadic POF (n = 142)	P	P adjusted for age at screening
Age at menarche, years	12.9 ± 1.4	13.2 ± 1.6	0.27	0.31
Age at secondary amenorrhea, years	32.8 ± 5.7	32.1 ± 8.6	0.48	0.42
Parity			0.46	0.67
0	31 (54)	87 (61)		
1	13 (22)	31 (22)		
≥ 2	14 (24)	24 (17)		
Age at first childbirth, years	29.3 ± 3.6	28.4 ± 4.1	0.54	0.53
Age at last childbirth, years	29.9 ± 3.2	29.7 ± 3.8	0.85	0.88
Incidence of miscarriages	3 (5)	19 (13)	0.10	0.07
Parity of mothers of index patients	3.1 ± 1.4	3.5 ± 1.9	0.10	0.08
Incidence of index patients being a single child	2 (3)	5 (4)	0.98	0.60
Age at menopause in mothers of index patients, years	41.0 ± 7.5	49.7 ± 2.6	<0.001	<0.001
Incidence of fertility problems in family	20 (35)	36 (25)	0.19	0.25
Incidence of ever use of oral contraceptives	54 (93)	122 (86)	0.16	0.16
Duration of use of oral contraceptives, years	11.2 ± 6.8	10.3 ± 6.7	0.35	0.96
Current hormone therapy	18 (31)	34 (24)	0.30	0.22
History of hormone therapy	7 (14)	21 (17)	0.56	0.28
Cycle before secondary amenorrhea			0.25	0.66
Unknown	5 (9)	26 (21)		
Regular	10 (19)	19 (15)		
Oligomenorrhea	25 (46)	44 (36)		
Shortened cycle	3 (6)	4 (3)		
Oral contraceptives	11 (20)	27 (22)		
Pregnancy	0	4 (3)		
Treatment for infertility	17 (29)	38 (27)	0.71	0.96
Vaginal ultrasound			0.10	0.71
Ovaries				
Ovaries both visible	33 (65)	76 (56)		
One ovary visible	3 (6)	25 (19)		
Ovaries not visible	15 (29)	34 (25)		
AFC[a]	0.5 ± 1.3	0.9 ± 1.8	0.22	0.32

Data are shown as mean ± SD or n (%).
POF, premature ovarian failure; AFC, antral follicle count.
a AFC was only calculated when both ovaries were visible.

Table 3. Laboratory findings of 200 women with POF

	Familial POF (n = 58)	Sporadic POF (n = 142)	P[a]	P adjusted for age at screening
Gonadotropins, sex hormones				
FSH, IU/L	82.3 ± 43.5	80.4 ± 41.5	0.78	0.80
LH, IU/L	41.9 ± 30.0	40.0 ± 20.5	0.93	0.97
E_2, pmol/L	133.7 ± 151.1	175.3 ± 230.8	0.88	0.89
Progesterone, nmol/L	3.00 ± 5.59	3.99 ± 6.82	0.39	0.33
17-OH progesterone, nmol/L	1.93 ± 2.87	1.78 ± 2.21	0.78	0.85
Prolactin, U/L	0.17 ± 0.06	0.19 ± 0.11	0.14	0.10
Testosterone, nmol/L	1.09 ± 0.37	1.10 ± 0.44	0.96	0.96
SHBG, nmol/L	73.6 ± 37.1	55.2 ± 26.9	0.002	0.005
FAI	1.89 ± 1.29	2.45 ± 1.47	0.008	0.022
Androstenedione, nmol/L	3.07 ± 0.99	3.43 ± 1.65	0.36	0.80
DHEA, nmol/L	13.6 ± 6.8	17.1 ± 11.6	0.12	0.51
DHEA-S, µmol/L	3.89 ± 1.81	4.54 ± 2.21	0.13	0.40
Cortisol, µmol/L	0.32 ± 0.10	0.32 ± 0.15	0.56	0.57
TSH, mU/L	1.76 ± 0.99	2.16 ± 2.06	0.43	0.54
Lipids				
LDL-C, mmol/L	2.96 ± 0.83	2.92 ± 0.78	0.78	0.77
HDL-C, mmol/L	1.81 ± 0.56	1.69 ± 0.42	0.32	0.53
Total Cholesterol, mmol/L	5.20 ± 0.97	5.03 ± 0.87	0.31	0.81
Triglycerides, mmol/L	0.94 ± 0.49	0.93 ± 0.46	0.92	0.76
Glucose, mmol/L	5.00 ± 0.49	5.18 ± 1.50	0.58	0.55
Insulin, mU/L	6.62 ± 5.14	7.85 ± 8.05	0.12	0.32

Data are shown as mean ± SD.
POF, premature ovarian failure; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E_2, estradiol; SHBG, sex hormone-binding globulin; FAI, free androgen index; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.
a Calculated using log transformed data value (data not shown).

Table 4. Etiological characteristics of 200 women with POF

	Familial POF (n = 58)	Sporadic POF (n = 142)	P	P adjusted for age at screening
Autoimmunity				
Anti-TPO positive[a,b]	6 (13)	20 (20)	0.31	0.30
Anti-adrenal positive[c]	0	2 (2)	0.33	1.00
Anti-ovarian positive[d]	0	1 (3)	0.51	1.00
Anti-parietal positive[e]	4 (4)	7 (9)	0.72	0.57
Anti-TSH receptor positive[f]	0	2 (29)	0.16	1.00
Genetic				
Cytogenetic abnormalities[g]				
None	39 (85)	89 (88)		
Mosaicism	6 (13)	6 (6)		
Other abnormality[h]	1 (2)	6 (6)	0.23	0.77
Fragile-X premutation present[i]	3 (8)	6 (7)	0.83	0.98

Data are presented as n (%).
TPO, thyroid peroxidase.
a Anti-TPO positive: ≥60 U/mL; b n = 143; c n = 142; d n = 107; e n = 117; f n = 13; g n = 147; h Other abnormalities include translocations and deletions; i n = 119.

Discussion

To our knowledge, the current series represents the most comprehensively phenotyped and largest study population of women with POF for which a comparison of familial and sporadic cases by standardized screening was performed. In the current group of 200 women with POF, maternal age at menopause was lower and SHBG concentrations were significantly higher in women with familial POF. Other descriptive and reproductive characteristics, laboratory findings, and etiological characteristics were similar among both groups.

We defined POF as familial when an index woman with POF had at least one relative with POF. Our definition is more restrictive compared to most other studies that also included a family member affected by menopause before 45 years of age (34,35). Studies using these definitions reported an incidence of familial POF of between 28.5% and 50% (33-38). We observed an incidence of familial POF of 29% in the current study population. We chose to exclude the POF cases where family members presented only with early menopause, because it remains uncertain whether early menopause represents a continuum with POF.

POF is a condition that greatly reduces fertility and thereby generates small families. A downside in the recruitment of familial POF patients is the possible overrepresentation of sporadic POF because families could simply be too small to

show a familial effect of POF. In addition, infertility and premature menopause may not be openly discussed in families and families with broken ties. The widespread use of oral contraceptives adds to the possible underdetection of familial POF. Taking this into consideration, careful family history taking and recruitment of affected and unaffected siblings for detailed evaluation are of great importance.

In our study group, maternal age at menopause was significantly lower in women with familial POF, which should come as no surprise because having a mother with menopause before 40 years of age fits the inclusion criteria for familial POF. Indeed, in our population, 32 (55%) women with familial POF had a mother affected by POF. This is in concordance with other reports in which heritability of menopause age was shown to be a risk factor for developing POF (3,42,43). These observations are plausible in view of a familial pattern of reproductive performance and age at menopause in particular. Another interesting phenomenon that has been described within POF families is the phenomenon genetic anticipation (30). This phenomenon could also account for the earlier presentation of POF in consecutive generations.

Conflicting data have been published regarding the influence of premenopausal risk profile on the development of POF, the incidence of cardiovascular disease and the lipid profile in POF women (8,9,40,44). We did not identify any differences in the incidence of familial cardiovascular disease or lipid profile comparing women with sporadic and women with familial POF, and all lipid levels were within the reference range. Moreover, the incidence of smoking and pack years of smoking did not differ between both groups. Interestingly, SHBG concentrations were significantly higher in women with familial POF. Because hormone therapy use and body mass index were similar in both groups, we currently do not have an explanation for this observation. Further investigation of the role of SHBG in relation to POF is needed.

Proven etiological factors for POF are autoimmunity, cytogenetic abnormalities, and the fragile-X premutation. If different genes underlie sporadic and familial POF, it may be expected that the incidence of autoimmunity in familial POF is different when compared to sporadic POF. Surprisingly, our findings again do not support this hypothesis. This also holds true for the incidence of cytogenetic abnormalities, although there seems to be a trend towards a higher incidence of numeric cytogenetic abnormalities in familial POF. The cytogenetic abnormalities account, in part, for the appearance of POF in our women with familial POF, which may be in concordance with earlier reports on familial mosaicism (45). It is possible that our series is still underpowered to show more subtle differences. We did not explore cytogenetic abnormalities within families because the aim of this study was to identify familial and sporadic cases according to phenotype characteristics of index women.

In a recently reported series of 357 women with POF, also including hypergonadotropic primary amenorrhea, similar findings for gonadotropins and ovarian and adrenal autoimmunity (2% and 1%, respectively) compared to ours were reported (46). However, the incidence of *FMR1* prematutation carriers (4%) in this population was surprisingly low compared to ours, which was 7% and 8% in the familial and sporadic groups, respectively. However, all findings fall within the already reported incidences in the literature (22,47). Addressing the same etiological factors as before, we were able to identify the genetic etiology of POF in 18 (12.7%) out of 142 sporadic cases and in 10 (17.2%) out of 58 familial cases, which is higher than reported earlier (7.8%) (46).

Future studies concerning the origin of this multigenic, complex disease are needed to unravel the genotype-phenotype relationships in the development of POF. As proposed recently, mutations in coding regions of candidate genes need to be further examined. However, because these genes account for small numbers women with POF, the regulatory regions and molecular pathways also need to be studied (24). Recent application of new techniques, such as genome-wide association studies, has provided promising results (30-32). The present study shows that there is no need to distinguish between familial and sporadic cases of POF when genome-wide association studies are performed.

An important issue is the recruitment of large cohorts, also including well-defined POF families, to be able to identify multiple low-risk genes. Only under those conditions can a possible relationship between genotype and phenotype be explored further.

Conclusion

The current study suggests that familial POF only differs in phenotype from sporadic POF with regard to maternal menopausal age and SHBG concentration. Therefore, it remains a difficult task counseling women with a positive family history for POF with regard to fertility in the future. There are no indications for different follow-up strategies in familial and sporadic idiopathic POF. In addition, the similar phenotype characteristics in sporadic and familial POF allow genetic studies to be done without subdivisions. Future studies on the origin of POF are needed, and institutional cooperation should be emphasized in order to create sufficient recruitment.

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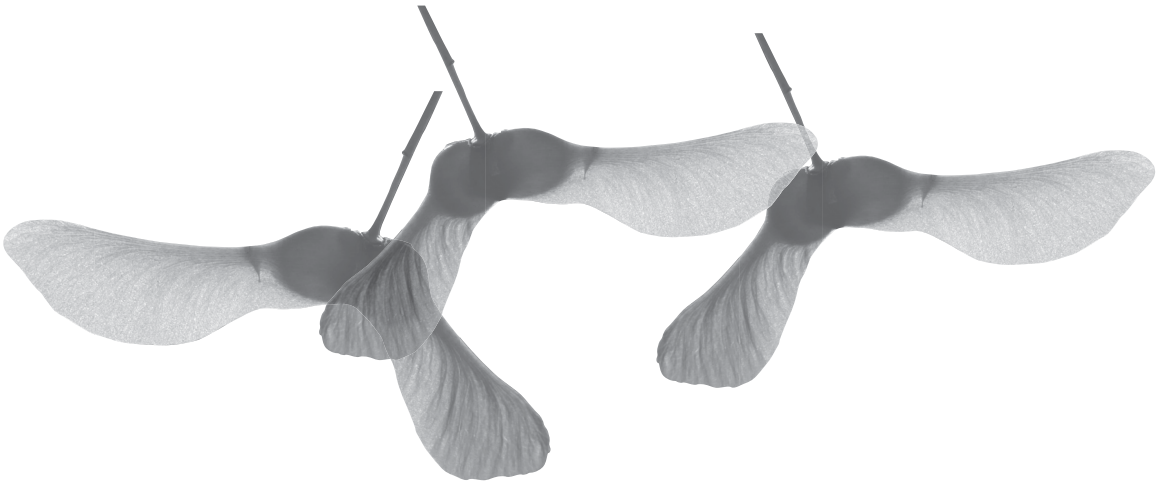
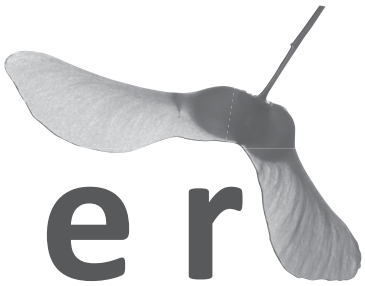
References

1. Treloar AE. Menstrual cyclicity and the pre-menopause. Maturitas 1981;3:249-264.
2. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986;67:604-606.
3. Torgerson DJ, Thomas RE, Reid DM. Mothers and daughters menopausal ages: is there a link? Eur J Obstet Gynecol Reprod Biol 1997;74:63-66.
4. Anasti JN, Kalantaridou SN, Kimzey LM, Defensor RA, Nelson LM. Bone loss in young women with karyotypically normal spontaneous premature ovarian failure. Obstet Gynecol 1998;91:12-15.
5. Uygur D, Sengul O, Bayar D, Erdinc S, Batioglu S, Mollamahmutoglu L. Bone loss in young women with premature ovarian failure. Arch Gynecol Obstet 2005;273:17-19.
6. van der Schouw YT, van der Graaf Y, Steyerberg EW, Eijkemans JC, Banga JD. Age at menopause as a risk factor for cardiovascular mortality. Lancet 1996;347:714-718.
7. Ossewaarde ME, Bots ML, Verbeek AL, Peeters PH, van der Graaf Y, Grobbee DE, van der Schouw YT. Age at menopause, cause-specific mortality and total life expectancy. Epidemiology 2005;16:556-562.
8. Kok HS, van Asselt KM, van der Schouw YT, van der Tweel I, Peeters PH, Wilson PW, Pearson PL, Grobbee DE. Heart disease risk determines menopausal age rather than the reverse. J Am Coll Cardiol 2006;47:1976-1983.
9. Atsma F, Bartelink ML, Grobbee DE, van der Schouw YT. Postmenopausal status and early menopause as independent risk factors for cardiovascular disease: a meta-analysis. Menopause 2006;13:265-279.
10. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, Melton LJ, III. Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. Neurology 2007;69:1074-1083.
11. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, Melton LJ, III. Increased risk of parkinsonism in women who underwent oophorectomy before menopause. Neurology 2008;70:200-209.
12. Liao KL, Wood N, Conway GS. Premature menopause and psychological well-being. J Psychosom Obstet Gynaecol 2000;21:167-174.
13. Coulam CB, Stringfellow S, Hoefnagel D. Evidence for a genetic factor in the etiology of premature ovarian failure. Fertil Steril 1983;40:693-695.
14. Hoek A, Schoemaker J, Drexhage HA. Premature ovarian failure and ovarian autoimmunity. Endocr Rev 1997;18:107-134.
15. Rebar RW. Premature ovarian “failure” in the adolescent. Ann NY Acad Sci 2008;1135:138-145.
16. Goswami D, Conway GS. Premature ovarian failure. Hum Reprod Update 2005;11:391-410.
17. Alper MM, Garner PR. Premature ovarian failure: its relationship to autoimmune disease. Obstet Gynecol 1985;66:27-30.

18. Schlessinger D, Herrera L, Crisponi L, Mumm S, Percesepe A, Pellegrini M, Pilia G, Forabosco A. Genes and translocations involved in POF. Am J Med Genet 2002;111:328-333.
19. Mattei MG, Mattei JF, Ayme S, Giraud F. X-autosome translocations: cytogenetic characteristics and their consequences. Hum Genet 1982;61:295-309.
20. Sherman SL. Premature ovarian failure in the fragile-X syndrome. Am J Med Genet 2000;97:189-194.
21. Mumm S, Herrera L, Waeltz PW, Scardovi A, Nagaraja R, Esposito T, Schlessinger D, Rocchi M, Forabosco A. X/autosomal translocations in the Xq critical region associated with premature ovarian failure fall within and outside genes. Genomics 2001;76:30-36.
22. Wittenberger MD, Hagerman RJ, Sherman SL, Conkie-Rosell A, Welt CK, Rebar RW, Corrigan EC, Simpson JL, Nelson LM. The *FMR1* premutation and reproduction. Fertil Steril 2007;87:456-465.
23. Rossetti R, Di Pasquale E, Marozzi A, Bione S, Toniolo D, Grammatico P, Nelson LM, Beck-Peccoz P, Persani L. *BMP15* mutations associated with primary ovarian insufficiency cause a defective production of bioactive protein. Hum Mutat 2009;30:804-810.
24. Laissue P, Vinci G, Veitia RA, Fellous M. Recent advances in the study of genes involved in non-syndromic premature ovarian failure. Mol Cell Endocrinol 2008;282:101-111.
25. Skillern A, Rajkovic A. Recent developments in identifying genetic determinants of premature ovarian failure. Sex Dev 2008;2:228-243.
26. Simpson JL. Genetic and phenotypic heterogeneity in ovarian failure: overview of selected candidate genes. Ann N Y Acad Sci 2008;1135:146-154.
27. Lourenço D, Brauner R, Lin L, De Perdigo A., Weryha G, Muresan M, Boudjenah R, Guerra-Junior G, iel-Guerra AT, Achermann JC, McElreavey K, Bashamboo A. Mutations in *NR5A1* associated with ovarian insufficiency. N Engl J Med 2009;360:1200-1210.
28. Lamberts SW, Uitterlinden AG. Genetic Testing in Clinical Practice. Annu Rev Med 2008;60:431-442.
29. Manolio TA. Cohort studies and the genetics of complex disease. Nat Genet 2009;41:5-6.
30. Oldenburg RA, van Dooren MF, de Graaf B, Simons E, Govaerts L, Swagemakers S, Verkerk JM, Oostra BA, Bertoli-Avella AM. A genome-wide linkage scan in a Dutch family identifies a premature ovarian failure susceptibility locus. Hum Reprod 2008;23:2835-2841.
31. Knauff EA, Franke L, van Es MA, van den Berg LH, van der Schouw YT, Laven JS, Lambalk CB, Hoek A, Goverde AJ, Christin-Maitre S, Hsueh AJ, Wijmenga C, Fauser BC. Genome-wide association study in premature ovarian failure patients suggests *ADAMTS19* as a possible candidate gene. Hum Reprod 2009;24:2372-2378.
32. van Dooren MF, Bertoli-Avella AM, Oldenburg RA. Premature ovarian failure and gene polymorphisms. Curr Opin Obstet Gynecol 2009;21:313-317.
33. Cramer DW, Xu H, Harlow BL. Family history as a predictor of early menopause. Fertil Steril 1995;64:740-745.
34. Vegetti W, Grazia Tibiletti M, Testa G, de Lauretis Yankowski L, Alagna F, Castoldi E, Taborelli M, Motta T, Bolis PF, Dalpra L, Crosignani PG. Inheritance in idiopathic premature ovarian failure: analysis of 71 cases. Hum Reprod 1998;13:1796-1800.

35. Tibiletti MG, Testa G, Vegetti W, Alagna F, Taborelli M, Dalpra L, Bolis PF, Crosignani PG. The idiopathic forms of premature menopause and early menopause show the same genetic pattern. Hum Reprod 1999;14:2731-2734.
36. van Kasteren YM, Hundscheid RD, Smits AP, Cremers FP, van ZP, Braat DD. Familial idiopathic premature ovarian failure: an overrated and underestimated genetic disease? Hum Reprod 1999;14:2455-2459.
37. Vegetti W, Marozzi A, Manfredini E, Testa G, Alagna F, Nicolosi A, Caliari I, Taborelli M, Tibiletti MG, Dalpra L, Crosignani PG. Premature ovarian failure. Mol Cell Endocrinol 2000;161:53-57.
38. Davis CJ, Davison RM, Payne NN, Rodeck CH, Conway GS. Female sex preponderance for idiopathic familial premature ovarian failure suggests an X chromosome defect: opinion. Hum Reprod 2000;15:2418-2422.
39. Rowe PJ, Comhaire FH, Hargreave TB, Mellows HJ. WHO Manual for the standardized investigation and diagnosis of the infertile couple. 1993;First Ed. Cambridge, UK: Press Syndicate of the University of Cambridge.
40. Knauff EA, Westerveld HE, Goverde AJ, Eijkemans MJ, Valkenburg O, van Santbrink EJ, Fauser BC, van der Schouw YT. Lipid profile of women with premature ovarian failure. Menopause 2008;15:919-923.
41. Knauff EAH, Eijkemans MJC, Lambalk CB, ten Kate-Booij MJ, Hoek A, Beerendonk CCM, Laven JSE, Goverde AJ, Broekmans FJM, Themmen APN, de Jong FH, Fauser BCJM, on behalf of the Dutch Premature Ovarian Failure Consortium. Anti-Mullerian Hormone, Inhibin B, and Antral Follicle Count in Young Women with Ovarian Failure. J Clin Endocrinol Metab 2009;94:786-792.
42. Cramer DW, Xu H. Predicting age at menopause. Maturitas 1996;23:319-326.
43. van Asselt KM, Kok HS, Pearson PL, Dubas JS, Peeters PH, te Velde ER, van Noord PA. Heritability of menopausal age in mothers and daughters. Fertil Steril 2004;82:1348-1351.
44. Kalantaridou SN, Naka KK, Papanikolaou E, Kazakos N, Kravariti M, Calis KA, Paraskevaidis EA, Sideris DA, Tsatsoulis A, Chrousos GP, Michalis LK. Impaired endothelial function in young women with premature ovarian failure: normalization with hormone therapy. J Clin Endocrinol Metab 2004;89:3907-3913.
45. Juberg RC, Holliday DJ, Hennessy VS. Familial sex chromosomal mosaicism. Am J Med Genet 1990;37:15-17.
46. Bachelot A, Rouxel A, Massin N, Dulon J, Courtillot C, Matuchansky C, Badachi Y, Fortin A, Paniel B, Lecuru F, Lefrere-Belda MA, Constancis E, Thibaud E, Meduri G, Mantel A, Misrahi M, Kuttenn F, Touraine P. Phenotyping and genetic studies of 357 consecutive patients presenting Premature Ovarian Failure. Eur J Endocrinol 2009;161:179-187.
47. Conway GS, Payne NN, Webb J, Murray A, Jacobs PA. Fragile-X premutation screening in women with premature ovarian failure. Hum Reprod 1998;13:1184-1187.





Chapter 3

Limited contribution of *NR5A1* (SF-1) mutations in women with primary ovarian insufficiency (POI)

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Abstract

Objective

To evaluate the significance of *NR5A1* mutations in a large, well-phenotyped cohort of women with primary ovarian insufficiency (POI). Mutations in the *NR5A1* gene (SF-1) were previously described in disorders of sexual development and adrenal insufficiency. Recently, a high frequency of *NR5A1* gene mutations was reported in a small group of women with POI.

Design

Cross-sectional cohort study.

Setting

University hospital.

Patients

Well-phenotyped women (n = 386) with secondary amenorrhea and diagnosed with POI, including women with familial POI (n = 77).

Interventions

None

Main outcome measures

The entire coding region and splice sites of the *NR5A1* gene were PCR-amplified and sequenced. The pathogenicity of identified mutations was predicted in silico by assessing Align-GVGD class and Grantham score.

Results

Sequencing was successful in 356 patients with POI. In total, 9 mutations were identified in 10 patients. Five of these mutations concerned novel nonconservative mutations occurring in 5 patients. Prediction of effect on protein function showed low to intermediate pathogenicity for all nonconservative mutations. The overall *NR5A1* gene mutation rate was 1.4%.

Conclusions

The current study demonstrates that mutations in the *NR5A1* gene are rare in women with POI. Primary ovarian insufficiency remains unexplained in the great majority of patients; therefore, continued efforts are needed to elucidate its underlying genetic factors.

Introduction

Menopause occurs at a mean age of 51 years (1). However, 1% of women experience menopause before the age of 40 years (2). Primary ovarian insufficiency (POI), also known as premature menopause or premature ovarian failure (3), is characterized by 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 (2). Primary ovarian insufficiency gives rise to infertility and increased risk for osteoporosis (4) and has been associated with a higher incidence of cardiovascular disease (5-7), depression (8), and possibly neurologic disease and impaired cognition (9). A positive family history for POI has been reported in 12% to 50% of patients (10-12).

Multiple factors may underlie the clinical entity of primary ovarian insufficiency. Primary ovarian insufficiency should therefore be considered a multifactorial heterogeneous condition. Gonadotoxic treatments using chemotherapeutic agents and irradiation, and extensive abdominal surgery and oophorectomy may cause iatrogenic POI. Furthermore, steroidogenic cell autoimmunity has been associated with autoimmune oophoritis in POI (13,14). A fair proportion of POI is caused by numerical or structural chromosomal abnormalities, including (full or mosaic) monosomy X (15). In addition, carrier status of the fragile-X premutation (*FMR1*) has a prevalence of 3% to 15% in women with POI (16). A recent study decribed mutations in the X-linked bone morphogenic protein 15 (*BMP15*) gene in 2% of patients with POI (17).

However, the search for other monogenetic causes of POI proved to be challenging. Besides syndromic forms of POI, caused by genes such as *FOXL2* or *GALT* (18-20), only rare gene mutations have been identified for nonsyndromic forms of POI. These include genes involved in folliculogenesis and follicle function: *GDF9*, *NOBOX*, FSH-receptor gene (*FSHR*) and LH-receptor gene (*LHR*) (21,22). Recently, high-throughput methods, using genomic variants such as single-nucleotide polymorphisms (SNPs) and copy number variants (CNVs), were applied to identify genetic risk loci in the complex genetic disease POI. A preliminary genome-wide association study performed by our group showed a possible role for the *ADAMTS19* gene (23). We also showed that CNVs on the X chromosome do not play a central role in the pathogenesis of POI (24). Up until now, no cause can be established in the majority of women with POI (12,25).

A recent report showed that in a sample of 40 women with sporadic POI, as much as 8% carried mutations in the nuclear receptor subfamily 5, group A, member 1 (*NR5A1*) gene (26). Moreover, the same study identified mutations in the *NR5A1* gene within four families with histories of both 46,XY sexual development disorders and 46,XX POI. It should be noted this study was different as compared to the studies mentioned before, in which an association was

studied between DNA marker such as SNPs and CNVs and the phenotype. In the latter study (26), the whole gene sequence was determined, thus identifying all sequence mutations and variants. The *NR5A1* gene is located on chromosome 9q33 and encodes the steroidogenic factor 1 (SF-1) protein, which regulates the transcription of genes associated with steroidogenesis, sexual development, and reproduction. Initially, *NR5A1* mutations were identified in 46,XY individuals with primary adrenal insufficiency and complete gonadal dysgenesis (27,28). Later, *NR5A1* mutations were associated with primary adrenal failure in a 46,XX woman with intact ovaries (29). Moreover, *NR5A1* mutations are relatively frequently present in patients with 46,XY disorders of sexual development (DSD) without adrenal insufficiency, such as severe hypospadias, or male infertility (26,30-33). At the moment over 30 different mutations are known.

The recently reported high mutation frequency of SF-1 mutations in a small group of women with POI in the previously published paper (26), prompted us to evaluate these results and to further establish the contribution of *NR5A1* mutations to POI. Therefore, we sequenced the complete coding regions of *NR5A1* in a large, well-phenotyped cohort of 356 women with POI, and we sought to identify and describe any new *NR5A1* mutations associated with POI.

Materials and methods

Patients

In 2005, a nationwide POI consortium for the screening and follow-up of patients with POI was initiated by the University Medical Center Utrecht, the Netherlands. Participating hospitals included all eight university hospitals in the Netherlands and 6 large regional hospitals. Women suspected to suffer from hypergonadotropic oligo- or amenorrhea were systematically evaluated in the outpatient clinic of each individual hospital, as has been described in previous studies from our group (7,12). For the current study, all women visiting the outpatient clinic for POI were identified (n = 378). Primary ovarian insufficiency was defined as spontaneous cessation of menses for at least 4 months in women younger than 40 years of age, along with repeated FSH concentrations exceeding 40 IU/L (2).

In short, data were gathered on reproductive and obstetric history, medical history with special attention for sexual development abnormalities and adrenal dysfunction, and family history for POI, sexual development disorders and adrenal hypoplasia. Family history covered three generations including paternal and maternal grandparents, paternal and maternal aunts and uncles, and the proband's parents and siblings. Familial POI was defined as when the index patient had at least one family member also affected by POI (12). Serum endocrine measurements included FSH and E_2. FSH concentrations were measured using

a chemoluminescence-based immunometric assay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA). Estradiol concentrations were measured using the Roche E170 Modular (Roche, Basel, Switzerland). Furthermore, androstenedione (AD), 17-hydroxyprogesterone (17-OH), DHEA, and DHEAS were measured. Androstenedione was measured after hexane-toluene extraction using an in-house RIA. Imprecision for the range 1-11 nmol/L was 7% to 9%. 17-Hydroxyprogesterone was measured using after toluene extraction using an in-house RIA employing a polyclonal anti-17a hydroxy-progesterone antibody (Bioconnect/Biogenesis), and 17a-hydroxy[1,2,6,7-3H]-progesterone (Perkin Elmer) was used as a tracer after chromatographic purification. Imprecision was 10.5%, 8%, and 8.5% at 3.5, 10, and 38 nmol/L, respectively (n = 75). Dehydroepiandrosterone was measured after diethylether extraction and Celite chromatography using an in-house RIA. Imprecision for the range 3,5-30 nmol/L was 6% to 12%. Dehydroepiandrosterone sulfate was measured using the Coat-A-Count DHEA-SO4 RIA (Siemens Diagnostics, Breda, Netherlands). Imprecision for the range 1,5-13 µmol/L was < 7%. Finally, karyotyping and screening for fragile-X premutation was performed. Mosaicism was defined by the presence of at least three or more mosaic cells per 32 analyzed cells. The study protocol was approved by the institutional review boards of the participating hospitals, and all women gave informed consent.

An additional eight patients with the diagnosis code POI were selected from the diagnostic database of the medical genetics department of the University Medical Center Utrecht, the Netherlands. All DNA samples were stored with informed consent for the purpose of future diagnostics and research.

Mutation analysis

For each patient, a blood sample was collected in a 10-mL EDTA tube. DNA was extracted from peripheral blood leukocytes using conventional techniques and frozen at -20°C until genotyping experiments were conducted.

The entire coding region (exon 2-7) and splice sites of the *NR5A1* (SF-1) gene were PCR-amplified with primers designed with primer3 software (http://www.broadinstitute.org/genome_software/other/primer3.html) (*Supplementary Table 1*). Exon 1 was not PCR-amplified, because it is a non-coding exon (34). Optimal PCR conditions were determined for each exon. After denaturation at 94°C for 5 minutes, the PCR amplification conditions for exon 2, 3, 4B and 6 were 15 cycles of 94 °C for 30 seconds, 65°C for 30 seconds (touchdown 0.5°C with each cycle), 72°C for 30 seconds, and 27 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72 °C for 30 seconds. For exon 6, an additional nested PCR with the same conditions was required. Optimal amplification for exon 4A was reached by 33 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C

for 30 seconds. Exon 5 was amplified by 33 cycles of 94°C for 30 seconds, 63°C for 30 seconds and 72° for C 30 seconds. For exon 7, optimal amplification conditions were 33 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. Amplification for all exons ended with a final elongation step of 72°C for 5 minutes. GC-rich DNA polymerase (AccuPrime by Invitrogen, Carlsbad, CA) and buffer (Buffer A by Invitrogen, Carlsbad, CA) were used. To verify the expected length of the amplified fragments, 2 µL was electrophoresed in a 2% agarose gel stained in ethidium bromide (0.5 µg/µL).

For sequencing of the amplified DNA fragments, 100 ng of purified DNA was mixed with 20 ng of primer and 1 µL BigDye Terminator, version 1.1 (Applied Biosystems, Foster City, CA), acting on the protocol. An ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) was used for automated sequencing. Data analysis was performed using Xming (Microsoft Corporation). To determine exact location and novelty of found mutations, the Ensembl database and the 1000Genomes database (www.ensembl.org, reference sequence: Genome Reference Consortium (GRCh37) and www.1000genomes.org) were consulted. In principle, only forward sequences were analyzed. Identified mutations were confirmed by sequencing the reverse strand. Because of the relatively short strands of the nested exon 6, every sample of this exon was analyzed in both forward and reverse sequences.

Confirmation of *NR5A1* mutations

When mutations were identified in *NR5A1*, a prediction of the effect of the mutation could be made by analyzing the effect on protein function. The possible pathogenicity of these mutations was predicted using the following prediction programs for mutation interpretation: Align-GVGD (available at agvgd.iarc.fr) (35) and Alamut (Interactive Biosoftware, Rouen, France). Grantham Variation (GV) score, Align-GVGD class, and splice site involvement was determined for each mutation. The GVGD (Grantham Variation – Grantham Deviation) classification is based on the chemical differences between each amino acid pair, polarity and molecular volume (GV) and sequence alignments (GD). GV scores below 62 are associated with variable-conservative pathogenicity of a mutation; mutations are considered variable- nonconservative when GV is higher than 62 (36). GVGD classes are the following (ranging from less likely to most likely that the amino acid substitution is to interfere with protein function): C0, C15, C25, C35, C45, C55, and C65.

We reconnected with women in whom nonconservative mutations were identified to inform them on the outcome of the analysis, and to perform additional *NR5A1* mutation analysis in their family members. Identical sequencing procedures were applied.

Statistical analysis

For baseline characteristics and laboratory findings, median and associated range of measurements were calculated. For categorical data, number and percentage are reported.

Results

A total of 386 women with POI were included in the current study. Baseline characteristics are reported in *Table 1*. Assessment of adrenal steroids identified normal adrenal function in all women. Seventy-seven women out of 229 with complete family history (34 %) were identified to have at least one family member with POI.

Table 1. Baseline characteristics for all women with POI (n = 386)

Age (years)	37.2 (15 - 60)
Age at secondary amenorrhea (years)	32.0 (12 - 40)
FSH (IU/L)	79 (40 - 200)
E_2 (pmol/L)	100 (< 20 - 200)
Adrenal dysfunction	0
Family history for POI	77 (34)[a]
Consanguineous parents	5 (1.4)[b]
Abnormal karyotype	21 (7.6)[c,d]
FMR1 premutation	15 (6.3)[e]

Note: Data shown as median (range) or n (%).
a Family history was known for n = 229; b Consanguinity was assessed in n = 345;
c Karyotype analysis was performed in n = 276; d n = 17 mosaicism, n = 4 translocation (see also *Supplementary table 2*); e *FMR1* premutation screening was performed in n = 234.

Sequencing was performed in all women, including those in whom an abnormal karyotype or carriership of the *FMR1* premutation was found considering the possibility of more than one genomic defect as a cause of POI. However, 30 patients (of whom 6 with familial POI), could not be included due to insufficient sequencing results (*Figure 1*). In the remaining 356 women (71 familial POI, 135 sporadic POI, 150 incomplete family history), 9 different mutations were identified in 10 women with POI. Four mutations were conservative, and 2 of them involved mutations in the intronic region (*Table 2*). Five novel nonconservative mutations of the *NR5A1* were identified in 5 patients. One of these women also showed 45,X[6]/47,XXX[4]/46,XX[121] mosaicism. None of them were fragile -X premutation carriers. Two women had a positive family history for POI. The mutation rate of *NR5A1* in this cohort of 356 women with POI was 1.4% of all cases, 2.2% in sporadic

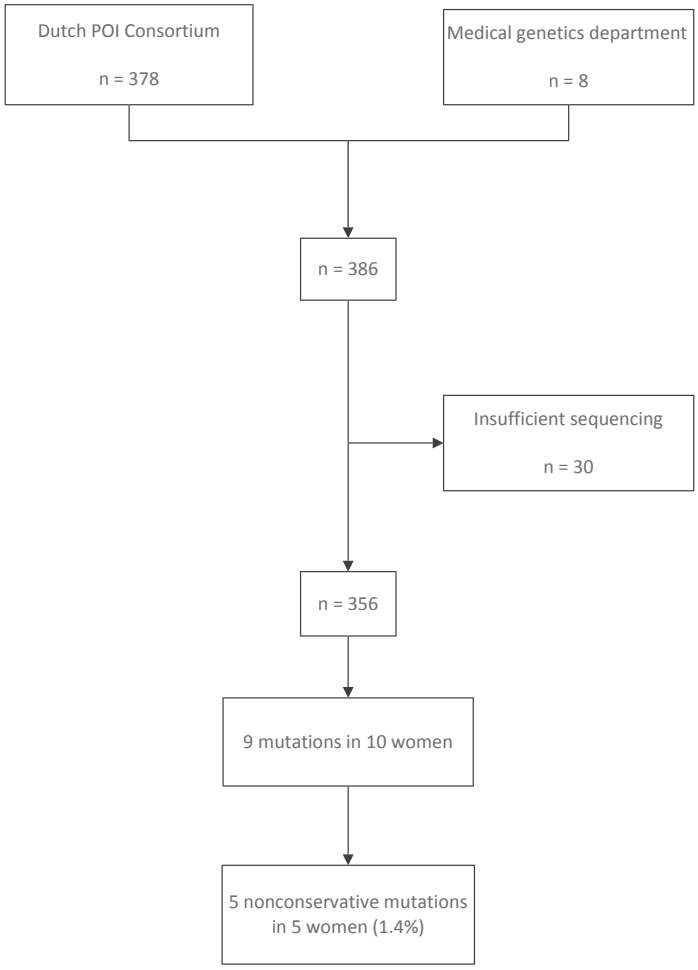

Figure 1. Flowchart of patient inclusion and subsequent mutation analysis.

cases (3 of 135), and 2.8% in familial cases (2 of 71) .

Evaluation of the pathogenicity of the nonconservative mutations is reported in *Table 3*. Changes in amino acid were predicted to result in modest effects on protein structure, varying from highly conserved proteins to weakly conserved proteins. None of the mutations were predicted to involve any splice site. Grantham scores varied from 29 to 113, and align-GVGD class was C0 (4 amino acids), C15 (1 amino acid), and C25 (1 amino acid), thus implying low predicted pathogenicity except for the latter 2 amino acids (intermediate pathogenicity).

We reconnected with all five women in whom a nonconservative mutation was

identified. Three of them were willing to get acquainted with the study's findings and to participate in the follow-up. Additional *NR5A1* sequencing was carried out in family members of two of these women, whereas all family members of the third woman lived abroad and were thus not able to participate. Sequencing was performed in the mother (also affected by POI) of the woman with mutation c.938G>A (patient 5), however, no mutation was identified. Additional sequencing of the unaffected mother of patient 2 (mutation c.407C>T) did not show any abnormalities.

Table 2. Results of the *NR5A1* mutation analysis and associated patient profile

Mutation analysis				
Patient	*Mutation*[a]	*Exon*	*Predicted amino acid change*[b]	*Effect*
1,2	c.407C>T	4A	P136L	Nonconservative
3	c.574G>T and c.575C>T	4B	A192F or A192S and A192V	Nonconservative
4	c.593C>T	4B	P198L	Nonconservative
5	c.938G>A	5	R313H	Nonconservative
6	c.244+55G>A	3-4[c]	None; no splice site	Conservative
7	c.991-27C>A	5-6[c]	None; no splice site	Conservative
8	c.825C>T	4B	S275S	Conservative
9,10	c.225G>C	2	T75T	Conservative

Patient profile				
Patient	*Family history POI*	*Ethnicity*	*FMR1*	*Karyotype*
1	No	Asian	normal	46,XX
2	No	Caucasian	normal	45,X[6]/47,XXX[4]/46,XX[121]
3	Yes	Mediterranean	normal	46,XX
4	No	Caucasian	normal	46,XX
5	Yes	Caucasian	normal	46,XX
6	NA	Caucasian	normal	46,XX
7	Yes	Caucasian	NA	46,XX
8	No	Black	normal	46,XX
9	No	Caucasian	normal	46,XX
10	No	Black	normal	46,XX

POI : primary ovarian insufficiency; NA : not available.
a Numbers refer to position of the nucleotide from the 5' end; c : coding region of the gene; > : substitution; first letter refers to wild type nucleotide; second letter refers to nucleotide which replaced the wild type.
b Number refers to position of the amino acid from the N terminus; first letter refers to one letter code of wild type amino acid; second letter refers to one letter code of the amino acid present in the mutation. A : Ala (Alanine); F : Phe (Phenylalanine); H : His (Histidine); L : Leu (Leucine); P : Pro (Proline); R : Arg (Arginine); S : Ser (Serine); T : Thr (Threonine); V : Val (Valine).
c Intronic region.

Table 3. Analysis of effect on protein function of nonconservative mutations using Grantham Score and Align-GVGD

Patient	Mutation[a]	Exon	Predicted amino acid change[b]	Protein conservation	Grantham Score	Align-GVGD Class[c]	Predicted pathogenicity
1 and 2	c.407C>T	4A	P136L	Weakly conserved	98	C0	Low
3	c.574G>T c.575C>T	4B	A192F	Weakly conserved	113	C15	Intermediate
			A192S		99	C0	Low
			A192V		64	C0	Low
4	c.593C>T	4B	P198L	Highly conserved	98	C0	Low
5	c.938G>A	5	R313H	Highly conserved	29	C25	Intermediate

a Numbers refer to position of the nucleotide from the 5'end; c : coding region of the gene; > : substitution; first letter refers to wild type nucleotide; second letter refers to nucleotide which replaced the wild type.
b Number refers to position of the amino acid from the N terminus; first letter refers to one letter code of wild type amino acid; second letter refers to one letter code of the amino acid present in the mutation. A : Ala (Alanine); F : Phe (Phenylalanine); H : His (Histidine); L : Leu (Leucine); P : Pro (Proline); R : Arg (Arginine); S : Ser (Serine); T : Thr (Threonine); V : Val (Valine).
c Align-GVGD classes: C0, C15, C25, C35, C45, C55 and C65, ranging from less likely to most likely to interfere with protein function.

Discussion

The current study aimed to investigate the frequency of *NR5A1* mutations in a large cohort of carefully phenotyped women diagnosed with spontaneous POI without adrenal insufficiency. The whole *NR5A1* gene was sequenced, thereby enabling the identification of all sequence mutations and variants. Five novel, nonconservative mutations were identified in five patients, 2 out of 71 (2.8%) familial cases and 3 out of 135 (2.2%) in sporadic cases, leading to an overall mutation rate of 1.4% (5 in 356). Analysis of amino acid changes showed that the pathogenicity of these mutations could be predicted to be low to intermediate. While a previous study suggested an *NR5A1* mutation rate of 8% in women with sporadic POI and perhaps even higher in familial cases (26), the current results do not confirm this finding.

Additional *NR5A1* sequencing in family members of nonconservative POI patients was carried out in patients 2 and 5. While the mother of patient 5 was also affected by POI, the daughter's age at onset of POI was much lower (20 years) compared with her mother's age at onset of POI (32 years). This family was previously studied in a genome-wide linkage analysis by another research group, and suggestive linkage was identified for three genomic regions on chromosomes 5, 14, and 18 (37). Because normal sequencing results were identified in the

mother, it may be hypothesized that the *NR5A1* mutation in the daughter had an amplifying effect to the severity of POI within this family. No mutation was identified in the unaffected mother of patient 2, which may indicate that the *NR5A1* mutation occurred de novo in this patient. However, the father of this patient died before the current study was begun and therefore this could not be verified completely.

The prediction of the pathogenicity of the identified nonconservative mutations in this study shows that these mutations only affect protein function mildly, if at all. This is in contrast with previous studies where identified *NR5A1* mutations mutant SF-1 protein was found to be mostly inactive: the "classic phenotype" of complete 46,XY gonadal dysgenesis and primary adrenal insufficiency, isolated 46,XY disorders of sex development, and isolated (primary) adrenal insufficiency in both genders (27-31,38). Therefore, it may be hypothesized that mild mutations lead to subtle sexual disorders, such as POI. This is supported by the findings of a recent publication suggesting that males with isolated oligozoospermia may also be affected by mild mutations of the *NR5A1* gene (33). Now that both the prevalence and the pathogenicity of the *NR5A1* mutations were much lower as compared to those previously described (26), we felt it would be of limited value to undertake additional in vitro experiments to assess their pathogenetic effect more precisely.

Several reasons may exist to account for the lower prevalence of *NR5A1* mutations in this cohort compared with a previous report (26). Firstly, our study cohort consisting of consecutive patients diagnosed with POI is much larger and therefore probably is a more reliable representation of the complete spectrum of POI. Only 25 women with spontaneous POI were included in the previous study and the recruitment procedure and inclusion and exclusion criteria were not clearly stated. Women with primary amenorrhea were not included in the current study, while in the previous study *NR5A1* mutations were only demonstrated in two prepubertal girls aged 12.5 years and 4 months, respectively. Moreover, both girls presented with abnormal phenotype characteristics: the first patient presented with short stature, and clitoral hypertrophia and elevated FSH were found in the second girl. Lastly, the Dutch cohort mainly comprises Caucasian women (77%), whereas the other study involved subjects of various ethnicities. Therefore, differences between both POI populations that were studied may explain the higher prevalence of *NR5A1* mutations in the previous study.

In conclusion, the findings of the current study suggest that *NR5A1* mutations are rare in women with POI. Therefore, there seems limited value in the inclusion of *NR5A1* mutation screening in the diagnostic workup of women affected by POI. Primary ovarian insufficiency etiology remains unexplained in the majority of women with POI, and studies concerning the origin of this multifactorial complex

disease are needed. As the concept of POI as a complex genetic disease arises, it is important to aim at unravelling the genetic variation underlying POI by performing next generation sequencing technologies in large, well-phenotyped cohorts for which international collaboration should be sought.

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Supplementary materials

Table S1. Primers used for PCR amplification of the *NR5A1* gene

Exon	Forward (5'-3')	Reverse (5'-3')
2	GCCTGGGCACAGAGAGG	CGTCTTGTCGATCTTGCAG
3	AGGTGTCCGGCTACCAC	ACTGGGCCCTAATGTCG
4A	TTGTTTGGAAAGGATCTGTG	AGAGAAGGGCTCTGGGTAG
4B	CACCGGACTACGTGCTG	CCAGGTACTGCCTGAAGC
5	GATGGGCACAGAGAGGTTAG	GATAATTAGGGTGCCCAGAG
6	TGACCTGCACCTCCAATC	CTACCTCTCCAGCCTCACC
6 nested	ACCCACGTCCTCTGACTG	GCTGTCTCCACCTCTCTGTC
7	TAACAGCCACCTCCTTGG	GTATTGGTGATGCTGGTGAC

Table S2. Detailed description of all cytogenetic abnormalities encountered in women with POI

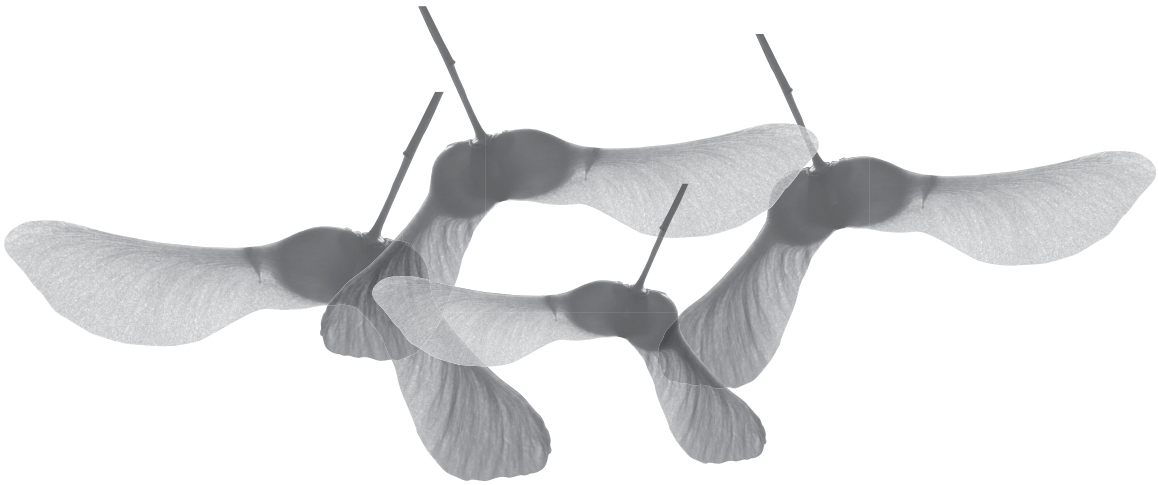
Chromosomal mosaicism	45,X[3]/46,XX[32] (2 patients)
	45,X[1]/47,XXX[3]/46,XX[26]
	45,X[3]/47,XXX[2]/46,XX[27]
	45,X[4]/46,XX[28]
	45,X[4]/46,XX[47]
	45,X[4]/46,XX[129]
	45,X[4]/47,XXX[1]/46,XX[45]
	45,X[5]/46,XX[31]
	45,X[5]/46,XX[95]
	45,X[6]/46,XX[144]
	45,X[6]/47,XXX[4]/46,XX[121]
	45,X[7]/46,XX[93]
	45,X[9]/46,XX[123]
	45,X[9]/46,XX[91]
	46,XX[5]/47,XXX[2]
	47XXX[3]/46,XX[29]
Chromosomal translocations	45,X,t(X;5)(q22.1;q22.1)
	t(9p;Xq)
	Translocation involving X chromosome, not further specified(2 patients)

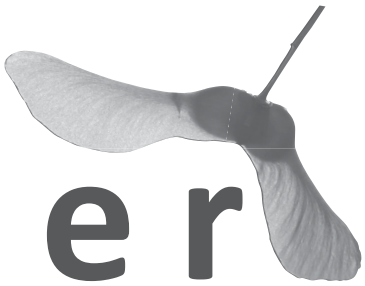
References

1. Treloar AE. Menstrual cyclicity and the pre-menopause. Maturitas 1981;3:249-264.
2. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986;67:604-606.
3. de Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. Lancet 2010;376:911-921.
4. Uygur D, Sengul O, Bayar D, Erdinc S, Batioglu S, Mollamahmutoglu L. Bone loss in young women with premature ovarian failure. Arch Gynecol Obstet 2005;273:17-19.
5. van der Schouw YT, van der Graaf Y, Steyerberg EW, Eijkemans JC, Banga JD. Age at menopause as a risk factor for cardiovascular mortality. Lancet 1996;347:714-718.
6. Kalantaridou SN, Naka KK, Papanikolaou E, Kazakos N, Kravariti M, Calis KA, Paraskevaidis EA, Sideris DA, Tsatsoulis A, Chrousos GP, Michalis LK. Impaired endothelial function in young women with premature ovarian failure: normalization with hormone therapy. J Clin Endocrinol Metab 2004;89:3907-3913.
7. Knauff EA, Westerveld HE, Goverde AJ, Eijkemans MJ, Valkenburg O, van Santbrink EJ, Fauser BC, van der Schouw YT. Lipid profile of women with premature ovarian failure. Menopause 2008;15:919-923.
8. van der Stege JG, Groen H, van Zadelhoff SJ, Lambalk CB, Braat DD, van Kasteren YM, van Santbrink EJ, Apperloo MJ, Weijmar Schultz WC, Hoek A. Decreased androgen concentrations and diminished general and sexual well-being in women with premature ovarian failure. Menopause 2008;15:23-31.
9. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, Melton LJ, III. Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. Neurology 2007;69:1074-1083.
10. Vegetti W, Grazia Tibiletti M, Testa G, de Lauretis Yankowski L, Alagna F, Castoldi E, Taborelli M, Motta T, Bolis PF, Dalpra L, Crosignani PG. Inheritance in idiopathic premature ovarian failure: analysis of 71 cases. Hum Reprod 1998;13:1796-1800.
11. van Kasteren YM, Hundscheid RD, Smits AP, Cremers FP, van ZP, Braat DD. Familial idiopathic premature ovarian failure: an overrated and underestimated genetic disease? Hum Reprod 1999;14:2455-2459.
12. Janse F, Knauff EA, Niermeijer MF, Eijkemans MJ, Laven JS, Lambalk CB, Fauser BC, Goverde AJ. Similar phenotype characteristics comparing familial and sporadic premature ovarian failure. Menopause 2010;17:758-765.
13. Bakalov VK, Anasti JN, Calis KA, Vanderhoof VH, Premkumar A, Chen S, Furmaniak J, Smith BR, Merino MJ, Nelson LM. Autoimmune oophoritis as a mechanism of follicular dysfunction in women with 46,XX spontaneous premature ovarian failure. Fertil Steril 2005;84:958-965.
14. Welt CK. Autoimmune oophoritis in the adolescent. Ann N Y Acad Sci 2008;1135:118-122.

15. Mattei MG, Mattei JF, Ayme S, Giraud F. X-autosome translocations: cytogenetic characteristics and their consequences. Hum Genet 1982;61:295-309.
16. Wittenberger MD, Hagerman RJ, Sherman SL, Conkie-Rosell A, Welt CK, Rebar RW, Corrigan EC, Simpson JL, Nelson LM. The *FMR1* premutation and reproduction. Fertil Steril 2007;87:456-465.
17. Rossetti R, Di Pasquale E, Marozzi A, Bione S, Toniolo D, Grammatico P, Nelson LM, Beck-Peccoz P, Persani L. *BMP15* mutations associated with primary ovarian insufficiency cause a defective production of bioactive protein. Hum Mutat 2009;30:804-810.
18. Kaufman F, Kogut MD, Donnell GN, Koch H, Goebelsmann U. Ovarian failure in galactosaemia. Lancet 1979;2:737-738.
19. Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G. The putative forkhead transcription factor *FOXL2* is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 2001;27:159-166.
20. Fridovich-Keil JL, Gubbels CS, Spencer JB, Sanders RD, Land JA, Rubio-Gozalbo E. Ovarian function in girls and women with *GALT*-deficiency galactosemia. J Inherit Metab Dis 2011;34:357-366.
21. Qin Y, Choi Y, Zhao H, Simpson JL, Chen ZJ, Rajkovic A. *NOBOX* homeobox mutation causes premature ovarian failure. Am J Hum Genet 2007;81:576-581.
22. Christin-Maitre S, Tachdjian G. Genome-wide association study and premature ovarian failure. Ann Endocrinol (Paris) 2010;71:218-221.
23. Knauff EA, Franke L, van Es MA, van den Berg LH, van der Schouw YT, Laven JS, Lambalk CB, Hoek A, Goverde AJ, Christin-Maitre S, Hsueh AJ, Wijmenga C, Fauser BC. Genome-wide association study in premature ovarian failure patients suggests *ADAMTS19* as a possible candidate gene. Hum Reprod 2009;24:2372-2378.
24. Knauff EA, Blauw HM, Pearson PL, Kok K, Wijmenga C, Veldink JH, van den Berg LH, Bouchard P, Fauser BC, Franke L. Copy number variants on the X chromosome in women with primary ovarian insufficiency. Fertil Steril 2011;95:1584-1588.
25. Bachelot A, Rouxel A, Massin N, Dulon J, Courtillot C, Matuchansky C, Badachi Y, Fortin A, Paniel B, Lecuru F, Lefrere-Belda MA, Constancis E, Thibaud E, Meduri G, Mantel A, Misrahi M, Kuttenn F, Touraine P. Phenotyping and genetic studies of 357 consecutive patients presenting Premature Ovarian Failure. Eur J Endocrinol 2009;161:179-187.
26. Lourenço D, Brauner R, Lin L, De Perdigo A., Weryha G, Muresan M, Boudjenah R, Guerra-Junior G, Maciel-Guerra AT, Achermann JC, McElreavey K, Bashamboo A. Mutations in *NR5A1* associated with ovarian insufficiency. N Engl J Med 2009;360:1200-1210.
27. Achermann JC, Ito M, Ito M, Hindmarsh PC, Jameson JL. A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. Nat Genet 1999;22:125-126.

28. Achermann JC, Ozisik G, Ito M, Orun UA, Harmanci K, Gurakan B, Jameson JL. Gonadal determination and adrenal development are regulated by the orphan nuclear receptor steroidogenic factor-1, in a dose-dependent manner. J Clin Endocrinol Metab 2002;87:1829-1833.
29. Biason-Lauber A, Schoenle EJ. Apparently normal ovarian differentiation in a prepubertal girl with transcriptionally inactive steroidogenic factor 1 (*NR5A1*/SF-1) and adrenocortical insufficiency. Am J Hum Genet 2000;67:1563-1568.
30. Lin L, Achermann JC. Steroidogenic factor-1 (SF-1, Ad4BP, *NR5A1*) and disorders of testis development. Sex Dev 2008;2:200-209.
31. Köhler B, Lin L, Mazen I, Cetindag C, Biebermann H, Akkurt I, Rossi R, Hiort O, Gruters A, Achermann JC. The spectrum of phenotypes associated with mutations in steroidogenic factor 1 (SF-1, *NR5A1*, Ad4BP) includes severe penoscrotal hypospadias in 46,XY males without adrenal insufficiency. Eur J Endocrinol 2009;161:237-242.
32. Lin L, Philibert P, Ferraz-de-Souza B, Kelberman D, Homfray T, Albanese A, Molini V, Sebire NJ, Einaudi S, Conway GS, Hughes IA, Jameson JL, Sultan C, Dattani MT, Achermann JC. Heterozygous missense mutations in steroidogenic factor 1 (SF1/Ad4BP, *NR5A1*) are associated with 46,XY disorders of sex development with normal adrenal function. J Clin Endocrinol Metab 2007;92:991-999.
33. Bashamboo A, Ferraz-de-Souza B, Lourenco D, Lin L, Sebire NJ, Montjean D, Bignon-Topalovic J, Mandelbaum J, Siffroi JP, Christin-Maitre S, Radhakrishna U, Rouba H, Ravel C, Seeler J, Achermann JC, McElreavey K. Human male infertility associated with mutations in *NR5A1* encoding steroidogenic factor 1. Am J Hum Genet 2010;87:505-512.
34. Oba K, Yanase T, Nomura M, Morohashi Ki, Takayanagi R, Nawata H. Structural Characterization of HumanAd4bp (SF-1)Gene. Biochemical and Biophysical Research Communications 1996;226:261-267.
35. Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, de Silva D, Zharkikh A, Thomas A. Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. J Med Genet 2006;43:295-305.
36. Bell J, Bodmer D, Sistermans E, Ramsden SC. Practice guidelines for the interpretation and reporting of unclassified variants (UVs) in clinical molecular genetics. UK Clinical Molecular Genetics Society (CMGS) and Dutch Society of Clinical Genetic Laboratory Specialists (VKGL). 2007. Available at: External link http://www.cmgs.org/BPGs/pdfs%20current%20bpgs/UV%20GUIDELINES%20ratified.pdf
37. Oldenburg RA, van Dooren MF, de Graaf B, Simons E, Govaerts L, Swagemakers S, Verkerk JM, Oostra BA, Bertoli-Avella AM. A genome-wide linkage scan in a Dutch family identifies a premature ovarian failure susceptibility locus. Hum Reprod 2008;23:2835-2841.
38. Lin L, Gu WX, Ozisik G, To WS, Owen CJ, Jameson JL, Achermann JC. Analysis of DAX1 (NR0B1) and steroidogenic factor-1 (*NR5A1*) in children and adults with primary adrenal failure: ten years' experience. J Clin Endocrinol Metab 2006;91:3048-3054.





Chapter 4

Normal and intermediate *FMR1* CGG repeat sizes are not associated with idiopathic primary ovarian insufficiency (POI)

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Submitted

Abstract

Objective

To assess the incidence of normal, intermediate and premutation *FMR1* alleles in well-phenotyped women with POI. In addition, a possible association of number of *FMR1* CGG repeats with POI severity was investigated.

Design

Cross-sectional cohort study

Setting

University hospital

Patients

144 women with amenorrhea diagnosed with overt POI, and 34 women with occult POI (defined as elevated FSH concentrations in women with regular or irregular menstrual cycles).

Interventions

PCR of the CGG-repeat region of the *FMR1* gene and subsequent fragment length determination.

Main outcome measures

Distribution of *FMR1* alleles. Association between the number of CGG repeats vs. age at diagnosis or vs. FSH concentrations.

Results

A total of 3 (2%) premutations were identified, which occurred only in women with overt POI. Overall, 6 (3%) intermediate alleles were found; 2 (6%) in occult POI and 4 (3%) in overt POI. A significant linear association between CGG repeat size and age at POI or FSH levels could not be demonstrated ($P = 0.93$ and $P = 0.09$, respectively).

Conclusions

The evaluation of normal- and intermediate *FMR1* repeat size seems of limited value in the diagnostic work-up of women affected by POI, or in women at risk for developing POI. The biological pathway and the clinical relevance of intermediate *FMR1* CGG repeat alleles remain to be elucidated.

Introduction

Menopause typically occurs at around 51 years of age (1). However, approximately 1% to 2% of women experience the cessation of menses before the age of 40 years (2). This condition - currently known as primary ovarian insufficiency (POI) (previously premature ovarian failure) - is characterized by amenorrhea for at least 4 months occurring prior to the age of 40 years, and repeatedly elevated follicle-stimulating hormone (FSH) concentrations above 40 IU/L along with decreased estradiol (E_2) levels (3,4).

The high heritability of age at menopause (5) together with the tendency for POI to run in families (6), implies a strong genetic component underlying POI. However, a clear Mendelian pattern of inheritance has not been identified. Furthermore, environmental factors such as smoking have also been shown to contribute to the variation in age of menopause (7,8). POI is therefore considered a multifactorial heterogeneous condition for which the specific cause remains unknown in the majority of cases (9,10). Genetic causes include numerical or structural chromosomal abnormalities including (full or mosaic) monosomy X (11), or relatively rare monogenetic causes such as mutations in the *FOXL2*, *BMP15*, *FSHR*, or *GDF9* gene (12-15). The most frequent monogenic cause of POI is the premutation of the fragile-X mental retardation gene 1 (*FMR1*) with an incidence reported between 0.8% to 13% (16).

The *FMR1* gene is located on the distal long arm of the X chromosome (Xq27.3) and consists of 17 exons (OMIM ID number 309550). Dysfunction of the *FMR1* gene is caused by the amplification of an unstable CGG triplet in the 5' untranslated region of the first exon (17). CGG repeat size exceeding 200 repeats is considered a full mutation, which leads to hypermethylation and subsequent silencing of the *FMR1* gene, and thereby results in the loss of fragile-X related mental retardation protein (FMRP) (18). The fragile-X syndrome is characterized by mental retardation, characteristic facial features, and behavioral problems such as autism and attention deficit disorder (OMIM ID number 300624). Both males and females may be affected by the fragile-X syndrome, although males are more severely affected because the mutation is X-linked. In women with full *FMR1* mutation, non-random X chromosome inactivation may regulate the FMRP production and thus the phenotype may be ameliorated, causing only one third of these women to suffer from decreased intelligence (19).

The full *FMR1* mutation may result from expansion of a premutation in maternal transmission from one generation to the next (20). The *FMR1* premutation is an amplification of the CGG repeat length to 55-200 repeats (19) (normal range < 45 repeats) (21). It is associated with POI (FXPOI) in young women, and with fragile-X associated tremor and ataxia syndrome (FXTAS), mostly occurring in the elderly (22). Furthermore, evidence points towards a higher incidence of

FMR1 premutation in women with occult POI (regular or irregular cycle, elevated FSH and/or decreased AMH) compared to controls (23). The incidence of *FMR1* premutation carriership varies from 1 in 178 to 1 in 468 in the general population (24-31). Approximately 21% of *FMR1* premutation carriers will develop FXPOI (16,32). The severity of ovarian dysfunction depends on the CGG repeat size in a non-linear fashion: women with mid-range repeats (80-100) experience POI earlier and more frequently than other carrier groups (33,34).

The intermediate CGG repeat length (45-54) (19,21) may expand to a full mutation after two or more generations (20). The role of intermediate CGG repeat size was recently investigated in POI and varying results were obtained. An increased frequency of intermediate alleles was identified in (occult) POI in several small sample size studies (23,35-38), but these findings could not be confirmed in a much larger sample size study (39). Yet another study suggested that any 5 CGG-count deviation from the most frequent CGG repeat size in the normal population (29-30 repeats) (40) would represent a possible risk factor for developing "occult" POI (41).

However, varying definitions for the description of intermediate CGG repeat length exist (19,21,42), and a lower boundary of *FMR1* repeat sizes which alter ovarian function has not yet been defined (37). The current study was undertaken to again assess the incidence of normal, intermediate and premutation *FMR1* alleles in women with POI. Moreover, we aimed to investigate a possible association of number of CGG repeats with age at POI as a parameter of POI severity.

Materials and methods

Patients

Since 2005, women suspected to suffer from a hypergonadotropic status presenting with a regular menstrual cycle, oligomenorrhea, or amenorrhea are systematically evaluated in the outpatient clinic of the University Medical Center Utrecht, the Netherlands, as has been described in previous studies from our group (10,43). For the current study, all consecutive women in this cohort diagnosed with overt POI or occult POI in whom data of the *FMR1* gene are known, were included (n = 144, and n = 34, respectively). Overt POI was defined as spontaneous cessation of menses for at least 4 months in women younger than 40 years of age, along with repeated FSH concentrations exceeding 40 IU/L (4). Women with primary amenorrhea were also included. Women with regular (previously: incipient ovarian failure) or irregular cycles (previously: transitional ovarian failure) were diagnosed with occult POI when early-follicular elevated FSH exceeded 12 IU/L (37,44). Exclusion criteria were the following: abnormal or unknown karyotype (n = 52), autoimmune (n = 42) or iatrogenic causes for POI (n = 1), and incomplete data on CGG repeat length (n = 16).

In short, data were gathered on reproductive and obstetric history, medical history, and family history for POI and mental retardation. For overt POI, age at diagnosis was defined as age at date of last menses, or age at date of last oral contraceptive pill (after which amenorrhea occurred, n = 39), or age at date of birth of the last child (after which menses did not return, n = 4). For women with occult POI, age at diagnosis was identical to the age at visit to the outpatient clinic, when the diagnosis was made. Family history covered three generations including paternal and maternal grandparents, paternal and maternal aunts and uncles, and the proband's parents and siblings. Familial POI was defined as when the index patient had at least one family member also affected by POI (10). Finally, FSH concentrations were measured using a chemoluminescence-based immunometric assay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA).

The study protocol was approved by the institutional review board of the University Medical Centre Utrecht, the Netherlands, and all women gave written informed consent (clinicaltrials.gov identifier: NCT01411644).

Genotyping

For each patient, a blood sample was collected in a 10 mL EDTA tube, and high molecular weight genomic DNA was extracted from blood samples using established procedures. Amplification was performed by PCR of the CGG-repeat region of the *FMR1* gene using Platinum Pfx DNA Polymerase according to the instruction of the manufacturer (Invitrogen). As forward primer PK2038 (5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3') and as reversed primer 5' FAM labeled PK2039 (5'-GCCCCGCACTTCCACCACCAGCTCCTCCA-3') were used. Fragment length determination was performed on an ABI 3130 sequencer (Applied Biosystems) using the GeneMarker genotype analysis software (SoftGenetics, State College, PA). Whenever a single allele length was observed, a Southern analysis was performed on Hind III en EclXI restricted DNA to confirm homozygosity for the number of CGG-repeats. Hybridization was performed using the PCR DIG Probe Synthesis Kit (Roche, Switzerland) with the primers PK3692-forward (5'-CGCCAAGAGGGCTTCAGGTCTCCT-3') and PK3693-reverse (5'-GAGACTGTTAAGAACATAAACGCGGG'-3').

The allele with the lowest triple repeat number was referred to as allele 1, while allele 2 was destined to represent the longest of both, consistent with prior publications (38,45). A normal CGG repeat count was defined as <45 repeats, the intermediate allele was defined as 45-54 CGG repeats, and the *FMR1* premutation was defined as 55-200 CGG repeats (19,21).

Statistical analysis

For continuous variables, means ± standard deviations (SDs) are reported. The differences between the overt and occult POI groups were compared using the Student's *t* test. For categorical data, number and percentage are reported, and chi-square tests were executed to assess statistical differences between both groups. The Mann-Whitney U test was performed to test for the CGG repeat distributions in women with overt POI vs. women with occult POI. To identify a possible non-linear association of number of CGG repeats in the allele with the longest triple repeat number (allele 2) and age at diagnosis, 3-knot restricted cubic splines were used. The restricted cubic splines did not indicate deviations from linearity. Therefore, linear regression analyses were performed to investigate the association between the number of CGG repeats in allele 2 vs. age at diagnosis, and vs. FSH concentrations. Because FSH is associated with age, age at diagnosis was entered as a covariate. Moreover, subgroup analyses for overt POI and for occult POI, and for familial and sporadic POI groups were performed. Furthermore, because age at diagnosis and the CGG repeats in allele 2 were not normally distributed, logarithmic transformation of all variables was performed before the analyses were executed. Analyses were performed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL) and R version 2.9.0. (http://www.r-project.org/).

Results

A total of 178 women, of whom 144 diagnosed with overt POI and 34 diagnosed with occult POI, were included for the current study. Baseline characteristics are reported in *Table 1*. Women with occult POI were older at screening and at diagnosis compared to women with overt POI. FSH was significantly higher in women with overt POI compared to women with occult POI. Forty-seven women had a positive family history for POI: 34% in overt POI and 28% in occult POI.

Details for the CGG repeat lengths for all women are reported in *Table 2*. The most common CGG repeat size of allele 2 was 30 repeats. Three *FMR1* premutations were identified in the entire study population (2%), which were all present in the overt POI group (2%). In total, 6 intermediate alleles were found (3%); 2 (6%) in occult POI and 4 (3%) in overt POI. The distributions of the CGG repeat size of allele 2 for each group are shown in *Figure 1*. Similar to the findings in the entire group, the most common repeat size was 30 in both subgroups, and ranging from 22 to 92 in overt POI and from 28 to 49 in occult POI. The distributions were not significantly different between both groups (P = 0.56).

Table 1. Baseline characteristics for all women included in the study

	Overt POI (n = 144)	Occult POI (n = 34)	Total (n = 178)
Age at diagnosis, years[a]	35.8 ± 7.6	36.2 ± 3.8	35.9 ± 7.0
Age at final menses, years	32.4 ± 6.7	NA	NA
Ever pregnant	72 (50%)	23 (67%)	95 (55%)
Parity	0.75 ± 1.0	0.91 ± 1.2	0.78 ± 1.1
Ever used oral contraception	128 (89%)	31 (91%)	159 (89%)
Ever used HT	12 (8%)	0	12 (7%)
Smoking			
Never	78 (54%)	21 (62%)	99 (56%)
Current	34 (24%)	5 (15%)	39 (22%)
Past	28 (19%)	8 (24%)	36 (20%)
BMI, kg/m²	24.6 ± 5.0	25.0 ± 6.6	24.7 ± 5.4
Family history for POI[b]	39 (34%)	8 (28%)	47 (33%)
FSH at diagnosis, IU/L[c]	86.8 ± 40.1	41.2 ± 25.2	78.0 ± 41.8

Data show as mean ± SD, or n (%).
POI, primary ovarian insufficiency; NA, not applicable; HT, hormone therapy; BMI, body mass index.
a Significant difference between overt and occult POI, P = 0.001.
b Family history complete for 164 women (92.1%). 20 Women had a family member affected by early menopause (between age 40 and 45 years). They were not included in this comparison, because it remains uncertain whether early menopause represents a continuum with POI.
c Significant difference between overt and occult POI, P < 0.001.

Table 2. *FMR1* CGG repeat length in women with overt or occult POI

	Overt POI (n = 144)	Occult POI (n = 34)	Total (n = 178)
***FMR1* CGG repeat size**			
FMR1 Allele 1: CGG repeats	27.3 ± 5.0	28.1 ± 3.5	27.4 ± 4.8
Median (range)	29 (8 – 41)	29 (20 – 31)	29 (8 – 41)
FMR1 Allele 2: CGG repeats	32.9 ± 8.6	32.3 ± 4.6	32.8 ± 8.0
Median (range)	30 (22 – 92)	30 (28 – 49)	30 (22 – 92)
Incidence of *FMR1* allelic form			
Normal (≤ 44 repeats)	137 (95%)	32 (94%)	169 (95%)
Intermediate (45-54 repeats)	4 (3%)	2 (6%)	6 (3%)
Premutation (55-200 repeats)	3 (2%)	0	3 (2%)

Data show as mean ± SD, median (range), or n (%).
POI, primary ovarian insufficiency; *FMR1* allele 1, allele with the lowest CGG repeat number; *FMR1* allele 2, allele with the highest CGG repeat number.

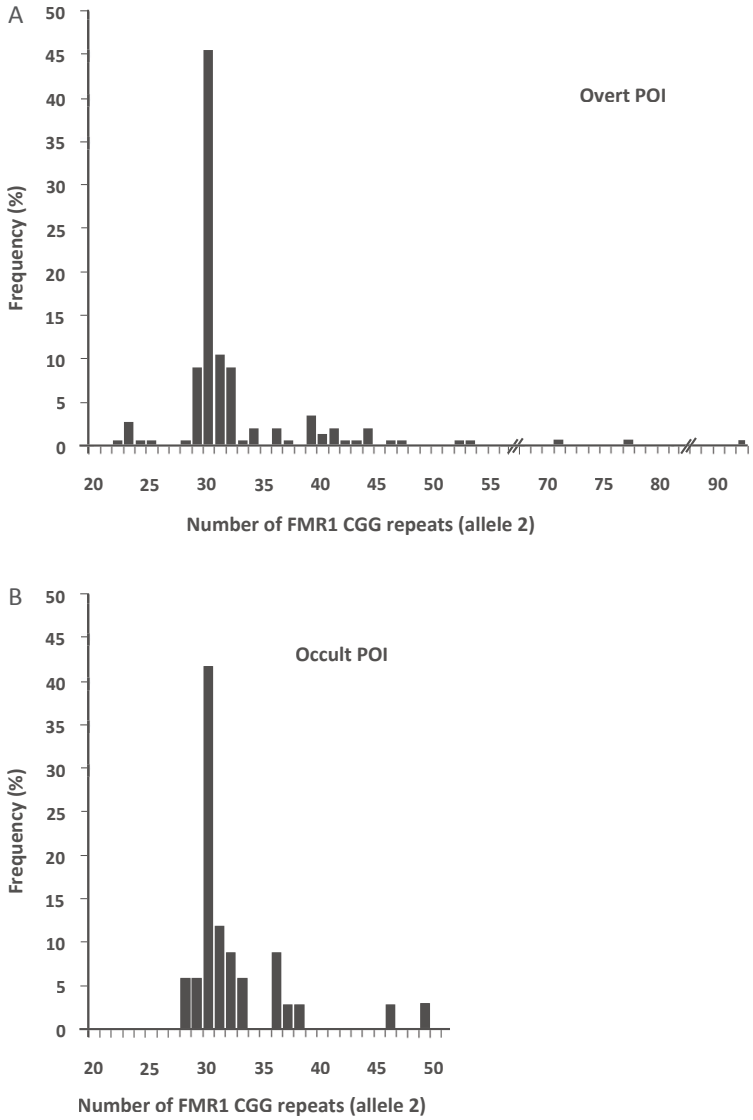

Figure 1.
A Distribution of *FMR1* CGG alleles in women with overt POI (n = 144).
Legend: POI, primary ovarian insufficiency.
B Distribution of *FMR1* CGG alleles in women with occult POI (n = 34).
Legend: POI, primary ovarian insufficiency.

Linear regression analysis for age at diagnosis vs. CGG repeat size of allele 2 did not identify a significant association (P = 0.93). The introduction of subgroups, i.e. overt POI and occult POI, did not change this finding (P = 0.99 and P = 0.39, respectively). Linear regression analysis for FSH vs. CGG repeat size was also not

statistically significant (P = 0.09). While no significant association between FSH and CGG repeat size was identified in occult POI (P = 0.73), borderline significance was identified in women with overt POI (P = 0.056). Subgroup analyses for familial vs. sporadic occurrence of POI did not change any of the findings (data not shown).

Discussion

The current study was undertaken to investigate the incidence of *FMR1* premutations and intermediate alleles in a considerable cohort of well-phenotyped Dutch women with overt or occult POI. Furthermore, a possible association of the number of CGG repeats and age at POI was investigated. The most common allele size was 30 CGG repeats in both groups. A total of 3 premutations was identified and occurred only in women with overt POI. A significant linear association between CGG repeat size and age at POI could not be demonstrated.

Gene transcription is significantly increased in *FMR1* premutation carriers. The resulting increased *FMR1* mRNA levels probably contribute to the pathogenesis of FXTAS through toxic gain-of-function effect (46). Up until now, a similar contribution of the *FMR1* premutation (or intermediate repeat size) to the pathogenesis of FXPOI has only been suggested (47). However, *FMR1* mRNA transcript levels are highly variable in CGG repeat numbers at the lower end of the premutation range (48), and this variability does not correlate to the variance of CGG triplet numbers in women with POI (49). In contrast, destabilization of the intermediate CGG stability (resulting from CGG repeats without AGG interspersions) could be another potential biological pathway in POI (36). However, AGG interruptions do not seem to decrease *FMR1* mRNA levels (50). Clearly, the possible biological mechanism of intermediate and premutation size CGG repeats in the pathogenesis of POI needs further study.

The first aim of the current study was to assess once again the incidences of normal or intermediate CGG repeats, and of the *FMR1* premutation in POI. The most frequent CGG repeat size was 30 in both overt and occult POI, which is similar to the general population (40). In total, three premutations were identified (1.7%), occurring in overt POI only (2.1%). These figures fall into the lower range of the published incidence of 0.8% to 7.5% for premutations in sporadic POI (51-54). Of the three premutation carriers in our study sample, two had a positive family history for POI. One woman had a mother affected by POI, and two maternal cousins who also carried the *FMR1* premutation, one of whom had a son affected by the fragile-X syndrome. The other woman had a sister also affected by POI. Intermediate *FMR1* alleles were identified in six women in total (3%); four in overt POI (3%) and two in occult POI (6%).

Up to now, only one study used identical definitions to the current study, and identified an incidence of 3.2% of intermediate alleles in occult POI (23).

Unfortunately, most previous publications in overt POI applied different definitions for intermediate alleles, although not concurrent with the existing guidelines (19,21,42). For the sake of comparison of those publications, we converted our findings to the other definitions: when intermediate CGG repeats are defined as 41-58 repeats, we found 12 intermediate alleles in overt POI (8.3%) and two in occult POI (5.9%); which is higher than the previously reported 3.8% to 4.7% in overt POI (36,39). Another rather wide definition of 34-54 repeats resulted in 26 (18.1%) in overt POI and seven (20.6%) in occult POI in our study sample, in contrast to the previously published incidence of 7.1% to 14.2% (35,39). Although the contribution of intermediate CGG repeat length in POI seems considerable using the two latter definitions, the clinical relevance of these repeat lengths needs further evaluation. Moreover, approximately 6% of the general female population carries alleles with 40-60 repeats (39,55), while the incidence of POI is only 1% to 2% (2). This suggests that low penetrance, variable expression, or the involvement of other genetic and/or environmental factors possibly play a role in the development of POI.

The second objective of the current study was to examine a possible association between CGG repeat size and age at diagnosis, as a measurement for the severity of POI. No significant association was identified by linear regression analysis. This finding is in contrast with a previously published study in Asian women (56), in which it was suggested that having > 38 CGG repeats was associated with younger age at onset of amenorrhea (56). However, the number of CGG repeats in Asians exhibits a different distribution compared to other ethnicities, with a secondary peak of 34-36 repeats in addition to the most frequent peak of 29 or 30 repeats (40,57,58). In the current study, the possibility of a non-linear relationship between CGG repeat length and age at diagnosis was excluded by 3-knot restricted cubic splines. Therefore, the results do not support a previously published non-linear relationship between CGG repeat length and the likelihood for women to develop occult POI (41). Finally, a possible association between CGG repeat size and FSH concentration was investigated. The analysis almost reached statistical significance in women with overt POI only. This is an interesting finding given the results of a recent study in which lower baseline FSH concentrations (30-52 IU/L) in women with overt POI were associated with a better prognosis for the resumption of ovarian function compared to higher baseline FSH concentrations (52-79 IU/L) (59). These associations need further study.

There may be some limitations in this study. Firstly, we chose to only perform analyses with continuous CGG repeat data to avoid the introduction of a bias due to multiple testing. Therefore, we cannot be certain whether a significant cut-off for CGG repeat length would exist in any of the investigated associations, although the current results do not support this hypothesis. Furthermore, analyses with

AMH as a measure of POI severity were not performed. The reason for this is that we have previously demonstrated that AMH concentrations are almost always undetectable in women with overt POI (44), and linear regression analysis in women with occult POI only was not feasible due to limited sample size.
In conclusion, the current study identified a low number of intermediate *FMR1* CGG repeat alleles and *FMR1* premutations in women with overt or occult POI according to the current *FMR1* guidelines. No significant association between CGG repeat size and age at POI diagnosis could be identified. The biological pathway and the clinical relevance of intermediate *FMR1* CGG repeat alleles remain to be elucidated. Therefore, there seems limited value in the evaluation of normal and intermediate *FMR1* repeat size in the diagnostic work-up of women affected by POI, or for prognostic purposes in women at risk for developing POI. However, although only a low incidence was identified in the current study, the identification of *FMR1* premutations remains important in women with POI because of the risk for expansion to a full mutation in their offspring.

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References

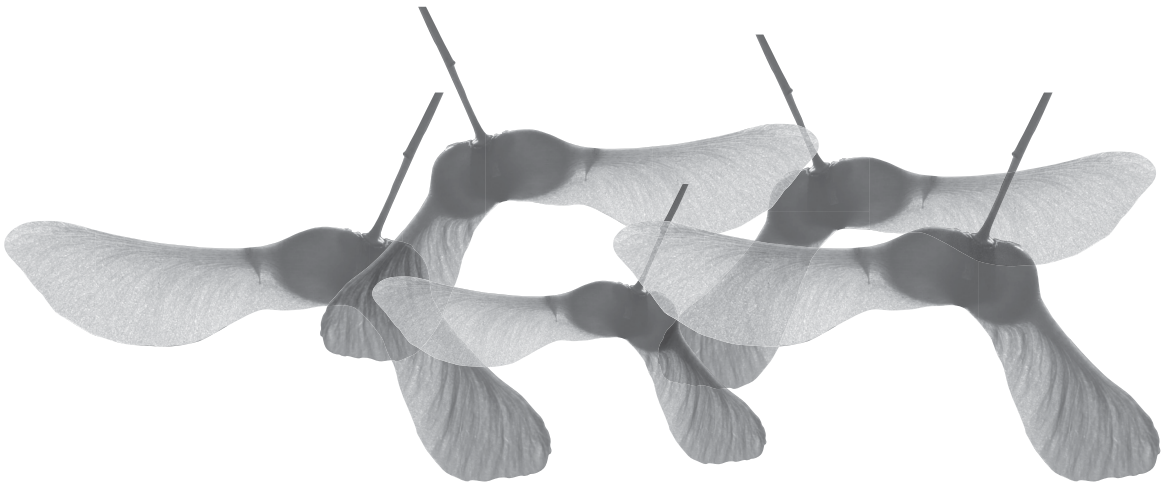
1. Treloar AE. Menstrual cyclicity and the pre-menopause. Maturitas 1981;3:249-264.
2. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986;67:604-606.
3. Cooper AR, Baker VL, Sterling EW, Ryan ME, Woodruff TK, Nelson LM. The time is now for a new approach to primary ovarian insufficiency. Fertil Steril 2010;95:1890-1897.
4. de Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. Lancet 2010;376:911-921.
5. van Asselt KM, Kok HS, Pearson PL, Dubas JS, Peeters PH, te Velde ER, van Noord PA. Heritability of menopausal age in mothers and daughters. Fertil Steril 2004;82:1348-1351.
6. van Kasteren YM, Hundscheid RD, Smits AP, Cremers FP, van Zonneveld P, Braat DD. Familial idiopathic premature ovarian failure: an overrated and underestimated genetic disease? Hum Reprod 1999;14:2455-2459.
7. Bromberger JT, Matthews KA, Kuller LH, Wing RR, Meilahn EN, Plantinga P. Prospective study of the determinants of age at menopause. Am J Epidemiol 1997;145:124-133.
8. van Asselt KM, Kok HS, van der Schouw YT, Grobbee DE, te Velde ER, Pearson PL, Peeters PH. Current smoking at menopause rather than duration determines the onset of natural menopause. Epidemiology 2004;15:634-639.
9. Bachelot A, Rouxel A, Massin N, Dulon J, Courtillot C, Matuchansky C, Badachi Y, Fortin A, Paniel B, Lecuru F, Lefrere-Belda MA, Constancis E, Thibaud E, Meduri G, Mantel A, Misrahi M, Kuttenn F, Touraine P. Phenotyping and genetic studies of 357 consecutive patients presenting Premature Ovarian Failure. Eur J Endocrinol 2009;161:179-187.
10. Janse F, Knauff EA, Niermeijer MF, Eijkemans MJ, Laven JS, Lambalk CB, Fauser BC, Goverde AJ. Similar phenotype characteristics comparing familial and sporadic premature ovarian failure. Menopause 2010;17:758-765.
11. Mattei MG, Mattei JF, Ayme S, Giraud F. X-autosome translocations: cytogenetic characteristics and their consequences. Hum Genet 1982;61:295-309.
12. Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S, Ristaldi MS, Marzella R, Rocchi M, Nicolino M, Lienhardt-Roussie A, Nivelon A, Verloes A, Schlessinger D, Gasparini P, Bonneau D, Cao A, Pilia G. The putative forkhead transcription factor *FOXL2* is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 2001;27:159-166.
13. Rossetti R, Di Pasquale E, Marozzi A, Bione S, Toniolo D, Grammatico P, Nelson LM, Beck-Peccoz P, Persani L. *BMP15* mutations associated with primary ovarian insufficiency cause a defective production of bioactive protein. Hum Mutat 2009;30:804-810.
14. Christin-Maitre S, Tachdjian G. Genome-wide association study and premature ovarian failure. Ann Endocrinol (Paris) 2010;71:218-221.

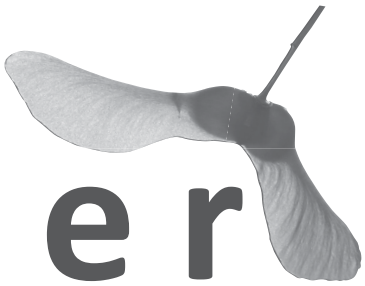
15. Fridovich-Keil JL, Gubbels CS, Spencer JB, Sanders RD, Land JA, Rubio-Gozalbo E. Ovarian function in girls and women with *GALT*-deficiency galactosemia. J Inherit Metab Dis 2011;34:357-366.
16. Wittenberger MD, Hagerman RJ, Sherman SL, Conkie-Rosell A, Welt CK, Rebar RW, Corrigan EC, Simpson JL, Nelson LM. The *FMR1* premutation and reproduction. Fertil Steril 2007;87:456-465.
17. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang FP, Eussen BE, van Ommen G-JB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA, Warren ST. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile-X syndrome. Cell 1991;65:905-914.
18. Oostra BA, Willemsen R. A fragile balance: *FMR1* expression levels. Hum Mol Genet 2003;12 Spec No 2:R249-R257.
19. Sherman S, Pletcher BA, Driscoll DA. Fragile-X syndrome: diagnostic and carrier testing. Genet Med 2005;7:584-587.
20. Nolin SL, Brown WT, Glicksman A, Houck GE, Jr., Gargano AD, Sullivan A, Biancalana V, Brondum-Nielsen K, Hjalgrim H, Holinski-Feder E, Kooy F, Longshore J, Macpherson J, Mandel JL, Matthijs G, Rousseau F, Steinbach P, Vaisanen ML, von Koskull H, Sherman SL. Expansion of the fragile-X CGG repeat in females with premutation or intermediate alleles. Am J Hum Genet 2003;72:454-464.
21. Kronquist KE, Sherman SL, Spector EB. Clinical significance of tri-nucleotide repeats in Fragile-X testing: a clarification of American College of Medical Genetics guidelines. Genet Med 2008;10:845-847.
22. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L, Brunberg JA, Greco C, Des Portes V, Jardini T, Levine R, Berry-Kravis E, Brown WT, Schaeffer S, Kissel J, Tassone F, Hagerman PJ. Fragile-X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. Am J Hum Genet 2003;72:869-878.
23. Karimov CB, Moragianni VA, Cronister A, Srouji S, Petrozza J, Racowsky C, Ginsburg E, Thornton KL, Welt CK. Increased frequency of occult fragile-X-associated primary ovarian insufficiency in infertile women with evidence of impaired ovarian function. Hum Reprod 2011;26:2077-2083.
24. Rousseau F, Rouillard P, Morel ML, Khandjian EW, Morgan K. Prevalence of carriers of premutation-size alleles of the FMRI gene--and implications for the population genetics of the fragile-X syndrome. Am J Hum Genet 1995;57:1006-1018.
25. Spence WC, Black SH, Fallon L, Maddalena A, Cummings E, Menapace-Drew G, Bick DP, Levinson G, Schulman JD, Howard-Peebles PN. Molecular fragile-X screening in normal populations. Am J Med Genet 1996;64:181-183.
26. Ryynanen M, Heinonen S, Makkonen M, Kajanoja E, Mannermaa A, Pertti K. Feasibility and acceptance of screening for fragile-X mutations in low-risk pregnancies. Eur J Hum Genet 1999;7:212-216.

27. Drasinover V, Ehrlich S, Magal N, Taub E, Libman V, Shohat T, Halpern GJ, Shohat M. Increased transmission of intermediate alleles of the *FMR1* gene compared with normal alleles among female heterozygotes. Am J Med Genet 2000;93:155-157.
28. Pesso R, Berkenstadt M, Cuckle H, Gak E, Peleg L, Frydman M, Barkai G. Screening for fragile-X syndrome in women of reproductive age. Prenat Diagn 2000;20:611-614.
29. Crawford DC, Meadows KL, Newman JL, Taft LF, Scott E, Leslie M, Shubek L, Holmgreen P, Yeargin-Allsopp M, Boyle C, Sherman SL. Prevalence of the fragile-X syndrome in African-Americans. Am J Med Genet 2002;110:226-233.
30. Cronister A, DiMaio M, Mahoney MJ, Donnenfeld AE, Hallam S. Fragile-X syndrome carrier screening in the prenatal genetic counseling setting. Genet Med 2005;7:246-250.
31. Hantash FM, Goos DM, Crossley B, Anderson B, Zhang K, Sun W, Strom CM. *FMR1* premutation carrier frequency in patients undergoing routine population-based carrier screening: insights into the prevalence of fragile-X syndrome, fragile-X-associated tremor/ataxia syndrome, and fragile-X-associated primary ovarian insufficiency in the United States. Genet Med 2011;13:39-45.
32. Sherman SL. Premature ovarian failure in the fragile-X syndrome. Am J Med Genet 2000;97:189-194.
33. Ennis S, Ward D, Murray A. Nonlinear association between CGG repeat number and age of menopause in *FMR1* premutation carriers. Eur J Hum Genet 2006;14:253-255.
34. Allen EG, Sullivan AK, Marcus M, Small C, Dominguez C, Epstein MP, Charen K, He W, Taylor KC, Sherman SL. Examination of reproductive aging milestones among women who carry the *FMR1* premutation. Hum Reprod 2007;22:2142-2152.
35. Bretherick KL, Fluker MR, Robinson WP. *FMR1* repeat sizes in the gray zone and high end of the normal range are associated with premature ovarian failure. Hum Genet 2005;117:376-382.
36. Bodega B, Bione S, Dalpra L, Toniolo D, Ornaghi F, Vegetti W, Ginelli E, Marozzi A. Influence of intermediate and uninterrupted *FMR1* CGG expansions in premature ovarian failure manifestation. Hum Reprod 2006;21:952-957.
37. Streuli I, Fraisse T, Ibecheole V, Moix I, Morris MA, de ZD. Intermediate and premutation *FMR1* alleles in women with occult primary ovarian insufficiency. Fertil Steril 2009;92:464-470.
38. Gleicher N, Weghofer A, Barad DH. A pilot study of premature ovarian senescence: I. Correlation of triple CGG repeats on the *FMR1* gene to ovarian reserve parameters FSH and anti-Mullerian hormone. Fertil Steril 2009;91:1700-1706.
39. Bennett CE, Conway GS, Macpherson JN, Jacobs PA, Murray A. Intermediate sized CGG repeats are not a common cause of idiopathic premature ovarian failure. Hum Reprod 2010;25:1335-1338.
40. Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, Holden JJ, Fenwick RG, Jr., Warren ST, Oostra BA, Nelson DL, Caskey CT. Variation of the CGG repeat at the fragile-X site results in genetic instability: resolution of the Sherman paradox. Cell 1991;67:1047-1058.
41. Gleicher N, Weghofer A, Oktay K, Barad D. Relevance of triple CGG repeats in the *FMR1* gene to ovarian reserve. Reprod Biomed Online 2009;19:385-390.

42. American College of Obstetricians and Gynecologists Committee on Genetics. ACOG committee opinion. No. 338: Screening for fragile-X syndrome. Obstet Gynecol 2006;107:1483-1485.
43. Knauff EA, Westerveld HE, Goverde AJ, Eijkemans MJ, Valkenburg O, van Santbrink EJ, Fauser BC, van der Schouw YT. Lipid profile of women with premature ovarian failure. Menopause 2008;15:919-923.
44. Knauff EAH, Eijkemans MJC, Lambalk CB, ten Kate-Booij MJ, Hoek A, Beerendonk CCM, Laven JSE, Goverde AJ, Broekmans FJM, Themmen APN, de Jong FH, Fauser BCJM, on behalf of the Dutch Premature Ovarian Failure Consortium. Anti-mullerian hormone, inhibin B, and antral follicle count in young women with ovarian failure. J Clin Endocrinol Metab 2009;94:786-792.
45. Ficicioglu C, Yildirim G, Attar R, Kumbak B, Yesildaglar N. The significance of the number of CGG repeats and autoantibodies in premature ovarian failure. Reprod Biomed Online 2010;20:776-782.
46. Garcia-Arocena D, Hagerman PJ. Advances in understanding the molecular basis of FXTAS. Hum Mol Genet 2010;19:R83-R89.
47. Willemsen R, Levenga J, Oostra BA. CGG repeat in the *FMR1* gene: size matters. Clin Genet 2011;80:214-225.
48. Allen EG, He W, Yadav-Shah M, Sherman SL. A study of the distributional characteristics of *FMR1* transcript levels in 238 individuals. Hum Genet 2004;114:439-447.
49. Schuettler J, Peng Z, Zimmer J, Sinn P, von Hagens C, Strowitzki T, Vogt PH. Variable expression of the Fragile-X Mental Retardation 1 (*FMR1*) gene in patients with premature ovarian failure syndrome is not dependent on number of (CGG)n triplets in exon 1. Hum Reprod 2011;26:1241-1251.
50. Peprah E, He W, Allen E, Oliver T, Boyne A, Sherman SL. Examination of *FMR1* transcript and protein levels among 74 premutation carriers. J Hum Genet 2010;55:66-68.
51. Conway GS, Payne NN, Webb J, Murray A, Jacobs PA. Fragile-X premutation screening in women with premature ovarian failure. Hum Reprod 1998;13:1184-1187.
52. Murray A, Webb J, Grimley S, Conway G, Jacobs P. Studies of FRAXA and FRAXE in women with premature ovarian failure. J Med Genet 1998;35:637-640.
53. Gersak K, Meden-Vrtovec H, Peterlin B. Fragile-X premutation in women with sporadic premature ovarian failure in Slovenia. Hum Reprod 2003;18:1637-1640.
54. Bussani C, Papi L, Sestini R, Baldinotti F, Bucciantini S, Bruni V, Scarselli G. Premature ovarian failure and fragile-X premutation: a study on 45 women. Eur J Obstet Gynecol Reprod Biol 2004;112:189-191.
55. Patsalis PC, Sismani C, Stylianou S, Ioannou P, Joseph G, Manoli P, Holden JJ, Hettinger JA. Genetic variation and intergenerational *FMR1* CGG-repeat stability in 100 unrelated three-generation families from the normal population. Am J Med Genet 1999;84:217-220.
56. Ishizuka B, Okamoto N, Hamada N, Sugishita Y, Saito J, Takahashi N, Ogata T, Itoh MT. Number of CGG repeats in the *FMR1* gene of Japanese patients with primary ovarian insufficiency. Fertil Steril 2011;96:1170-1174.

57. Chen SH, Schoof JM, Buroker NE, Scott CR. The identification of a (CGG)6AGG insertion within the CGG repeat of the *FMR1* gene in Asians. Hum Genet 1997;99:793-795.
58. Otsuka S, Sakamoto Y, Siomi H, Itakura M, Yamamoto K, Matumoto H, Sasaki T, Kato N, Nanba E. Fragile-X carrier screening and *FMR1* allele distribution in the Japanese population. Brain Dev 2010;32:110-114.
59. Bidet M, Bachelot A, Bissauge E, Golmard JL, Gricourt S, Dulon J, Coussieu C, Badachi Y, Touraine P. Resumption of ovarian function and pregnancies in 358 patients with premature ovarian failure. J Clin Endocrinol Metab 2011;96:3864-3872.





Chapter 5

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

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Abstract

Background

Increasing age and postmenopausal 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 (T) 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 T assays. The current meta-analysis was conducted to evaluate current literature reporting serum total T concentrations under these conditions, including stratification for various T assays.

Methods

A systematic review and meta-analysis of controlled observational studies was performed. The electronic databases of Pubmed, Embase and the Cochrane Library were systematically searched until October 2011 for comparative studies on total T concentrations in women with spontaneous POI or iatrogenic menopause compared to controls. 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 1,358 for iatrogenic menopause were reviewed, of which 9 and 17 papers, respectively, were selected for final analysis. Both groups demonstrated significantly lower total T concentrations compared to controls (WMD (95% CI) -0.38 (-0.55 to -0.22) nmol/L, and -0.29 (-0.39 to -0.18) nmol/L, respectively), yet with substantial between-study heterogeneity. Subgroup analysis for assay type was statistically significant in spontaneous POI only. Sensitivity analyses of high-quality studies did not change results, and resulted in a substantial decrease in heterogeneity in spontaneous POI.

Conclusions

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

Introduction

While spontaneous menopause typically occurs around the age of 51 years (1), approximately 1% to 2% of women experience menopause before the age of 40 years (2). 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 follicle-stimulating hormone (FSH) concentrations in the postmenopausal range and a hypo-estrogenic state (3,4). Spontaneous POI may be caused by steroidogenic cell autoimmunity (5), numerical or structural chromosomal abnormalities, such as monosomy X (6), or monogenic causes, including the fragile-X premutation (7) and other single gene mutations (8-10). In the great majority of women diagnosed with POI, however, 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 (11).

Another group of postmenopausal 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 (12), the incidence of women with iatrogenic menopause due to both benign and malignant diseases has now increased to 3.4% to 4.5% (13).

Spontaneous POI and iatrogenic menopause are associated with infertility, and increased risk for osteoporosis (14,15), cardiovascular disease (16-18), and diminished emotional well-being (19). 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 (T) concentrations (20-22). Up until now, it is debated whether the postmenopausal ovary remains a significant source of androgen production. There is conflicting evidence indicating that hypoandrogenism in postmenopausal women may derive from a gradual decrease in both ovarian and adrenal production of androgens with increasing age (23), while others suggest that only ovarian androgen production further decreases during menopausal transition (24,25). 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: (1) diminished sense of well-being or dysphoric mood, (2) persistent, unexplained fatigue, and (3) sexual function changes (20). Besides FAIS, decreased T concentrations

have been associated with multiple general health consequences in peri- and postmenopausal women. These include increased risks for dyslipidemia (26) and coronary heart disease events (27,28). These associations remain controversial, however, because other studies found no or even reverse effects on risks for metabolic syndrome (29,30) or cardiovascular disease (31). In contrast, from existing literature it becomes clear that lower total T concentrations are associated with increased fracture risk (32) and decreased bone density (33). Furthermore, associations between low T concentrations and decreased verbal fluency (34), and increased frailty in the elderly (35) have been described.

Although some studies (36,37) and guidelines (38) 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 (39).

The diagnoses of FAIS and other health risks possibly associated with hypoandrogenemia are complicated by the lack of reliable T assays and the need for age-adjusted reference values (40,41). 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 to other assays (42,43). The addition of extraction and chromatography procedures before use of RIA removes these interfering proteins and cross-reacting steroids, but extraction is labor intensive and time-consuming (40). A third type of T 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 (44). Finally, most circulating T is biologically inactive due to binding to serum proteins, primarily sex hormone-binding protein (SHBG) and albumin (45). Free T (FT) is the unbound component of total T, while bioavailable T is defined as the concentration of T 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 T (46). Instead of measuring FT concentrations itself, the Free Androgen Index (FAI; T/SHBG x 100) is often calculated. However, FAI is also highly dependent on the quality of T and SHBG assay measurements (47).

Multiple studies have investigated serum total T 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 T assays were applied and control groups were not uniform. The current systematic review

and meta-analysis was aimed to investigate total T 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 constitution of control group and T assay that was used.

Materials and 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 title and abstract. 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 T concentrations in spontaneous POI, and one for T concentrations in iatrogenic menopause.

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

The search for T 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 "free testosterone"[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 follicle-stimulating hormone (FSH) to a menopausal level and decreased estradiol (E_2) concentrations (2,3). Iatrogenic menopause was defined as women who underwent bilateral salpingo-oophorectomy (BSO) or became postmenopausal due to gonadotoxic treatment (such as chemotherapy or irradiation) before natural menopause occurred (48). Controls were required to be women without POI or iatrogenic menopause. Both similar-aged, cycling controls as well as naturally postmenopausal controls were considered eligible.

Exclusion criteria were hyperandrogenemia, BSO or gonadotoxic treatment performed after menopause had occurred, 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 galactosemia, 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.T.) to avoid selection bias (*Figure 1*). Final inclusion occurred after the examination of full text. Any disagreement was resolved by consensus or a third reviewer (B.F.).

Data extraction

From each study included for review, the following information was extracted by two investigators independently (F.J. and S.T.) using a standardized data extraction form: author, year of publication, study design, patient population characteristics (criteria, sample size, age, body mass index (BMI), time since POI/iatrogenic menopause), constitution of controls, and a description of the applied total T assay (*Tables 1 and 2*). Total T concentrations were also extracted from relevant studies. When data were presented in subgroups within a study, pooled means and pooled mean standard deviations (SDs) were calculated, using the following formula: $SD_{pooled}^{\ 2} = [(n_1 - 1) \times SD_1^{\ 2} + (n_2 - 1) \times SD_2^{\ 2} + (m_1^{\ 2} + m_2^{\ 2} - 2 \times m_1 \times m_2) \times n_1 \times n_2/(n_1 + n_2)]/(n_1 + n_2 - 1)$, where n is the sample size, SD is standard deviation, and m is the mean. Any T concentrations reported as conventional

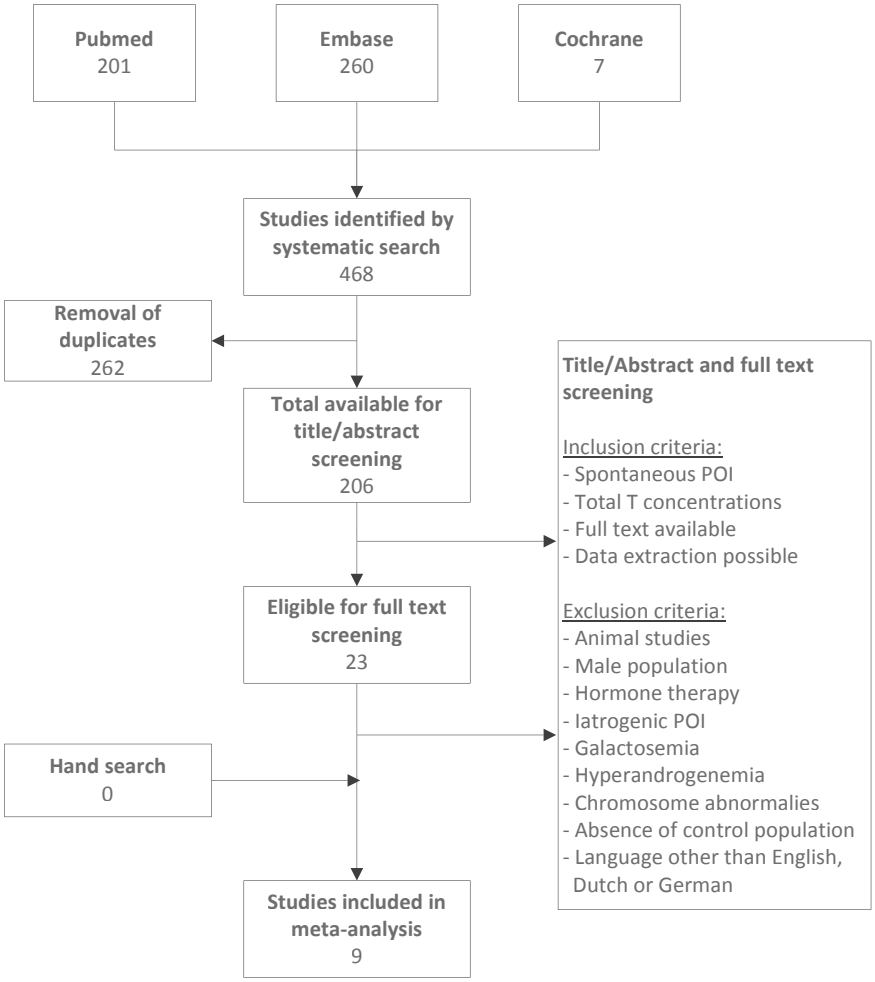


Figure 1A Search and selection for studies on total testosterone concentrations in women with spontaneous POI.

units (ng/mL or ng/dL) were converted to SI units (nmol/L) by multiplication of the data by 3.467 or 0.03467, respectively. Reported standard errors of the mean (SEM) were converted to SD with the following formula: SD = SEM x √n, where SD is the standard deviation, SEM is standard error of the mean, and n is the sample size. Geometric means and 95% confidence intervals were converted to 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.

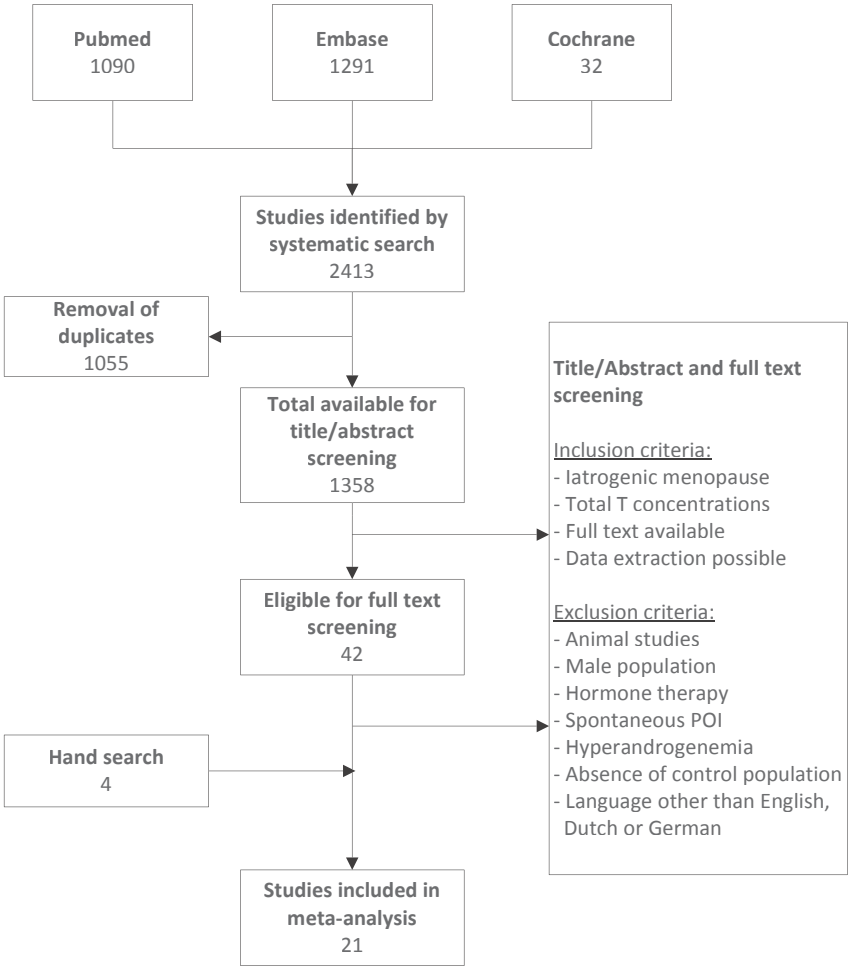


Figure 1B Search and selection for studies on total testosterone concentrations in women with iatrogenic menopause.

Critical appraisal

Included studies were critically appraised by two independent reviewers (F.J. and S.T.) according to the Newcastle-Ottawa Scale for meta-analysis of observational studies (49) and following the Cochrane risk of bias assessment (50) (*Table 3* and *Supplementary Tables 1 and 2*). 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 T concentration, such as age, BMI and duration of amenorrhea, were considered

as higher quality studies. Furthermore, validity of outcome measurement (T 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% confidence intervals (95% CIs) were calculated for the comparison of T 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 T assay and for constitution of controls were conducted to examine their contribution to T concentrations. Finally, sensitivity analyses of high-quality studies were performed. High-quality studies were defined as a high Newcastle-Ottawa score and studies in which confounding factors for T 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 2,413 studies for spontaneous POI and iatrogenic menopause, respectively (*Figure 1*). Duplicates were removed using Reference Manager, which resulted in 206 and 1,358 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 1,316 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 (51) was excluded due to identical results as a previously published study (52), 2 studies were excluded because POI diagnosis did not meet the predefined criteria (53,54), 3 studies did not include female controls (18,55,56), 1 study did not measure serum T concentrations (57), 1 study focused only on women with galactosemia (58), 1 study was a review paper (59), 2 studies were conference abstracts (60,61), and 3 studies were excluded because of language other than English, Dutch or German (62-64).

For iatrogenic menopause, the following studies were excluded (n = 25): 1 study (65) was excluded due to identical results as a previously published study (66), 3 studies were excluded because postmenopausal oophorectomy was performed

Table 1. Summary of studies assessing total testosterone concentrations in women with spontaneous POI and controls

Author	Study Design	POI	Control	Total testosterone assay
Bermudez 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 Intraassay CV 5% Interassay CV NR LLOD NR
Hartmann 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, postmenopausal, age 53.1 ± 2.6 years, BMI 24.5 ± 2.6 kg/m^2, duration of amenorrhea 2.6 ± 1.4 years	Direct RIA Intraassay CV 6.5% Interassay CV 11.2% LLOD 0.14 nmol/L
Elias 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 Intraassay CV <11% Interassay CV <12% LLOD < 0.04 nmol/L
Doldi 1998	Cross-sectional Case control	N = 25, age 30.2 years, BMI 22.4 kg/m2. 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 Interassay 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 Interassay CV NR LLOD NR

Table 1 continued

Author	Study Design	POI	Control	Total testosterone assay
Benetti-Pinto 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 postmenopausal women, age 55.1 ± 3.9 years, duration of amenorrhea 5.4 ± 3.8 years	Direct RIA Intra-assay CV NR Interassay CV: 5.6%. LLOD NR
Kalantaridou 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. Intraassay CV 6.1% Interassay CV 15% LLOD 0.10 nmol/L
Van der Stege 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 Intraassay CV 4.9-7.1% Interassay CV 14-19% LLOD NR
Janse 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 Intraassay CV 3.5-3.8 % Interassay 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; LOD: limit of detection; NR: not reported.

Table 2. Summary of studies assessing total testosterone concentrations in women with iatrogenic menopause and controls

Author	Study Design	Iatrogenic menopause	Control	Testosterone assay
Lamb 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 Intraassay CV NR Interassay 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 Intraassay CV NR Interassay 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 Intraassay CV NR Interassay CV NR LOD NR
Studd 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 Intraassay CV NR Interassay CV NR LOD NR
Beksaç 1983	Prospective cohort Case control	N = 27 women undergoing BSO and hysterectomy, measurement 7 days postoperatively. Mean age and BMI NR	N = 25 premenopausal women undergoing hysterectomy without oophorectomy, measurement 7 days postoperatively. Mean age and BMI NR	Method of RIA not specified Intraassay CV NR Interassay CV NR LOD NR
Sherwin 1985	Prospective cohort Case control	N = 10 women undergoing hysterectomy with BSO for benign causes, mean age 45.9 ± 3.3 years, measurement 8 months postoperatively (placebo for HT)	N = 10 women undergoing hysterectomy without BSO for benign causes, mean age 36.3 ± 2.2 years, measurement 8 months postoperatively	Direct RIA Intraassay CV NR Interassay CV NR LOD NR

Table 2. Continued I

Author	Study Design	Iatrogenic menopause	Control	Testosterone assay
Inskip 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 (≥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 Intraassay CV 6% Interassay CV 10% LOD 0.07 nmol/L
Wakatsuki 1995	Cross-sectional Case control	N = 10 BSO, age 43.67 ± 2.55 years, >2 years after BSO	N = 10 natural menopause, age 65.63 ± 5.58 years, >1 years after final menstrual period	Extraction/chromatography RIA Intraassay CV NR Interassay CV NR LOD NR
Laughlin 2000	Cross-sectional Case control	N = 123 BSO, age 73 ± 7 years, BMI 24.5 ± 3.8 kg/m^2, age at BSO 46 ± 9 years	N = 438 natural menopause, age 74 ± 8 years, BMI 24.1 ± 3.7 kg/m^2, age at menopause 49 ± 5 years	Extraction/chromatography RIA Intraassay CV 4.0% Interassay CV 4.9% LOD 0.011 nmol/L
Sowers 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 Intraassay CV 15.5% Interassay CV 15.5% LOD NR
Couzinet 2001	Cross-sectional Case control	N = 15 BSO, age 53 ± 3 years, time since BSO 15.3 ± 2.1 years	N = 15 natural menopause, age 57 ± 4 years, time since menopause 13.3 ± 2.0 years	Extraction/chromatography RIA Intraassay CV 15% Interassay CV 17% LOD 0.17 nmol/L
García-Pérez 2004	Cross-sectional Case control	N = 35 BSO, age 49.31 ± 6.52 years, BMI 25.60 ± 3.64 kg/m^2, time since BSO 4.31 ± 4.13 years	N = 112 natural menopause, age 54.23 ± 5.48 years, BMI 25.95 ± 4.14 kg/m^2, time since menopause 6.24 ± 5.29 years	Direct RIA Intraassay CV 4% Interassay CV 9% LOD NR

Table 2. Continued II

Author	Study Design	Iatrogenic menopause	Control	Testosterone assay
Davison 2005	Cross-sectional Case control	N = 27 BSO, age 55-75 years	N = 183 natural menopause, age 55-75 years.	Extraction/chromatography RIA Intraassay CV 4.2-10.5% Interassay 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 postoperative 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 postoperative day 7	Direct RIA Intraassay CV 2.1% Interassay CV 2.5%. LOD NR
Cappola 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% Interassay 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 Intraassay CV 8.9% Interassay CV 19.1% LOD 0.10 nmol/L
Korse 2009	Cross-sectional Case control	N = 35 BRCA1/2 carriers after prophylactic BSO, age 45.9 ± 6.1 years	N = 40 naturally postmenopausal BRCA1/2 carriers, age 56.5 ± 5.9 years	Direct RIA Intraassay CV NR Interassay CV NR LOD 0.07 nmol/L
Danforth 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 Intraassay CV 6-13.6 % Interassay 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 postoperatively	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 Intraassay CV NR Interassay CV 4-5 % LOD 0.027 nmol/L

Table 2. Continued III

Author	Study Design	Iatrogenic menopause	Control	Testosterone assay
Labrie 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 Interassay CV 2.9% Interassay 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 postoperatively	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 Intraassay CV NR Interassay 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.

(67-69), in 1 study it was unclear whether the gonadotoxic treatment resulted in a postmenopausal status (70), 12 studies did not include female controls (57,71-81), 2 studies did not measure total T (82,83), in 1 study women used hormone therapy (84), 1 study did not report post-oophorectomy T values (66), 1 study was a conference record (85), and 3 studies were excluded because of language other than English, Dutch or German (86-88).
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 1*. In most studies, POI diagnosis was reported as a postmenopausal FSH concentration > 40 IU/L. Two studies did not adhere to this strict cut-off for FSH, due to describing FSH as 'elevated' (89) or using a cut-off of only 25.6 IU/L (90), 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 2 also included a postmenopausal control group (52,91). Direct radioimmunoassay (RIA) was applied in 5 studies, while 4 studies incorporated extraction/chromatography steps before RIA was applied.
Details for the included studies for iatrogenic menopause are described in *Table 2*. 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 (92). Reasons for BSO were benign or prophylactic in 5 studies, while BSO was performed because of cancer in 3 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 (93). Eleven studies applied extraction/chromatography and RIA, 2 used (isotope dilution-) liquid chromatography- tandem mass spectrometry (LC-MS/MS), 1 used gas chromatography- mass spectrometry (GC-MS), and the remaining studies used direct RIA (n = 6). One study did not mention the type of T assay that was used (94).

Methodological quality

The quality assessment for studies selected for the comparison of T concentrations in spontaneous POI vs. controls is reported in *Table 3* (summary) and in *Supplementary Table 1*. All but 2 studies adjusted for the important confounders age and BMI, or duration of menopause and BMI. However, in 5 out of 9 studies selection bias could not be excluded because recruitment procedures for women

Table 3. Summary of critical appraisal of included studies using the Newcastle-Ottawa Quality Assessment Scale for case-control studies

Study ID	Selection *(max 4 stars)*	Comparability *(max 2 stars)*	Exposure *(max 3 stars)*
Spontaneous POI			
Bermudez 1993	**	-	*
Hartmann 1997	**	**	**
Elias 1997	*	*	*
Doldi 1998	**	**	**
Falsetti 1999	**	**	*
Benetti-Pinto 2005	**	**	*
Kalantaridou 2006	****	**	**
Van der Stege 2008	***	**	**
Janse 2011	***	**	**
Iatrogenic menopause			
Lamb 1964	****	-	**
Abraham 1969	*	*	*
Vermeulen 1976	**	*	**
Studd 1978	**	-	**
Beksaç 1983	**	-	**
Sherwin 1985	***	*	**
Inskip 1994	**	**	**
Wakatsuki 1995	*	*	*
Laughlin 2000	****	**	**
Sowers 2001	****	*	**
Couzinet 2001	**	**	*
García-Pérez 2004	**	**	*
Davison 2005	***	*	*
Hassa 2006	***	*	*
Cappola 2007	***	-	*
McTiernan 2008	*	*	*
Korse 2009	****	**	***
Danforth 2010	*	**	*
Bui 2010	**	-	*
Labrie 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, 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, c) no description.
4. Definition of controls. a) no history of disease (endpoint)*, 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)*, 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, 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, c) rate different and no designation.

with spontaneous POI and controls were not described. Of the remaining 4 studies, 2 included all consecutive POI patients, while 2 invited these women with advertisements, internet, or through recruitment letters to physicians. In these 4 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 T assays were employed in all case-control comparisons. The influence of reporting bias could not be assessed. In summary, 3 studies (19,95,96) scored 7 points or higher on the Newcastle-Ottawa scale, indicating good methodological quality.

For the comparison of T concentrations in iatrogenic menopause vs. controls, the quality assessment for included studies are reported in *Table 3* (summary) and in *Supplementary Table 2*. In most studies (n = 12) iatrogenic menopause was confirmed by medical records, however, 4 studies relied on self report only and 3 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 6 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 5 studies no matching or adjustment was performed at all. Moreover, recruitment procedures of cases or controls were not described in 9 and 6 studies, respectively. Eight studies 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 T assays in all case-control comparisons. Assessment of reporting bias was not possible. Summarizing the Newcastle-Ottawa scale assessment, 3 studies (97-99) scored 7 points or higher, and were therefore considered as the best available evidence.

Testosterone concentrations in spontaneous POI

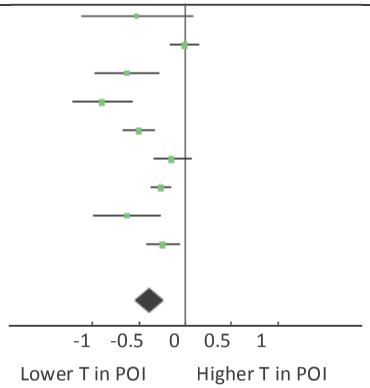
The 9 studies included in the meta-analysis reported total T concentrations in 529 women with spontaneous POI and in 319 controls. Women with spontaneous POI demonstrated significantly lower total T concentrations compared with controls (9 studies); WMD (95% CI) -0.38 (-0.55 to -0.22) nmol/L. However, substantial between-study heterogeneity was identified (I^2 = 81%) (*Figure 2*). Subgroup analyses for comparison between the associations with cycling controls and postmenopausal controls identified that total T concentrations in spontaneous

POI are significantly lower in the first control group (WMD (95% CI): -0.38 (-0.55 to -0.22) nmol/L), but not in the latter control group (WMD (95% CI): 0.07 (-0.17 to 0.32) nmol/L). This between-subgroup difference reached statistical significance (P = 0.002) (*Supplementary Figure 1*). 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 Figure 2*). In the sensitivity analysis, the difference in total T concentrations between women with spontaneous POI and controls remained robust (3 studies, WMD (95% CI): -0.31 (-0.46 to -0.15) nmol/L), and resulted in a substantial decrease in heterogeneity (I^2 = 51%) (*Figure 3*).

Testosterone concentrations in iatrogenic menopause

Due to incomplete data and nonresponsive authors, the following studies could not be included in the meta-analysis (n = 4): in 1 study only mean ratios without original data were reported (92), 2 studies reported geometric means without 95% CIs and data could therefore not be converted into means and SDs (100,101), and 1 study did not mention SD for cases (102). The 17 studies included in the meta-analysis reported data on 558 women with iatrogenic menopause and 2,425 controls. In women with iatrogenic menopause, total T 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%) (*Figure 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) vs. postmenopausal or postmenopausally 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 not reach statistical significance (P = 0.09) (*Supplementary Figure 3*). Subgroup analysis for assay type did not identify statistical differences between T measurement by direct RIA (WMD (95% CI): -0.49 (-0.83 to 0.14) nmol/L) vs. extraction/chromatography RIA or LC-MS/MS or GC-MS (WMD (95% CI): -0.18 (-0.27 to -0.10) nmol/L) in this population (P = 0.09) (*Supplementary Figure 4*). Sensitivity analyses performed for the best-quality studies confirmed the overall findings (3 studies, WMD (95% CI): -0.24 (-0.29 to -0.18) nmol/L), and between-study heterogeneity remained substantial (I^2 = 83%) (*Figure 5*).

	Spontaneous POI			Controls					
Study	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean Difference, Random, 95% CI
Bermudez 1993	7	0.55	0.31	6	1.07	0.69		5.1%	-0.52 (-1.12 - 0.08)
Elias 1997	29	0.96	0.38	29	0.97	0.19		13.6%	-0.01 (-0.16 - 0.14)
Hartmann 1997	33	0.87	0.55	33	1.5	0.83		9.3%	-0.63 (-0.97 - -0.29)
Doldi 1998	25	1.2	0.4	18	2.1	0.6		9.8%	-0.90 (-1.22 - -0.58)
Falsetti 1999	40	1.0	0.3	30	1.5	0.4		13.2%	-0.50 (-0.67 - -0.33)
Benetti-Pinto 2005	30	0.79	0.36	30	0.93	0.43		12.5%	-0.14 (-0.34 - 0.06)
Kalantaridou 2006	130	0.81	0.32	65	1.07	0.36		14.5%	-0.26 (-0.36 - -0.16)
van der Stege 2008	27	2.34	0.79	63	2.97	0.78		9.0%	-0.63 (-0.98 - -0.28)
Janse 2011	208	1.12	0.41	45	1.36	0.58		13.1%	-0.24 (-0.42 - -0.06)
Total (95% CI)	**529**			**319**				**100.0%**	**-0.38 (-0.55 - -0.22)**

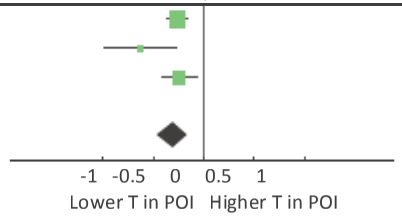


Heterogeneity: $I^2 = 81\%$

Test for overall effect: Z = 4.58 ($P < 0.00001$)

Figure 2. Meta-analysis of 9 comparative studies on total testosterone concentrations in women with spontaneous POI compared with controls.

	Spontaneous POI			Controls					
Study	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean Difference, Random, 95% CI
Kalantaridou 2006	130	0.81	0.32	65	1.07	0.36		50.8%	-0.26 (-0.36 - -0.16)
van der Stege 2008	27	2.34	0.79	63	2.97	0.78		14.4%	-0.63 (-0.98 - -0.28)
Janse 2011	208	1.12	0.41	45	1.36	0.58		34.7%	-0.24 (-0.42 - -0.06)
Total (95% CI)	**365**			**173**				**100.0%**	**-0.31 (-0.46 - -0.15)**



Heterogeneity: $I^2 = 51\%$

Test for overall effect: Z = 3.94 ($P < 0.0001$)

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

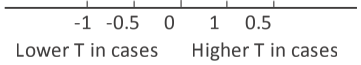
Discussion

The current systematic review of the literature and subsequent meta-analysis was set up to assess for the first time whether serum total testosterone (T) 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 T concentrations were significantly lower compared to controls: WMD (95% CI) -0.38 (-0.55 to -0.22) nmol/L. The difference between pooled total T concentrations in women with iatrogenic menopause compared to 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, and 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 T to estradiol by fatty tissue), and possibly to diminished well-being and sexual health (20), and increased risk for cardiovascular disease (28), it is relevant to evaluate the T 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. There is increasing evidence in the general female population that advancing age per se is associated with decreasing total T concentrations (41,103,104). The decline in circulating T may also occur as a result of atrophy of the aging adrenal zona reticularis (diminished production of T precursors), which may lead to decreased peripheral conversion of DHEA-sulfate (through DHEA) to T (23,105). However, it is debated whether menopause as such is associated with serum T alterations. It has been shown that T levels do not fall abruptly in women undergoing natural menopause due to the preservation of androgen producing theca cells along with elevated LH concentrations (69,106), although the ovarian contribution to circulating T in postmenopausal women remains the topic of extensive debate (25,93). 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 T is decreased, but the underlying mechanisms remain to be discovered.

To control or adjust for the two possible confounders age and BMI (107,108) was an important part of the quality assessment in the current review of total T 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.

	Iatrogenic menopause			Controls					
Study	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean difference, Random, 95% CI
Abraham 1969	6	1.7	0.42	6	2.1	0.54		2.5%	-0.40 (-0.95 - 0.15)
Vermeulen 1976	8	0.24	0.21	19	1.03	0.6		4.7%	-0.79 (-1.10 - -0.48)
Beksaç 1983	27	1.7	0.32	25	2.01	0.26		6.6%	-0.31 (-0.47 - -0.15)
Sherwin 1985	10	1.55	0.11	10	3.08	0.16		7.0%	-1.53 (-1.65 - -1.41)
Wakatsuki 1995	10	1.98	0.28	10	2.17	0.61		3.5%	-0.19 (-0.61 - 0.23)
Laughlin 2000	123	0.29	0.14	438	0.56	0.26		7.7%	-0.27 (-0.30 - -0.24)
Couzinet 2001	15	0.55	0.12	15	0.62	0.27		6.7%	-0.07 (-0.22 - 0.08)
Sowers 2001	33	0.53	0.02	509	0.78	0.01		7.8%	-0.25 (-0.26 - -0.24)
García-Pérez 2004	35	1.77	0.9	112	1.59	0.75		4.4%	0.18 (-0.15 - 0.51)
Davison 2005	27	0.39	0.24	183	0.69	0.45		7.1%	-0.30 (-0.41 - -0.19)
Hassa 2006	35	0.95	0.47	57	1.1	0.81		5.2%	-0.15 (-0.41 - 0.11)
Cappola 2007	56	0.45	0.42	219	0.69	0.66		6.8%	-0.24 (-0.38 - -0.10)
McTiernan 2008	24	0.47	0.16	241	0.74	0.32		7.5%	-0.27 (-0.35 - -0.19)
Korse 2009	35	0.97	0.42	40	0.89	0.46		6.1%	0.08 (-0.12 - 0.28)
Bui 2010	8	0.82	0.42	16	0.86	0.39		4.2%	-0.04 (-0.39 - 0.31)
Labrie 2011	71	0.38	0.17	442	0.49	0.28		7.6%	-0.11 (-0.16 - -0.06)
Alarslan 2011	35	1.88	0.85	83	1.87	0.77		4.4%	0.01 (-0.32 - 0.34)
Total (95% CI)	**558**			**2425**				**100.0%**	**-0.29 (-0.39 - -0.18)**

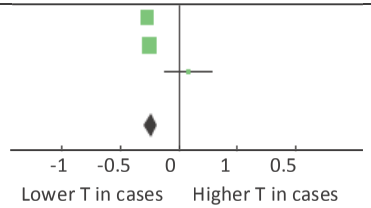


Heterogeneity: $I^2 = 97\%$

Test for overall effect: Z = 5.39 ($P < 0.00001$)

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

	Iatrogenic menopause			Controls					
	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean difference, Random, 95% CI
Laughlin 2000	123	0.29	0.14	438	0.56	0.26		42.6%	-0.27 (-0.30 - -0.24)
Sowers 2001	33	0.53	0.02	509	0.78	0.01		50.8%	-0.25 (-0.26 - -0.24)
Korse 2009	35	0.97	0.42	40	0.89	0.46		6.7%	0.08 (-0.12 - 0.28)
Total (95% CI)	**191**			**987**				**100.0%**	**-0.24 (-0.29 - -0.18)**



Heterogeneity: $I^2 = 83\%$

Test for overall effect: Z = 8.39 ($P < 0.00001$)

Figure 5. Sensitivity analysis of the 3 best-quality studies included in the meta-analysis on total testosterone in women with spontaneous POI.

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- vs. postmenopausal 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-heterogeneity may be that none of the studies applied identical T assays. The measurement of T in women is challenging due to lack of trueness, precision and sensitivity of various available T assays (40), and between-study comparability of results is therefore problematic. Subgroup analyses for direct RIA vs. extraction/chromatography RIA or LC-MS/MS identified that heterogeneity between studies became less important when the latter assay types were applied, and a significant difference 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 T assays (40,43,109). Finally, most applied T assays were reported to have high intra- and interassay 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 T assays need to be improved before T measurements could be used in a clinical setting to distinguish a women with hypoandrogenism from normoandrogenic women.

Only a limited number of studies have directly investigated the consequences of decreased T 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 T concentrations were identified in these women with POI, an independent role for T concentration could not be identified (19). 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 (HSDD) (110,111), diminished health-related quality of life (112), osteoporosis (14) and fracture risk (113), parkinsonism (114) or cognitive impairment (115). These findings are suggestive of a common pathological pathway, but the exact association with decreased T 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 investigated androgen therapy in co-treatment with estrogen therapy (116,117), and effects of higher serum T concentrations on the remission of symptoms, such as memory function, are not clear (118).

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 lead 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 in the best quality studies only (please see *Figures 3 and 5*). Furthermore, the search was restricted to total T concentrations only. While SHBG, bioavailable T, and FT have also been shown to correlate with clinical state such as metabolic syndrome (107), it is unclear which of these measurement best reflects the availability of T at tissue level (119).

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

Acknowledgements

We like to thank the authors of the original publications who took the effort to provide us with additional data or clarifications of their studies (19,120). Furthermore, we would like to thank Dr. Angelique Goverde for her critically reading the manuscript.

Supplementary materials

Table S1. Critical appraisal of included studies for spontaneous POI using the Newcastle-Ottawa

	Selection			
Study ID	*1. Is case definition adequate?*	*2. Cases*	*3. Selection of Controls*	*4. Definition of controls*
Bermudez 1993	*	- no description	- no description	* Normal ovarian function
Hartmann 1997	* medical record	- Patients at outpatient clinic; unclear whether collected consecutively	- healthy volunteers for OC study (cycling women) and hospital controls (postmenopausal)	* cycling: regular cycle, normal FSH menopausal: medical record
Elias 1997	* elevated FSH (not specified)	- no description	- no description	- no description of source ("normal control subjects")
Doldi 1998	* FSH > 25.6 IU/L	- no description	- Hospital controls	* medical record
Falsetti 1999	* medical record	- no description	- no description	* medical record
Benetti-Pinto 2005	* medical record	- no description	- no description	* Control 1: regular menstrual cycle Control 2: Postmenopausal
Kalantaridou 2006	* medical record	* Recruitment letters to physicians, internet.	* General population recruitment by advertisement.	* medical record
Van der Stege 2008	* medical record	- Invitation, response rate 47%	* General population recruitment by advertisement	* medical record, hormonal screening
Janse 2011	* medical record	* consecutive patients visiting outpatient clinic	- Hospital controls	* medical record, FSH < 12 IU/L

Abbreviations: POI: primary ovarian insufficiency; FSH: follicle-stimulating hormone; BMI: body mass index; MP: menopause

Selection

1. Is the case definition adequate? a) yes, with independent validation*, b) yes, e.g. record linkage or based on self reports, 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, c) no description.
4. Definition of controls. a) no history of disease (endpoint)*, b) no description of source.

Quality Assessment Scale

Comparability	Exposure		
1. Comparability of cases & controls	*1. Ascertainment of exposure*	*2. Same tests for groups*	*3. Non-response rate*
-	* medical record	- not reported	- not reported
** cycling: age- and BMI-matched menopausal: BMI- and time since menopause-matched	* medical record	* outpatient clinic	- not reported
* Age-matched, no information on BMI	- not described for controls	- not reported	- not reported
** Similar age and BMI	* medical record	* outpatient clinic	- not reported
* * Similar age and BMI	* medical record	- not reported	- not reported
** young controls: matched for age and BMI menopause controls: matched for duration of amenorrhea	* medical record	- not reported	- not reported
* * Similar age and BMI, adjusted for ethnicity	* medical record	*	- not reported
** Adjusted for age and BMI	* medical record	*	- not reported
** Adjusted for age and BMI	* medical record	*	- not reported

Comparability

1. Comparability of cases and controls on the basis of the design or analysis. a) study controls for(select most important factor)*, 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, 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, c) rate different and no designation.

Table S2. Critical appraisal of included studies for iatrogenic menopause using the Newcastle-Ottawa

	Selection			
Study ID	*1. Is case definition adequate?*	*2. Cases*	*3. Selection of Controls*	*4. Definition of controls*
Lamb 1964	* medical record	*	*	*
Abraham 1969	- no description	- not stated	- no description	*
Vermeulen 1976	* medical record, FSH	- not stated	- no description	* medical record and FSH
Studd 1978	* medical record	- Not representative	- Not representative	*
Beksaç 1983	* medical record	- no description	- no description	* medical record
Sherwin 1985	* medical record	* Consecutive cases	- Hospital controls	* medical record
Inskip 1994	- menopausal status unknown	*	- Hospital controls	* Hysterectomy only
Wakatsuki 1995	- no description	- not stated	- no description	*
Laughlin 2000	* self report and medical record	* Participants of heart disease cohort	* Participants of heart disease cohort; response rate 82%	* self report
Sowers 2001	* medical record	* community based sample	* community based sample	* medical record
Couzinet 2001	* medical record	- not stated	- no description	* medical record
García-Pérez 2004	* medical record	- population regularly at menopause unit. Possibility for selection bias	- hospital controls, regularly attending menopause unit.	* natural menopause, medical record
Davison 2005	- self report; unclear whether BSO caused iatrogenic MP	* community based sample	* community based sample	* self report and FSH and E_2 concentrations

Quality Assessment Scale

Comparability	**Exposure**		
1. Comparability of cases & controls	*1. Ascertainment of exposure*	*2. Same tests for groups*	*3. Non-response rate*
- Not performed	*	*	- not reported
* BMI-adjusted, but not adjusted for age	- not described	*	- not reported
* Comparable age, but no information on BMI	* medical record	*	- not reported
Not performed	*	*	- not reported
- Age unknown; not adjusted for any variable	* medical records	*	- not reported
* Adjusted for age, but no information on BMI	* medical records	*	- not reported
** Adjusted for age and BMI	*	*	- not reported
* not adjusted for age, but similar in BMI	- no description	*	- not reported
** Age- and BMI-matched	* medical record checked for concordance	*	- not reported for individual groups
* Similar age, but BMI in subgroups unknown	* surgical record	* community sample	- not reported
** BMI- and time since MP matched, Controlled for age	* medical record	- not stated	- not reported
** Similar BMI, controlled for age and time since MP	-	*	- Participation rate controls 62%, cases 81%
* Age-matched, but no information on BMI or time since menopause	- self report	* community sample	- not reported

Table S2. Continued

Study ID	Selection			
	1. Is case definition adequate?	*2. Cases*	*3. Selection of Controls*	*4. Definition of controls*
Hassa 2006	* medical record	* representative cases at hospital	- hospital controls	* medical record
Cappola 2007	- Self report	* community based sample	* community based sample	* Self report
McTiernan 2008	- Self report	- Potential for selection bias	- Potential for selection bias	* questionnaire
Korse 2009	* medical record	* consecutive cases	* consecutive cases	* medical record
Danforth 2009	- questionnaire	- not stated	- all nurses, not community controls	* questionnaire
Bui 2010	* medical record	- Potential for selection bias	- selection of women in other longitudinal study	*
Labrie 2011	* medical history	- not stated	- no description	* medical history
Alarslan 2011	- not reported	*	- hospital controls, visiting for routine gynaecological care.	*

Abbreviations: FSH: follicle-stimulating hormone; BMI: body mass index; MP: menopause.

Selection

1. Is the case definition adequate? a) yes, with independent validation*, b) yes, e.g. record linkage or based on self reports, 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, c) no description.
4. Definition of controls. a) no history of disease (endpoint)*, b) no description of source.

Comparability	Exposure		
1. Comparability of cases & controls	*1. Ascertainment of exposure*	*2. Same tests for groups*	*3. Non-response rate*
* Adjusted for age, but not for BMI	*	- not reported	- not reported
- Not controlled for age, BMI, time since menopause	- Self report	* community sample	- not reported for individual groups
* Adjusted for BMI	- Self report	*	- not reported
** Controlled for age, NS difference time since MP	* medical record	*	* all women were included
** Adjusted for age, BMI, and time since menopause	- questionnaire	*	- not reported
- Not controlled	*	- no	- not reported
* Similar age, but not adjusted for BMI, and time since menopause	- Medical history, not blinded	*	- not reported
* Matched for age and BMI, but duration of postmenopause sign higher in cases	*	*	- not reported

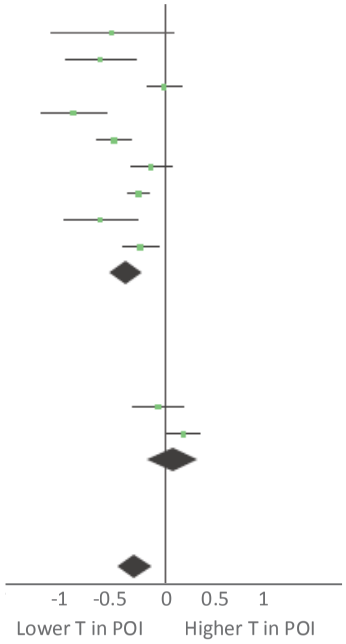
Comparability

1. Comparability of cases and controls on the basis of the design or analysis. a) study controls for (select most important factor)*, 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, 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, c) rate different and no designation.

	Spontaneous POI			Controls					
Study	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean difference, Random, 95% CI
Cycling controls									
Bermudez 1993	7	0.55	0.31	6	1.07	0.69		4.7%	-0.52 (-1.12 - 0.08)
Hartmann 1997	33	0.87	0.55	33	1.5	0.83		7.9%	-0.63 (-0.97 - -0.29)
Elias 1997	25	0.96	0.38	18	0.97	0.19		10.3%	-0.01 (-0.18 - 0.16)
Doldi 1998	25	1.2	0.4	18	2.1	0.6		8.2%	-0.90 (-1.22 - -0.58)
Falsetti 1999	40	1.0	0.3	30	1.5	0.4		10.3%	-0.50 (-0.67 - -0.33)
Benetti-Pinto 2005	30	0.79	0.36	30	0.93	0.43		9.9%	-0.14 (-0.34 - 0.06)
Kalantaridou 2006	130	0.81	0.32	65	1.07	0.36		11.1%	-0.26 (-0.36 - -0.16)
van der Stege 2008	27	2.34	0.79	63	2.97	0.78		7.6%	-0.63 (-0.98 - -0.28)
Janse 2011	208	1.12	0.41	45	1.36	0.58		10.2%	-0.24 (-0.42 - -0.06)
Subtotal (95% CI)	**525**			**308**				**80.2%**	**-0.38 (-0.55 - -0.22)**
Heterogeneity: $I^2 = 80\%$									
Test for overall effect: Z = 4.64 ($P < 0.00001$)									
Postmenopausal controls									
Hartmann 1997	33	0.87	0.55	32	0.94	0.45		9.3%	-0.07 (-0.31 - 0.17)
Benetti-Pinto 2005	30	0.79	0.35	30	0.61	0.27		10.5%	0.18 (0.02 - 0.34)
Subtotal (95% CI)	**63**			**62**				**19.8%**	**0.07 (-0.17 - 0.32)**
Heterogeneity: $I^2 = 65\%$									
Test for overall effect: Z = 0.59 ($P = 0.56$)									
Total (95% CI)	**588**			**370**				**100.0%**	**-0.30 (-0.47 - -0.13)**



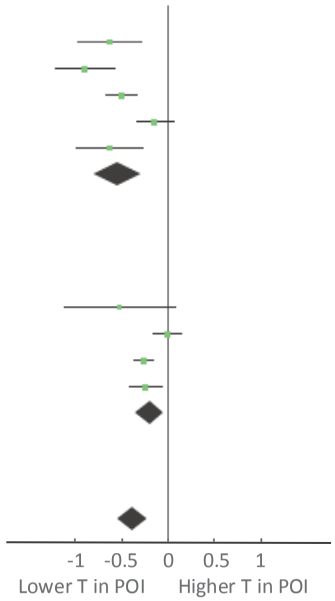
Heterogeneity: $I^2 = 86\%$

Test for overall effect: Z = 3.53 ($P = 0.0004$)

Test for subgroup differences: $P = 0.002$

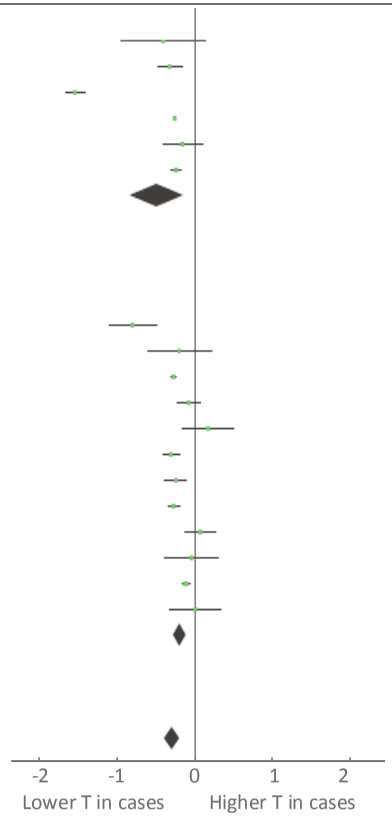
Supplementary figure 1. Subgroup analysis for constitution of control group (pre- vs. postmenopausal women) in the meta-analysis on total testosterone in women with spontaneous POI.

	Spontaneous POI			Controls					
Study	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean Difference, Random, 95% CI
Direct RIA									
Hartmann 1997	33	0.87	0.55	33	1.5	0.83		9.3%	-0.63 (-0.97 - -0.29)
Doldi 1998	25	1.2	0.4	18	2.1	0.6		9.8%	-0.90 (-1.22 - -0.58)
Falsetti 1999	40	1.0	0.3	30	1.5	0.4		13.2%	-0.50 (-0.67 - -0.33)
Benetti-Pinto 2005	30	0.79	0.36	30	0.93	0.43		12.5%	-0.14 (-0.34 - 0.06)
van der Stege 2008	27	2.34	0.79	63	2.97	0.78		9.0%	-0.63 (-0.98 - -0.28)
Subtotal (95% CI)	**155**			**174**				**53.8%**	**-0.54 (-0.79 - -0.29)**
Heterogeneity: $I^2 = 79\%$									
Test for overall effect: Z = 4.16 ($P < 0.0001$)									
Extraction/chromatography RIA									
Bermudez 1993	7	0.55	0.31	6	1.07	0.69		5.1%	-0.52 (-1.12 - 0.08)
Elias 1997	29	0.96	0.38	29	0.97	0.19		13.6%	-0.01 (-0.16 - 0.14)
Kalantaridou 2006	130	0.81	0.32	65	1.07	0.36		14.5%	-0.26 (-0.36 - -0.16)
Janse 2011	208	1.12	0.41	45	1.36	0.58		13.1%	-0.24 (-0.42 - -0.06)
Subtotal (95% CI)	**374**			**145**				**46.2%**	**-0.19 (-0.35 - -0.04)**
Heterogeneity: $I^2 = 64\%$									
Test for overall effect: Z = 2.49 ($P = 0.01$)									
Total (95% CI)	**529**			**319**				**100.0%**	**-0.38 (-0.55 - -0.22)**
Heterogeneity: $I^2 = 81\%$									
Test for overall effect: Z = 4.58 ($P < 0.00001$)									
Test for subgroup differences: $P = 0.02$									



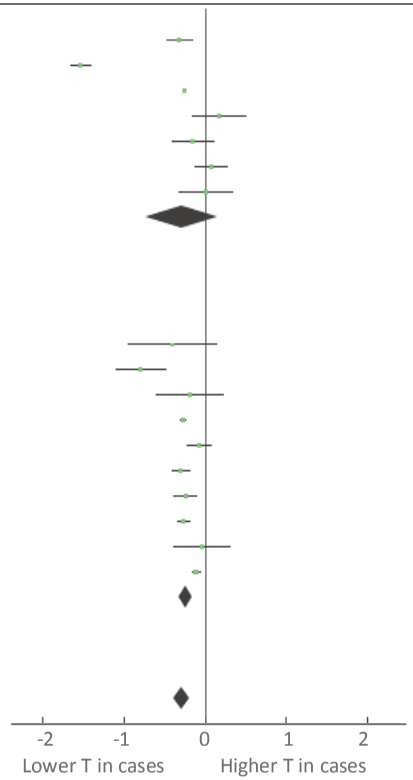
Supplementary figure 2. Subgroup analysis for assay type (direct RIA vs. extraction/chromatography RIA or LC-MS/MS) in the meta-analysis on total testosterone in women with spontaneous POI.
Legend: RIA: radioimmunoassay; LC-MS/MS: liquid chromatography- tandem mass spectrometry.

	Iatrogenic menopause			Controls					
	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean difference, Random, 95% CI
Cycling controls OR premenopausal hysterectomy only									
Abraham 1969	6	1.7	0.42	6	2.1	0.54		2.2%	-0.40 (-0.95 - 0.15)
Beksaç 1983	27	1.7	0.32	25	2.01	0.26		6.2%	-0.31 (-0.47 - -0.15)
Sherwin 1985	10	1.55	0.11	10	3.08	0.16		6.6%	-1.53 (-1.65 - -1.41)
Sowers 2001	33	0.53	0.02	509	0.78	0.01		7.4%	-0.25 (-0.26 - -0.24)
Hassa 2006	35	0.95	0.47	57	1.1	0.81		4.8%	-0.15 (-0.41 - 0.11)
Labrie 2011	442	0.38	0.17	47	0.62	0.24		7.1%	-0.24 (-0.31 - -0.17)
Subtotal (95% CI)	**553**			**654**				**34.2%**	**-0.49 (-0.83 - -0.14)**
Heterogeneity: I^2 = 99%									
Test for overall effect: Z = 2.76 (*P* = 0.006)									
Natural menopause OR postmenopausal hysterectomy only									
Vermeulen 1976	8	0.24	0.21	19	1.03	0.6		4.3%	-0.79 (-1.10 - -0.48)
Wakatsuki 1995	10	1.98	0.28	10	2.17	0.61		3.1%	-0.19 (-0.61 - 0.23)
Laughlin 2000	123	0.29	0.14	438	0.56	0.26		7.3%	-0.27 (-0.30 - -0.24)
Couzinet 2001	15	0.55	0.12	15	0.62	0.27		6.3%	-0.07 (-0.22 - 0.08)
García-Pérez 2004	35	1.77	0.9	112	1.59	0.75		4.0%	0.18 (-0.15 - 0.51)
Davison 2005	27	0.39	0.24	183	0.69	0.45		6.7%	-0.30 (-0.41 - -0.19)
Cappola 2007	56	0.45	0.42	219	0.69	0.66		6.4%	-0.24 (-0.38 - -0.10)
McTiernan 2008	24	0.47	0.16	241	0.74	0.32		7.0%	-0.27 (-0.35 - -0.19)
Korse 2009	35	0.97	0.42	40	0.89	0.46		5.6%	0.08 (-0.12 - 0.28)
Bui 2010	8	0.82	0.42	16	0.86	0.39		3.8%	-0.04 (-0.39 - 0.31)
Labrie 2011	71	0.38	0.17	442	0.49	0.28		7.2%	-0.11 (-0.16 - -0.06)
Alarslan 2011	35	1.88	0.85	83	1.87	0.77		4.0%	0.01 (-0.32 - 0.34)
Subtotal (95% CI)	**447**			**1818**				**65.8%**	**-0.18 (-0.27 - -0.10)**
Heterogeneity: I^2 = 84%									
Test for overall effect: Z = 4.31 (*P* < 0.0001)									
	1000			**2472**				**100.0%**	**-0.29 (-0.38 - -0.19)**
Total (95% CI)									
Heterogeneity: I^2 = 97%									
Test for overall effect: Z = 5.78 (*P* < 0.00001)									
Test for subgroup differences: *P* = 0.09									



Supplementary figure 3. Subgroup analysis for constitution of control group (pre- vs. postmenopausal women) in the meta-analysis on total testosterone in women with iatrogenic menopause.

	Iatrogenic menopause			Controls					
	N	Mean (nmol/L)	SD	N	Mean (nmol/L)	SD	WMD Random, 95% CI	Weight	Mean difference, Random, 95% CI
Direct RIA									
Beksaç 1983	27	1.7	0.32	25	2.01	0.26		6.6%	-0.31 (-0.47 - -0.15)
Sherwin 1985	10	1.55	0.11	10	3.08	0.16		7.0%	-1.53 (-1.65 - -1.41)
Sowers 2001	33	0.53	0.02	509	0.78	0.01		7.8%	-0.25 (-0.26 - -0.24)
García-Pérez 2004	35	1.77	0.9	112	1.59	0.75		4.4%	0.18 (-0.15 - 0.51)
Hassa 2006	35	0.95	0.47	57	1.1	0.81		5.2%	-0.15 (-0.41 - 0.11)
Korse 2009	35	0.97	0.42	40	0.89	0.46		6.1%	0.08 (-0.12 - 0.28)
Alarslan 2011	35	1.88	0.85	83	1.87	0.77		4.4%	0.01 (-0.32 - 0.34)
Subtotal (95% CI)	**210**			**836**				**41.6%**	**-0.29 (-0.74 - 0.16)**
Heterogeneity: I^2 = 99%									
Test for overall effect: Z = 1.27 (*P* = 0.20)									
Extraction/chromatography RIA or LC-MS/MS									
Abraham 1969	6	1.7	0.42	6	2.1	0.54		2.5%	-0.40 (-0.95 - 0.15)
Vermeulen 1976	8	0.24	0.21	19	1.03	0.6		4.7%	-0.79 (-1.10 - -0.48)
Wakatsuki 1995	10	1.98	0.28	10	2.17	0.61		3.5%	-0.19 (-0.61 - 0.23)
Laughlin 2000	123	0.29	0.14	438	0.56	0.26		7.7%	-0.27 (-0.30 - -0.24)
Couzinet 2001	15	0.55	0.12	15	0.62	0.27		6.7%	-0.07 (-0.22 - 0.08)
Davison 2005	27	0.39	0.24	183	0.69	0.45		7.1%	-0.30 (-0.41 - -0.19)
Cappola 2007	56	0.45	0.42	219	0.69	0.66		6.8%	-0.24 (-0.38 - -0.10)
McTiernan 2008	24	0.47	0.16	241	0.74	0.32		7.5%	-0.27 (-0.35 - -0.19)
Bui 2010	8	0.82	0.42	16	0.86	0.39		4.2%	-0.04 (-0.39 - 0.31)
Labrie 2011	71	0.38	0.17	442	0.49	0.28		7.6%	-0.11 (-0.16 - -0.06)
Subtotal (95% CI)	**348**			**1589**				**58.4%**	**-0.24 (-0.32 - -0.16)**
Heterogeneity: I^2 = 82%									
Test for overall effect: Z = 5.67 (*P* < 0.00001)									
Total (95% CI)	**558**			**2425**				**100.0%**	**-0.29 (-0.39 - -0.18)**
Heterogeneity: I^2 = 97%									
Test for overall effect: Z = 5.39 (*P* < 0.00001)									
Test for subgroup differences: *P* = 0.82									



Supplementary figure 4. Subgroup analysis for assay type (direct RIA vs. extraction/chromatography RIA or LC-MS/MS or GC-MS) in the meta-analysis on total testosterone in women with spontaneous POI.
Legend: RIA: radioimmunoassay; LC-MS/MS: liquid chromatography- tandem mass spectrometry; GC-MS: gas chromatography- tandem mass spectrometry.

References

1. Treloar AE. Menstrual cyclicity and the pre-menopause. Maturitas 1981;3:249-264.
2. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986;67:604-606.
3. Cooper AR, Baker VL, Sterling EW, Ryan ME, Woodruff TK, Nelson LM. The time is now for a new approach to primary ovarian insufficiency. Fertil Steril 2010;95:1890-1897.
4. de Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. Lancet 2010;376:911-921.
5. Bakalov VK, Anasti JN, Calis KA, Vanderhoof VH, Premkumar A, Chen S, Furmaniak J, Smith BR, Merino MJ, Nelson LM. Autoimmune oophoritis as a mechanism of follicular dysfunction in women with 46,XX spontaneous premature ovarian failure. Fertil Steril 2005;84:958-965.
6. Mattei MG, Mattei JF, Ayme S, Giraud F. X-autosome translocations: cytogenetic characteristics and their consequences. Hum Genet 1982;61:295-309.
7. Wittenberger MD, Hagerman RJ, Sherman SL, Conkie-Rosell A, Welt CK, Rebar RW, Corrigan EC, Simpson JL, Nelson LM. The *FMR1* premutation and reproduction. Fertil Steril 2007;87:456-465.
8. Qin Y, Choi Y, Zhao H, Simpson JL, Chen ZJ, Rajkovic A. *NOBOX* homeobox mutation causes premature ovarian failure. Am J Hum Genet 2007;81:576-581.
9. Rossetti R, Di Pasquale E, Marozzi A, Bione S, Toniolo D, Grammatico P, Nelson LM, Beck-Peccoz P, Persani L. *BMP15* mutations associated with primary ovarian insufficiency cause a defective production of bioactive protein. Hum Mutat 2009;30:804-810.
10. Janse F, de With LM, Duran KJ, Kloosterman WP, Goverde AJ, Lambalk CB, Laven JS, Fauser BC, Giltay JC. Limited contribution of *NR5A1* (SF-1) mutations in women with primary ovarian insufficiency (POI). Fertil Steril 2012;97:141-146.
11. Knauff EA, Franke L, van Es MA, van den Berg LH, van der Schouw YT, Laven JS, Lambalk CB, Hoek A, Goverde AJ, Christin-Maitre S, Hsueh AJ, Wijmenga C, Fauser BC. Genome-wide association study in premature ovarian failure patients suggests *ADAMTS19* as a possible candidate gene. Hum Reprod 2009;24:2372-2378.
12. Altekruse SF, Kosary CL, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlader N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK. SEER Cancer Statistics Review, 1975-2007. Available at seer.cancer.gov/csr/1975_2007/2010.
13. Graziottin A. Menopause and sexuality: key issues in premature menopause and beyond. Ann N Y Acad Sci 2010;1205:254-261.
14. Yildiz A, Sahin I, Gol K, Taner Z, Uluturk A, Biberoglu K. Bone loss rate in the lumbar spine: a comparison between natural and surgically induced menopause. Int J Gynaecol Obstet 1996;55:153-159.
15. Uygur D, Sengul O, Bayar D, Erdinc S, Batioglu S, Mollamahmutoglu L. Bone loss in young women with premature ovarian failure. Arch Gynecol Obstet 2005;273:17-19.

16. van der Schouw YT, van der Graaf Y, Steyerberg EW, Eijkemans JC, Banga JD. Age at menopause as a risk factor for cardiovascular mortality. Lancet 1996;347:714-718.
17. Kalantaridou SN, Naka KK, Papanikolaou E, Kazakos N, Kravariti M, Calis KA, Paraskevaidis EA, Sideris DA, Tsatsoulis A, Chrousos GP, Michalis LK. Impaired endothelial function in young women with premature ovarian failure: normalization with hormone therapy. J Clin Endocrinol Metab 2004;89:3907-3913.
18. Knauff EA, Westerveld HE, Goverde AJ, Eijkemans MJ, Valkenburg O, van Santbrink EJ, Fauser BC, van der Schouw YT. Lipid profile of women with premature ovarian failure. Menopause 2008;15:919-923.
19. van der Stege JG, Groen H, van Zadelhoff SJ, Lambalk CB, Braat DD, van Kasteren YM, van Santbrink EJ, Apperloo MJ, Weijmar Schultz WC, Hoek A. Decreased androgen concentrations and diminished general and sexual well-being in women with premature ovarian failure. Menopause 2008;15:23-31.
20. Bachmann G, Bancroft J, Braunstein G, Burger H, Davis S, Dennerstein L, Goldstein I, Guay A, Leiblum S, Lobo R, Notelovitz M, Rosen R, Sarrel P, Sherwin B, Simon J, Simpson E, Shifren J, Spark R, Traish A. Female androgen insufficiency: the princeton consensus statement on definition, classification, and assessment. Fertil Steril 2002;77:660-665.
21. Davis SR. When to suspect androgen deficiency other than at menopause. Fertil Steril 2002;77 Suppl 4:S68-S71.
22. Rivera-Woll LM, Papalia M, Davis SR, Burger HG. Androgen insufficiency in women: diagnostic and therapeutic implications. Hum Reprod Update 2004;10:421-432.
23. Davison SL, Bell R, Donath S, Montalto JG, Davis SR. Androgen Levels in Adult Females: Changes with Age, Menopause, and Oophorectomy. J Clin Endocrinol Metab 2005;90:3847-3853.
24. Rannevik G, Jeppsson S, Johnell O, Bjerre B, Laurell-Borulf Y, Svanberg L. A longitudinal study of the perimenopausal transition: altered profiles of steroid and pituitary hormones, SHBG and bone mineral density. Maturitas 1995;21:103-113.
25. Couzinet B, Meduri G, Lecce MG, Young J, Brailly S, Loosfelt H, Milgrom E, Schaison G. The postmenopausal ovary is not a major androgen-producing gland. J Clin Endocrinol Metab 2001;86:5060-5066.
26. Khatibi A, Agardh CD, Shakir YA, Nerbrand C, Nyberg P, Lidfeldt J, Samsioe G. Could androgens protect middle-aged women from cardiovascular events? A population-based study of Swedish women: The Women's Health in the Lund Area (WHILA) Study. Climacteric 2007;10:386-392.
27. Patel SM, Ratcliffe SJ, Reilly MP, Weinstein R, Bhasin S, Blackman MR, Cauley JA, Sutton-Tyrrell K, Robbins J, Fried LP, Cappola AR. Higher serum testosterone concentration in older women is associated with insulin resistance, metabolic syndrome, and cardiovascular disease. J Clin Endocrinol Metab 2009;94:4776-4784.

28. Laughlin GA, Goodell V, Barrett-Connor E. Extremes of endogenous testosterone are associated with increased risk of incident coronary events in older women. J Clin Endocrinol Metab 2010;95:740-747.
29. Bell RJM, Donath SM, Davison SLM, Davis SRM. Endogenous androgen levels and well-being: differences between premenopausal and postmenopausal women. Menopause 2006;13:65-71.
30. Janssen I, Powell LH, Kazlauskaite R, Dugan SA. Testosterone and visceral fat in midlife women: the Study of Women's Health Across the Nation (SWAN) fat patterning study. Obesity (Silver Spring) 2010;18:604-610.
31. Kalyani RR, Franco M, Dobs AS, Ouyang P, Vaidya D, Bertoni A, Gapstur SM, Golden SH. The association of endogenous sex hormones, adiposity, and insulin resistance with incident diabetes in postmenopausal women. J Clin Endocrinol Metab 2009;94:4127-4135.
32. Lee JS, LaCroix AZ, Wu L, Cauley JA, Jackson RD, Kooperberg C, Leboff MS, Robbins J, Lewis CE, Bauer DC, Cummings SR. Associations of serum sex hormone-binding globulin and sex hormone concentrations with hip fracture risk in postmenopausal women. J Clin Endocrinol Metab 2008;93:1796-1803.
33. Rariy CM, Ratcliffe SJ, Weinstein R, Bhasin S, Blackman MR, Cauley JA, Robbins J, Zmuda JM, Harris TB, Cappola AR. Higher serum free testosterone concentration in older women is associated with greater bone mineral density, lean body mass, and total fat mass: the cardiovascular health study. J Clin Endocrinol Metab 2011;96:989-996.
34. Drake EB, Henderson VW, Stanczyk FZ, McCleary CA, Brown WS, Smith CA, Rizzo AA, Murdock GA, Buckwalter JG. Associations between circulating sex steroid hormones and cognition in normal elderly women. Neurology 2000;54:599-603.
35. Wu IC, Lin XZ, Liu PF, Tsai WL, Shiesh SC. Low serum testosterone and frailty in older men and women. Maturitas 2010;67:348-352.
36. Somboonporn W, Davis S, Seif MW, Bell R. Testosterone for peri- and postmenopausal women. Cochrane Database Syst Rev 2005;CD004509.
37. Traish A, Guay AT, Spark RF. Are the Endocrine Society's Clinical Practice Guidelines on Androgen Therapy in Women misguided? A commentary. J Sex Med 2007;4:1223-1234.
38. North American Menopause Society. The role of testosterone therapy in postmenopausal women: position statement of The North American Menopause Society. Menopause 2005;12:496-511.
39. Wierman ME, Basson R, Davis SR, Khosla S, Miller KK, Rosner W, Santoro N. Androgen Therapy in Women: An Endocrine Society Clinical Practice Guideline. Journal of Clinical Endocrinology & Metabolism 2006;91:3697-3710.
40. Rosner W, Vesper H. Toward excellence in testosterone testing: a consensus statement. J Clin Endocrinol Metab 2010;95:4542-4548.

41. Haring R, Hannemann A, John U, Radke D, Nauck M, Wallaschofski H, Owen L, Adaway J, Keevil BG, Brabant G. Age-Specific Reference Ranges for Serum Testosterone and Androstenedione Concentrations in Women Measured by Liquid Chromatography-Tandem Mass Spectrometry. Journal of Clinical Endocrinology & Metabolism 2012;97:408-415.
42. Boots LR, Potter S, Potter D, Azziz R. Measurement of total serum testosterone levels using commercially available kits: high degree of between-kit variability. Fertil Steril 1998;69:286-292.
43. Stanczyk FZ, Cho MM, Endres DB, Morrison JL, Patel S, Paulson RJ. Limitations of direct estradiol and testosterone immunoassay kits. Steroids 2003;68:1173-1178.
44. Vesper HW, Botelho JC, Shacklady C, Smith A, Myers GL. CDC project on standardizing steroid hormone measurements. Steroids 2008;73:1286-1292.
45. Dunn JF, Nisula BC, Rodbard D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 1981;53:58-68.
46. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab 2007;92:405-413.
47. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 1999;84:3666-3672.
48. Wulf H. Menopause-related definitions. International Congress Series 2004;1266:133-138.
49. Wells GA, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses. Proceedings of the 3rd Symposium on Systematic Reviews Beyond the Basics: Improving Quality and Impact 2000;3-5.
50. Higgins JPT, Green Se. Cochrane Handbook for Systematic Reviews of Interventions. 2011;Version 5.1.0 [updated March 2011].
51. Bernardi F, Hartmann B, Casarosa E, Luisi S, Stomati M, Fadalti M, Florio P, Santuz M, Luisi M, Petraglia F, Genazzani AR. High levels of serum allopregnanolone in women with premature ovarian failure. Gynecol Endocrinol 1998;12:339-345.
52. Hartmann BW, Kirchengast S, Albrecht A, Laml T, Soregi G, Huber JC. Androgen serum levels in women with premature ovarian failure compared to fertile and menopausal controls. Gynecol Obstet Invest 1997;44:127-131.
53. Aloia JF, Cohn SH, Vaswani A. Risk factors for postmenopausal osteoporosis. Am J Med 1985;78:95-100.
54. Ludwig M, Wolters U. Premature ovarian failure and androgen levels. Geburtshilfe Frauenheilkd 2007;67:1238-1242.
55. Duignan NM, Shaw RW, Glass MR, Butt WR, Edwards RL. Sex hormone levels and gonadotrophin release in premature ovarian failure. Br J Obstet Gynaecol 1978;85:862-867.

56. Bachelot A, Meduri G, Massin N, Misrahi M, Kuttenn F, Touraine P. Ovarian steroidogenesis and serum androgen levels in patients with premature ovarian failure. J Clin Endocrinol Metab 2005;90:2391-2396.
57. Mason A, Wallace AM, Macintyre H, Teoh YP, Bath LE, Critchley HO, Kelnar CJ, Wallace WH, Ahmed SF. Undetectable salivary testosterone in young women with premature ovarian failure. Clin Endocrinol (Oxf) 2006;64:711-714.
58. Kaufman FR, Donnell GN, Lobo RA. Ovarian androgen secretion in patients with galactosemia and premature ovarian failure. Fertil Steril 1987;47:1033-1034.
59. Arlt W. Management of the androgen-deficient woman. Growth Horm IGF Res 2003;13:S85-S89.
60. Grech HG, Panay N. Premature ovarian failure (analysis of a tertiary referral centre database). Menopause Int 2008;14:188-192.
61. Woodman J, Grech HG, Panay N. Androgen replacement therapy in premature ovarian failure. Maturitas 2009;63:S30.
62. Stanosz S, Kuligowski D, Pieleszek A. Concentration of dihydroepiandrosterone, dihydroepiandrosterone sulphate and testosterone during premature menopause in women chronically exposed to carbon disulfide. Med Pr 1995;46:337-340.
63. Berlier P, Nicolino N, Bertrand AM, Chatelain P. Primary ovarian failure (Turner syndrome excluded). Presenting symptoms and outcome. Ann Pediatr 1999;46:514-517.
64. Skalba P, Bednarska-Czerwinska A, Wojtowicz M, Lewandowski P. Serum androgens concentrations in women with idiopathic premature ovarian failure. Wiad Lek 2006;59:52-57.
65. Oriana S, Secreto G, Severini A. Bilateral ovariectomy in premenopausal patients with advanced breast cancer, after the evaluation of estrogen receptors and urinary androgen excretion. Breast Cancer Research and Treatment 1982;2:101-104.
66. Preda F, Pizzocaro G, Oriana S. Correlation between clinical response to bilateral oophorectomy, estrogen receptors and urinary androgen excretion in 49 patients with advanced breast cancer. Tumori 1979;65:325-330.
67. Lukanova A, Lundin E, Zeleniuch-Jacquotte A, Muti P, Mure A, Rinaldi S, Dossus L, Micheli A, Arslan A, Lenner P, Shore RE, Krogh V, Koenig KL, Riboli E, Berrino F, Hallmans G, Stattin P, Toniolo P, Kaaks R. Body mass index, circulating levels of sex-steroid hormones, IGF-I and IGF-binding protein-3: A cross-sectional study in healthy women. Eur J Endocrinol 2004;150:161-171.
68. Antoniucci DM, Sellmeyer DE, Cauley JA, Ensrud KE, Schneider JL, Vesco KK, Cummings SR, Melton III LJ. Postmenopausal bilateral oophorectomy is not associated with increased fracture risk in older women. J Bone Miner Res 2005;20:741-747.
69. Fogle RH, Stanczyk FZ, Zhang X, Paulson RJ. Ovarian Androgen Production in Postmenopausal Women. J Clin Endocrinol Metab 2007;92:3040-3043.
70. Eby NL, Boice J, Gold EB, Hoover RN, Loriaux DL. Estrogen and androgen levels in women treated with radiation for cervical cancer - Possible influence on breast cancer risk. American Journal of Epidemiology 1989;129:527-532.

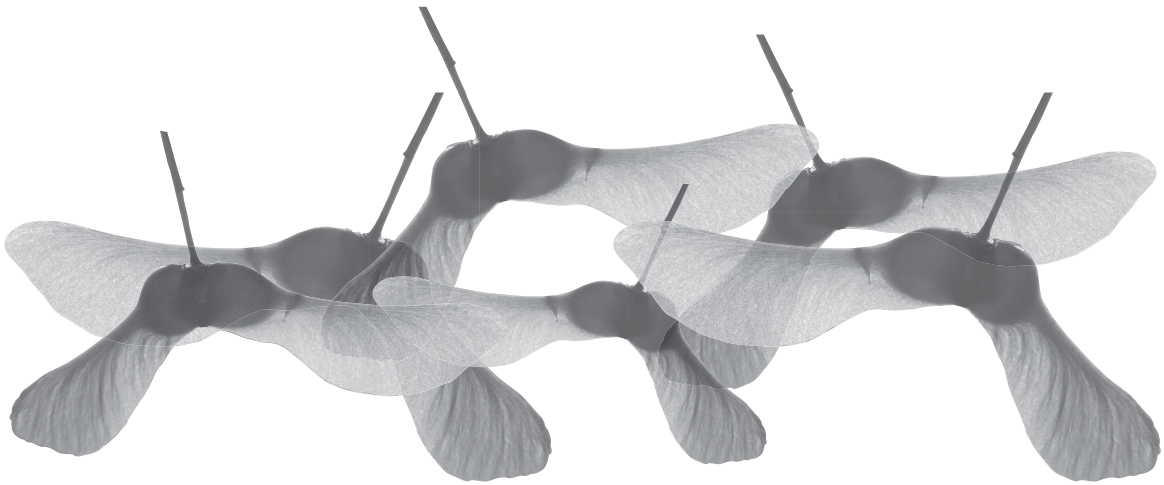
71. Horton R, Romanoff E, Walker J. Androstenedione and testosterone in ovarian venous and peripheral plasma during ovariectomy for breast cancer. The Journal of clinical endocrinology and metabolism 1966;26:1267-1269.
72. Judd HL, Lucas WE, Yen SS. Effect of oophorectomy on circulating testosterone and androstenedione levels in patients with endometrial cancer. Am J Obstet Gynecol 1974;118:793-798.
73. Chakravarti S, Collins WP, Newton JR, Oram DH, Studd JW. Endocrine changes and symptomatology after oophorectomy in premenopausal women. Br J Obstet Gynaecol 1977;84:769-775.
74. Dennerstein L, Wood C, Hudson B, Burrows G. Clinical features and plasma hormone levels after surgical menopause. Aust N Z J Obstet Gynaecol 1978;18:202-205.
75. Dowsett M, Cantwell B, Lal A, Jeffcoate SL, Harris AL. Suppression of postmenopausal ovarian steroidogenesis with the luteinizing hormone-releasing hormone agonist goserelin. J Clin Endocrinol Metab 1988;66:672-677.
76. Hughes CL, Jr., Wall LL, Creasman WT. Reproductive hormone levels in gynecologic oncology patients undergoing surgical castration after spontaneous menopause. Gynecol Oncol 1991;40:42-45.
77. De Leo V, la Marca A, Talluri B, D'Antona D, Morgante G. Hypothalamo-pituitary-adrenal axis and adrenal function before and after ovariectomy in premenopausal women. Eur J Endocrinol 1998;138:430-435.
78. Azzena A, Rulli R, Bernardi C, Zannoli M, Zen T, Cardascia L. Hormone replacement therapy and Lipoprotein (a) plasma levels in women in reproductive age with hysterectomy and bilateral oophorectomy. Ital J Gynaecol Obstet 2002;14:27-30.
79. Nozaki M, Hashimoto K, Nakano H. Relationship between bone resorption and adrenal sex steroids and their derivatives in oophorectomized women. Fertil Steril 2004;82:1556-1560.
80. Nar A, Demirtas E, Ayhan A, Gurlek A. Effects of bilateral ovariectomy and estrogen replacement therapy on serum leptin, sex hormone binding globulin and insulin like growth factor-I levels. Gynecol Endocrinol 2009;25:773-778.
81. Kalyan S, Hitchcock CL, Pudek M, Prior JC. Acute effects of premenopausal hysterectomy with bilateral oophorectomy on serum lipids, hormonal values, inflammatory markers, and metabolism. J Gynecol Surg 2011;27:9-15.
82. Forsling ML, Harriet C, Anderson M, Wheeler MJ, Shanti Raju K. The effect of oophorectomy and hormone replacement on neurohypophyseal hormone secretion in women. Clin Endocrinol 1996;44:39-44.
83. Acar B, Uslu T, Topuz A, Osma E, Ercal T, Posaci C, Erata Y, Mumcu A. Relation between bone mineral content and clinical, hormonal and biochemical parameters in postmenopausal women. Arch Gynecol Obstet 1998;261:121-128.

84. Schmitt-Robe B, Eicher W, Muck AO, Klinga K. Subjective complaints and hormonal response in the first 6 weeks after hysterectomy. Zentralbl Gynakol 1992;114:579-586.
85. Bucuras D, Anastasiu D, Craina M. Physiological versus surgical menopause-Impact of female sexual satisfanction. J Sex Med 2010;7:451-452.
86. Bassalyk LS, Koposova TL, Murav'eva NI, Gershtein ES, Smirnova KD. Effect of radiation and drug therapy on the hormonal status of patients with breast cancer, taking into consideration the receptor level of the tumor. Med Radiol (Mosk) 1986;31:48-52.
87. Halerz-Nowakowska B. Effect of ovariectomy and natural menopause on levels of selected pituitary hormones, 17-beta estradiol and lipid profile in blood serum. Ginekol Pol 1995;66:553-560.
88. Kulak J, Urbanetz AA, Kulak CA, Borba VZ, Boguszewski CL. Serum androgen concentrations and bone mineral density in postmenopausal ovariectomized and non-ovariectomized women. Arq Bras Endocrinol Metabol 2009;53:1033-1039.
89. Elias AN, Pandian MR, Rojas FJ. Serum levels of androstenedione, testosterone and dehydroepiandrosterone sulfate in patients with premature ovarian failure to age-matched menstruating controls. Gynecol Obstet Invest 1997;43:47-48.
90. Doldi N, Belvisi L, Bassan M, Fusi FM, Ferrari A. Premature ovarian failure: steroid synthesis and autoimmunity. Gynecol Endocrinol 1998;12:23-28.
91. Benetti-Pinto CL, Bedone AJ, Magna LA. Evaluation of serum androgen levels in women with premature ovarian failure. Fertil Steril 2005;83:508-510.
92. Inskip PD, Eby NL, Cookfair D, Freedman RS, Richardson GS, Wactawski-Wende J, Hoover RN, Boice JD, Jr. Serum estrogen and androgen levels following treatment for cervical cancer. Cancer Epidemiol Biomarkers Prev 1994;3:37-45.
93. Labrie F, Martel C, Balser J. Wide distribution of the serum dehydroepiandrosterone and sex steroid levels in postmenopausal women: role of the ovary? Menopause 2011;18:30-43.
94. Beksac MS, Kisnisci HA, Cakar AN, Beksac M. The endocrinological evaluation of bilateral and unilateral oophorectomy in premenopausal women. Int J Fertil 1983;28:219-224.
95. Kalantaridou SN, Calis KA, Vanderhoof VH, Bakalov VK, Corrigan EC, Troendle JF, Nelson LM. Testosterone deficiency in young women with 46,XX spontaneous premature ovarian failure. Fertil Steril 2006;86:1475-1482.
96. Janse F, Eijkemans MJ, Goverde AJ, Lentjes EG, Hoek A, Lambalk CB, Hickey TE, Fauser B, Norman R. Assessment of androgen concentrations in women: liquid chromatography- tandem mass spectrometry and extraction radioimmunoassay show comparable results. Eur J Endocrinol 2011;165:925-933.
97. Laughlin GA, Barrett-Connor E, Kritz-Silverstein D, von Muhlen D. Hysterectomy, Oophorectomy, and Endogenous Sex Hormone Levels in Older Women: The Rancho Bernardo Study. J Clin Endocrinol Metab 2000;85:645-651.

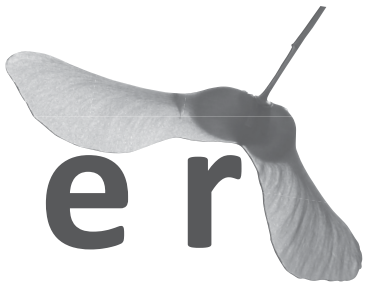
98. Sowers MF, Beebe JL, McConnell D, Randolph J, Jannausch M. Testosterone Concentrations in Women Aged 25-50 Years: Associations with Lifestyle, Body Composition, and Ovarian Status. Am J Epidemiol 2001;153:256-264.
99. Korse CM, Bonfrer JM, van BM, Verheijen RH, Rookus MA. Estradiol and testosterone levels are lower after oophorectomy than after natural menopause. Tumour Biol 2009;30:37-42.
100. Studd JW, Chakravarti S, Collins WP. Plasma hormone profiles after the menopause and bilateral oophorectomy. Postgrad Med J 1978;54 Suppl 2:25-30.
101. Danforth KN, Eliassen AH, Tworoger SS, Missmer SA, Barbieri RL, Rosner BA, Colditz GA, Hankinson SE. The association of plasma androgen levels with breast, ovarian and endometrial cancer risk factors among postmenopausal women. Int J Cancer 2010;126:199-207.
102. Lamb EJ, Dignam WJ, Pion RJ, Simmer HH. Plasma androgens in women. I. Normal and non-hirsute females, oophorectomized and adrenalectomized patients. Acta Endocrinol (Copenh) 1964;45:243-253.
103. Labrie F, Belanger A, Cusan L, Gomez JL, Candas B. Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. J Clin Endocrinol Metab 1997;82:2396-2402.
104. Parker CR, Jr., Slayden SM, Azziz R, Crabbe SL, Hines GA, Boots LR, Bae S. Effects of Aging on Adrenal Function in the Human: Responsiveness and Sensitivity of Adrenal Androgens and Cortisol to Adrenocorticotropin in Premenopausal and Postmenopausal Women. J Clin Endocrinol Metab 2000;85:48-54.
105. Panzer C, Guay A. Testosterone replacement therapy in naturally and surgically menopausal women. J Sex Med 2009;6:8-18.
106. Braunstein GD. Androgen insufficiency in women: summary of critical issues. Fertil Steril 2002;77 Suppl 4:S94-S99.
107. Santoro N, Torrens J, Crawford S, Allsworth JE, Finkelstein JS, Gold EB, Korenman S, Lasley WL, Luborsky JL, McConnell D, Sowers MF, Weiss G. Correlates of circulating androgens in mid-life women: the study of women's health across the nation. J Clin Endocrinol Metab 2005;90:4836-4845.
108. Cappola AR, Ratcliffe SJ, Bhasin S, Blackman MR, Cauley J, Robbins J, Zmuda JM, Harris T, Fried LP. Determinants of serum total and free testosterone levels in women over the age of 65 years. J Clin Endocrinol Metab 2007;92:509-516.
109. Herold DA, Fitzgerald RL. Immunoassays for testosterone in women: better than a guess? Clin Chem 2003;49:1250-1251.
110. Nappi RE, Lello S, Melis GB, Albani F, Polatti F, Genazzani AR. LEI (Lack of tEstosterone Impact) survey in a clinical sample with surgical menopause. Climacteric 2009;12:533-540.
111. Castelo-Branco C, Palacios S, Combalia J, Ferrer M, Traveria G. Risk of hypoactive sexual desire disorder and associated factors in a cohort of oophorectomized women. Climacteric 2009;12:525-532.

112. Bhattacharya SM. Health-related quality of life following surgical menopause and following gonadotrophin-releasing hormone analogue-induced pseudomenopause. Gynecol Endocrinol 2009;25:621-623.
113. Melton LJt, Khosla S, Malkasian GD, Achenbach SJ, Oberg AL, Riggs BL. Fracture risk after bilateral oophorectomy in elderly women. J Bone Miner Res 2003;18:900-905.
114. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, Melton LJ, III. Increased risk of parkinsonism in women who underwent oophorectomy before menopause. Neurology 2008;70:200-209.
115. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, Melton LJ, III. Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. Neurology 2007;69:1074-1083.
116. Shifren JL, Braunstein GD, Simon JA, Casson PR, Buster JE, Redmond GP, Burki RE, Ginsburg ES, Rosen RC, Leiblum SR, Caramelli KE, Mazer NA. Transdermal testosterone treatment in women with impaired sexual function after oophorectomy. N Engl J Med 2000;343:682-688.
117. Buster JE, Kingsberg SA, Aguirre O, Brown C, Breaux JG, Buch A, Rodenberg CA, Wekselman K, Casson P. Testosterone patch for low sexual desire in surgically menopausal women: a randomized trial. Obstet Gynecol 2005;105:944-952.
118. Moller MC, Bartfai AB, Radestad AF. Effects of testosterone and estrogen replacement on memory function. Menopause 2010;17:983-989.
119. Matsumoto AM, Bremner WJ. Serum testosterone assays--accuracy matters. J Clin Endocrinol Metab 2004;89:520-524.
120. Bermudez JA, Moran C, Herrera J, Barahona E, Perez MC, Zarate A. Determination of the steroidogenic capacity in premature ovarian failure. Fertil Steril 1993;60:668-671.
121. Falsetti L, Scalchi S, Villani MT, Bugari G. Premature ovarian failure. Gynecol Endocrinol 1999;13:189-195.
122. Abraham GE, Lobotsky J, Lloyd CW. Metabolism of testosterone and androstenedione in normal and ovariectomized women. J Clin Invest 1969;48:696-703.
123. Vermeulen A. The hormonal activity of the postmenopausal ovary. J Clin Endocrinol Metab 1976;42:247-253.
124. Sherwin BB, Gelfand MM. Sex steroids and affect in the surgical menopause: a double-blind, cross-over study. Psychoneuroendocrinology 1985;10:325-335.
125. Wakatsuki A, Sagara Y. Lipoprotein metabolism in postmenopausal and oophorectomized women. Obstetrics & Gynecology 1995;85:523-528.
126. Garcia-Perez MA, Moreno-Mercer J, Tarin JJ, Cano A. Bone Turnover Markers and PTH Levels in Surgical Versus Natural Menopause. Calcif Tissue Int 2004;74:143-149.
127. Hassa H, Tanir HM, Ardic N. Early postoperative changes in testosterone, dehydroepiandrosterone sulfate, and sex hormone-binding globulin after hysterectomy with or without concomitant oophorectomy. Fertil Steril 2006;86:981-989.

128. McTiernan A, Wu L, Barnabei VM, Chen C, Hendrix S, Modugno F, Rohan T, Stanczyk FZ, Wang CY. Relation of demographic factors, menstrual history, reproduction and medication use to sex hormone levels in postmenopausal women. Breast Cancer Res Treat 2008;108:217-231.
129. Bui HN, Struys EA, Martens F, de RW, Thienpont LM, Kenemans P, Verhoeven MO, Jakobs C, Dijstelbloem HM, Blankenstein MA. Serum testosterone levels measured by isotope dilution-liquid chromatography-tandem mass spectrometry in postmenopausal women versus those in women who underwent bilateral oophorectomy. Ann Clin Biochem 2010;47:248-252.
130. Alarslan D, Sarandol A, Cengiz C, Develioglu OH. Androgens and sexual dysfunction in naturally and surgically menopausal women. J Obstet Gynaecol Res 2011;37:1027-1034.



Chapter 6



Assessment of androgen concentration in women: liquid chromatography-tandem mass spectrometry and extraction radioimmunoassay show comparable results

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Abstract

Objective

The measurement of serum testosterone in women is challenging due to lack of trueness, precision, and sensitivity of various available testosterone assays. Accurate assessment of testosterone in women is crucial especially in conditions associated with alleged over- or underproduction of testosterone, such as in polycystic ovary syndrome (PCOS) or primary ovarian insufficiency (POI). The aim of this study was to measure and compare androgen concentrations in women with PCOS, POI, and female controls and to evaluate the performance of extraction RIA and liquid chromatography- tandem mass spectrometry (LC-MS/MS) in these women.

Design

Cross-sectional study.

Methods

Carefully phenotyped women with POI (n = 208) or PCOS (n = 200), and 45 healthy, regularly cyclic female controls were included. Method comparison analyses were performed for total testosterone, androstenedione (AD), and DHEA, as measured by LC-MS/MS and extraction RIA.

Results

All androgen levels were significantly elevated in women with PCOS compared with POI patients ($P < 0.05$) and controls ($P < 0.05$). Women with POI presented with similar androgen concentrations as controls, except for AD. Compared to measurements by extraction RIA, testosterone, DHEA, and AD concentrations measured by LC-MS/MS were systematically lower. However, extraction RIA and LC-MS/MS, testosterone, DHEA, and AD measurements were shown to have good agreement as assessed by Bland-Altman analysis and intraclass correlation coefficient: 0.95 (95% confidence interval 0.94-0.91), 0.83 (0.79-0.86), and 0.96 (0.95-0.97), respectively.

Conclusions

LC-MS/MS, compared with a labor-intensive extraction RIA, shows good precision, sensitivity, and high accuracy for measuring female testosterone, DHEA, and AD concentrations under various clinical conditions. LC-MS/MS, therefore, represents a convenient and reliable assay for both clinical and research purposes, where androgen measurement in women is required.

Introduction

The measurement of serum testosterone has proven to be a great challenge due to lack of precision and sensitivity of various commercially available testosterone assays, resulting in limited utility (1). Testosterone assays were originally developed to measure testosterone concentrations in the normal male range (2). However, women exhibit testosterone levels well below this range, and therefore, reliable measurement of female testosterone concentrations is particularly problematic due to trueness and precision problems (3). In women of reproductive age, 25% of testosterone biosynthesis occurs in the ovarian theca cells. Another 25% is produced by the adrenal gland, and the remaining 50% of circulating testosterone derives from peripheral conversion of testosterone precursors (DHEA and androstenedione (AD)). DHEA and its sulfate metabolite (DHEAS) are exclusively produced by the adrenal glands, whereas AD is produced by both the adrenal and the ovary (4,5). Most circulating testosterone is biologically inactive as it binds to serum proteins, primarily sex hormone-binding protein (SHBG) and albumin (2,6).

The most widely used methods for measuring serum testosterone are the simple, relatively inexpensive RIA and chemiluminescence immunoassay, performed directly in serum. Although the immunoassays show on average good precision, they often show more bias. This is especially true for the lower range, where they can be subject to increased interference and overestimation of steroid concentrations compared with other assays (7,8). Using extraction and chromatography methods preceding RIA has the advantage of removing interfering proteins and cross-reacting steroids. Although extraction RIA seems preferable over direct assays, it is infrequently used in clinical practice because proper validation is lacking and extraction is labor intensive and time consuming (1,9,10). A commonly used practical approach to estimate bioactive testosterone is the calculation of the free androgen index (FAI). While FAI was shown to correlate quite well with physical separation measures of female free testosterone (not male), FAI is highly dependent on the quality of testosterone and SHBG assay measurements (2).

In order to overcome these difficulties, tandem mass spectrometry (MS/MS) preceded by gas or liquid chromatography (GC-MS/MS and LC-MS/MS) assays for steroid measurement is emerging (11). LC-MS/MS has some advantages over the use of immunoassays, especially compared with those performed on platforms. LC-MS/MS shows equal or better precision, and these assays do not suffer from interferences due to chromatographic separation and mass spectrometry analysis. Interferences can cause serious bias at low testosterone concentrations, which may be encountered in postmenopausal women and children. However, the high costs of mass spectrometry equipment may be a possible drawback for routine clinical use. Although LC-MS/MS assays show higher precision and better accuracy than immunoassays, there is still a difference in performance (sensitivity, specificity and trueness) among LC-MS/MS methods and thus there is a need for standardization (12).

Reliable assessment of testosterone in women is crucial for the diagnosis of conditions associated with alleged overproduction of testosterone such as in polycystic ovary syndrome (PCOS), or conditions that may incur androgen deficiency, such as postmenopause, oophorectomy, or primary ovarian insufficiency (POI) (13-15). However, analysis of serum testosterone by commercial assays fails to identify biochemical hyperandrogenism in 20 to 40% of women with PCOS, while hyperandrogenism is one of the criteria on which the diagnosis is based (16,17). Moreover, female androgen insufficiency remains controversial due to the lack of reliable serum testosterone assays to define this condition (18). Clinical guidelines concerning indications for androgen therapy in case of hypo-androgenism cannot be established (19). These examples illustrate the need for a reliable testosterone measurement, which is easily available at reasonable cost for both clinical and research purposes.
The current study was aimed at evaluating the measurement of testosterone and other androgens by LC-MS/MS in comparison to measurement by extraction RIA in women with normal and presumably elevated and decreased androgens.

Materials and methods

Subjects

In 2004, a study involving standardized phenotyping and follow-up was initiated in the University Medical Center Utrecht, the Netherlands, in which all women suspected of a menstrual cycle disturbance and referred to our outpatient clinic participated. Details of the systematic evaluation, approved by the local ethical committee, have been described previously (20,21). For the current study, 169 women with POI and 200 women affected by PCOS, who visited the outpatient clinic until July 2009, were included. POI was defined as secondary amenorrhea for at least 4 months prior to age 40 years, along with repeated FSH concentrations exceeding 40 IU/L (22). PCOS was diagnosed according to the Rotterdam consensus criteria if at least two of the following criteria were present: i) oligo/anovulation, ii) clinical and/or biochemical hyperandrogenism (testosterone >2.0 nmol/L, assessed by our in-house extraction RIA), and/or iii) polycystic ovaries on ultrasonography (16). Additionally, 39 women with POI visiting four other centers (participating in the Dutch POI Consortium (21,23)) were also included in the current study.
As controls, 45 women visiting the preconceptional clinic prior to starting IVF/ICSI treatment because of severe male infertility between October 2006 and July 2008 were included. Severe male infertility was defined as semen analysis with volume x concentration x motility below 2.0 million. All women were healthy and experienced regular menstrual cycles. Exclusion criteria were PCOS, previous poor response after ovarian hyperstimulation, age > 38 years, early follicular FSH concentrations > 12 IU/L, and thyroid dysfunction. All included study subjects gave written informed consent.

Measurements

Serum samples were collected during the early follicular phase of the menstrual cycle in women with regular cycles. In case of a cycle disturbance, samples were drawn at a random cycle day and a progesterone concentration was measured concomitantly to ensure preovulatory state. None of the women had used any hormonal medication for at least two weeks. After collection and aliquoting, one fresh sample was analyzed immediately while the remaining aliquots were stored at -20 °C. In Utrecht, the fresh testosterone measurement was performed using an in-house extraction RIA, after diethylether extraction of 500 µL sample, using a polyclonal anti-testosterone antibody (Dr. Pratt AZG 3290, Department of Nuclear Medicine, University Medical Center Groningen). [1,2-3H(N)]-testosterone (NET-387, DuPont NEN B.V., Dordrecht, The Netherlands) was used as a tracer following chromatographic verification of its purity. A small amount of recovery tracer was added to the sample to correct for losses during extraction. The lower limit of quantitation (LLQ), i.e. the concentration which can be determined with 20% coefficient of variation (CV; estimated from a precision profile), was 0.10 nmol/L and inter-assay variation was 10, 6, and 7% at 0.85, 2.6, and 12 nmol/L, respectively (n = 100). Intra-assay CVs at 2 and 10 nmol/L were 3.8 and 3.5%, respectively. There was no interference detected from dihydrotestosterone, AD, and DHEA. Recent comparison of the in-house method with a dilution liquid (ID)-GC/MS method using 20 human serum samples of the Dutch EQAS gave following correlation: RIA = 1.13 x (ID-GC/MS) + 0.33 nmol/L (tested range was 0.8-33 nmol/L; 95% confidence limit of the slope: 1.04-1.20 and of the intercept: -1.04 to 0.91)(24). Futhermore, the following measurements were performed on fresh serum for women with POI and PCOS: estradiol (E_2), SHBG, DHEA, DHEAS, and AD. E_2 concentrations were measured using the Roche E170 Modular (Roche). SHBG concentrations were quantified using an Immulite platform (Diagnostic Products Corporation, Breda, The Netherlands). DHEA was measured after diethylether extraction and Celite chromatography using an in-house RIA. DHEAS was measured using the Coat-A-Count DHEA-SO4 RIA (Siemens Diagnostics, Breda, The Netherlands). AD was measured after hexane-toluene extraction using an in-house RIA. Interassay CVs for E_2 were 13, 5.7, and 3.4% at 66, 200, and 600 pmol/L (n = 100) respectively; for SHBG 4.2, 3.9, and 4.4% at 10, 36, and 120 nmol/L (n = 93) respectively; for DHEA 7.7, 7.6, and 8.8% at 1.4, 4.2, and 12.5 nmol/L (n = 29) respectively, for DHEAS 8.5, 5.5, and 6.8% at 1.3, 5.3, and 12.8 µmol/L (n = 60) respectively; and for AD 11.5, 5.7, and 6.7% at 1, 5 and 11 nmol/L (n = 50) respectively. Intra-assay CVs for DHEA were 9 and 5% at 5 and 20 nmol/L, respectively, for AD it was 4% at 7 nmol/L. For E_2, DHEAS, and SHBG, the intra-assay CVs were within the assay documentation. Testosterone, AD, and DHEA were calibrated against gravimetrically determined quantities of pure

standard in steroid-free BSA. For the other assays, we used the calibration of the immunoanalyzers. All measurements were performed in duplicate. The reference range for cycling women in this laboratory were the following: for testosterone: 0.50-2.00 nmol/L, for DHEA 10-30 nmol/L, and for AD: 3.0-7.0 nmol/L. Serum SHBG concentrations were also measured in female controls, using identical assays.

Testosterone, AD, and DHEA were also measured in each individual sample at the CPR Pharma Pty Ltd laboratory, organized by the University of Adelaide, Australia. Frozen aliquots were sent to Australia and thawed just before use. Testosterone, AD, DHEA, internal standards (d3-testosterone (IS-testosterone), and 19-d3-androstenedione (IS-AD)) were extracted using liquid-liquid extraction with hexane-dichloromethane solvent mixture. The analytes were separated by HPLC on an Alltima HP C18HL column, and the eluates monitored by an API5500 MS/MS detector in positive multiple reaction monitoring (MRM) mode. The single charged Q1/Q3 transition is 289.3/96.9, 287.1/97.0, and 289.2/253.2 amu for testosterone, AD, and DHEA, respectively, and 292.2/97.0 and 290.2/100.0 amu for IS-testosterone and IS-AD respectively (see *Supplementary Figure 1*). The extract was then assayed against a calibration curve, and data were acquired and processed by the data acquisition system, Analyst 1.5 linked directly to the API5500 MS/MS detector. The method's range is from 0.175 to 69.6 nmol/L for testosterone and AD, and 4.34-1740 nmol/L for DHEA. Measurements were performed in duplicate. Accuracy for testosterone against nine validated pure standards was assessed as above and ranged from -2.0% to +3.8% across the complete standard range (eight replicates) and between -2.0% and +1.6% in the range 0.173-3.47 nmol/L. Precision for testosterone ranged between 1.5% and 7.5% across the whole range and was 1.5-9.3% between 0.173 and 3.47 nmol/L. Results were similar for all analytes measured. The following inter-assay CVs were identified: in controls: 5.6% (testosterone), 8.4% (DHEA), and 6.4% (AD); in PCOS: 4.3% (testosterone), 5.9% (DHEA), and 4.5% (AD). The limit of detection was determined by repeated measures of the three lowest standards used in the assay. A precision dose profile was established and statistical significance between the standards shown by clear separation of the doses. The run time was 10 min per sample.

FAI was calculated for both testosterone measurements: testosterone/SHBG x 100 (25) using the SHBG measurement performed in Utrecht.

Statistical analysis

Median values and range for each individual group of women were compared using one-way analysis of covariance and Bonferroni post-hoc tests for significance, correcting for age and body mass index (BMI; possible confounders)(26). Method comparison analyses were performed by constructing scatter plots and

Bland-Altman plots to evaluate the extent to which testosterone, DHEA and AD measurements agreed in both methods. Bland-Altman plots show the percentage difference between two corresponding measurements plotted against the measurements' mean.

For all Bland-Altman plots, the mean percentage difference between the methods (mean bias) with associated 95% confidence intervals (CI) and limits of agreement (± 2 SD) were calculated. Intraclass correlation coefficients (ICC) with 95% CIs were calculated for testosterone, DHEA and AD. Large ICC (maximum 1) indicates variation among both assays is equal to zero (perfect agreement) (27,28). Because androgen concentrations were not normally distributed, logarithmic transformation of variables was applied before execution of analyses. When testosterone or DHEA concentrations were undetectable, 0.5 x LLOD was used (29).

To further compare the performance of extraction RIA and LC-MS/MS for the measurement of testosterone, DHEA and AD in this population, correlations between various androgens and E_2 were assessed. To this end, linear regression analyses were performed, where testosterone (RIA) and testosterone (LC-MS/MS) were entered separately and similarly repeated, entering the variables in the opposite order. Univariate standardized β coefficients with associated S.E. are reported. Larger univariate standardized β coefficients represent stronger associations with the steroids. Univariate standardized β coefficients of both testosterone assays were compared using a bootstrapping procedure with 2000 replications, because both testosterone measurements were performed in identical samples. 95% CIs were calculated to identify significant differences between the correlations with androgens and E_2 versus the testosterone measurements by RIA and LC-MS/MS assays. Identical analyses were performed for DHEA (RIA) and DHEA (LC-MS/MS), and for AD (RIA) and AD (LC-MS/MS). Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, Il) and R version 2.9.0. (http://www.r-project.org/).

Results

Median concentrations of all measurements are shown per patient group in *Table 1*. In women with PCOS, all hormone concentrations were significantly higher compared to those in POI patients and in controls, with exception of SHBG. Androgens (except AD), E_2 and SHBG concentrations were similar for women with POI and controls.

The scatter and Bland-Altman plots for both testosterone, DHEA, and AD measurements are depicted in *Figures 1-3*. All scatter plots show high correlation between extraction RIA and LC-MS/MS with correlation coefficients of 0.960 for testosterone and AD and 0.830 for DHEA. The ICC for testosterone, DHEA, and AD

Table 1. Baseline characteristics for each patient group.

	Controls (n = 45)	POI (n = 208)	PCOS (n = 200)	P
Age (years)	33.1 (20.3 - 37.8)	37.6 (15.8 - 62.9)	29.3 (17.9 - 41.0)	*<0.001*
BMI (kg/m^2)	23.4 (17.1 - 36.9)	23.4 (17.1 - 37.3)	24.2 (16.2 - 55.9)	**0.003**
T RIA (nmol/L)	1.20 (0.61 - 3.90)	1.10 (0.29 - 3.00)	2.00 (0.64 - 6.30)	**<0.001**
T LC-MS/MS (nmol/L)[a]	0.87 (0.31 - 2.78)	0.76 (0.09 - 2.30)	1.55 (0.34 - 5.48)	**<0.001**
E_2 RIA (pmol/L)	N/A	40 (20 - 1500)[b]	200 (70 - 2250)	*<0.001*
SHBG RIA (nmol/L)	60 (12 - 199)	55 (17 - 201)	47 (7 - 191)	0.04*
DHEA RIA (nmol/L)	N/A	11.0 (1.10 - 43.0)[c]	18.0 (2.00 - 67.0)	*0.02*
DHEA LC-MS/MS (nmol/L)[d]	16.0 (8.74 - 58.9)	12.1 (4.34 - 43.0)	15.8 (4.34 - 65.2)	NS
DHEAS RIA (µmol/L)	N/A	3.90 (0.41 - 12.0)[c]	4.80 (0.77 - 14.0)	NS
AD RIA (nmol/L)	N/A	2.90 (0.80 - 8.90)[e]	7.20 (2.40 - 22.0)	*<0.001*
AD LC-MS/MS (nmol/L)	3.29 (1.31 - 10.9)	2.23 (0.33 - 8.46)	5.87 (1.81 - 15.9)	*<0.005*
FAI RIA	1.83 (0.75 - 15.8)	1.76 (0.29 - 8.24)	4.65 (0.68 - 26.2)	**<0.001**
FAI LC-MS/MS	1.29 (0.48 - 10.7)	0.56 (0.02 - 2.11)	3.36 (0.60 - 21.0)	**<0.001**

Analysis of covariance was carried out on logarithmically transformed variables to study differences between patient groups. Results are shown as median (range). All P values are adjusted for age and BMI.
Italic values in P column indicate that all groups were significantly different from each other; bold values in P column indicate that PCOS was significantly different from controls and POI; *, indicates that PCOS was significantly different from controls; N/A, not available; NS, not significant; LLOD, lower limit of detection
a Measurements below LLOD: in POI n = 1 (0.4%); b n = 195; c n = 162; d Measurements below LLOD: in controls, n = 15 (33%); POI, n = 58 (28%); and PCOS, n = 14 (7%); e n = 164.

were 0.95 (95% CI 0.94-0.96), 0.83 (0.79-0.86), and 0.96 (0.95-0.97), respectively, indicating good agreement between both extraction RIA and LC-MS/MS assays. It becomes evident from the Bland-Altman plots that the average percent difference (i.e. mean bias) in serum testosterone, DHEA and AD levels between LC-MS/MS and extraction RIA were -31.5, -7.1, and -20.9%, respectively. This indicates that LC-MS/MS yields significantly lower serum testosterone and AD results than extraction RIA, which may possibly be explained by interfering substances in the latter assay. In 24 (5.3%) of testosterone, 22 (6.0%) of DHEA, and 16 (4.4%) of AD, the differences were beyond the limits of agreement (mean difference +/- 2SD), with the majority of these outliers occurring in the lower concentration ranges. Small, insignificant differences may incur a great percentage difference when calculated in the low range, which is dramatically shown in a Bland-Altman plot. In conclusion, the Bland-Altman plots suggest that the two methods are in good agreement with testosterone, DHEA and AD. Finally, correlations between testosterone, DHEA, and AD vs. related steroids (i.e. other androgens and E_2)

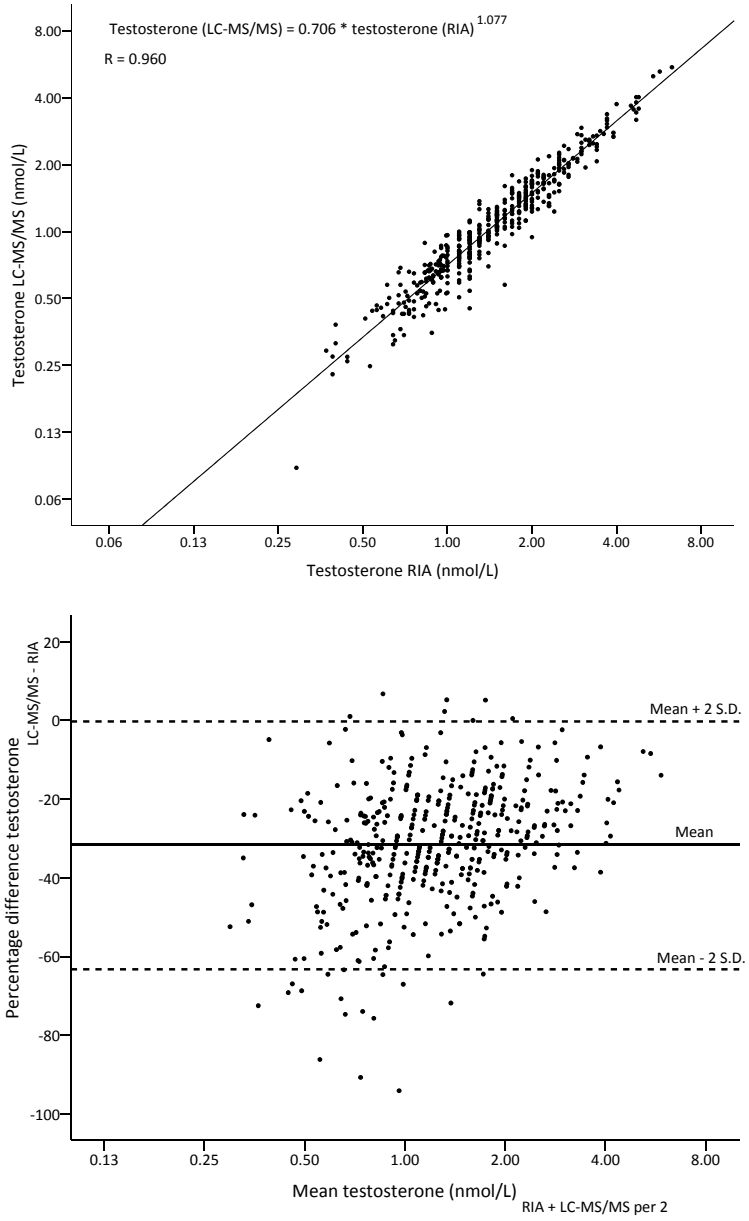

Figure 1. Scatter plot (upper panel) and Bland-Altman plot (bottom panel) to show the extent to which testosterone measurements agreed in both methods.
Legend: The solid line represents the mean percentage difference between the methods (mean bias) and the dashed lines 2 SD of the mean percentage difference (limits of agreement). Filled circles represent individual measurements.

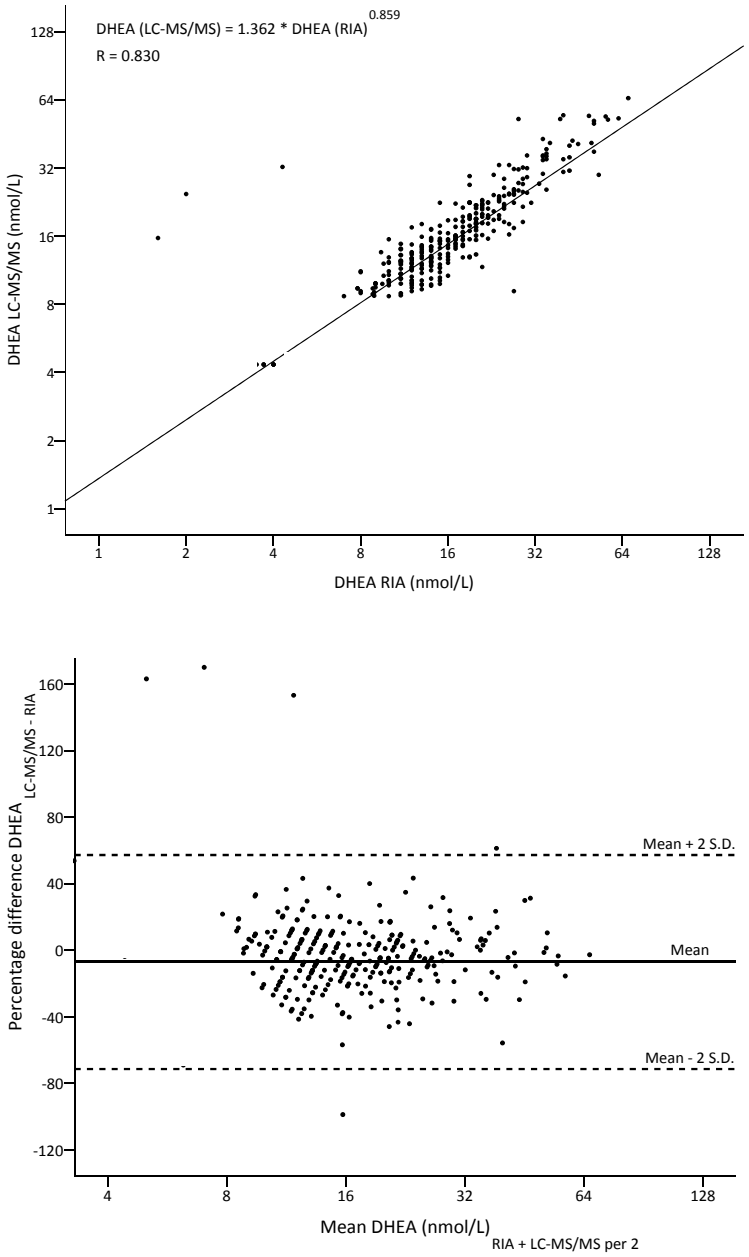

Figure 2. Scatter plot (upper panel) and Bland-Altman plot (bottom panel) to show the extent to which DHEA measurements agreed in both methods.
The solid line represents the mean percentage difference between the methods (mean bias) and the dashed lines 2 SD of the mean percentage difference (limits of agreement). Filled circles represent individual measurements.

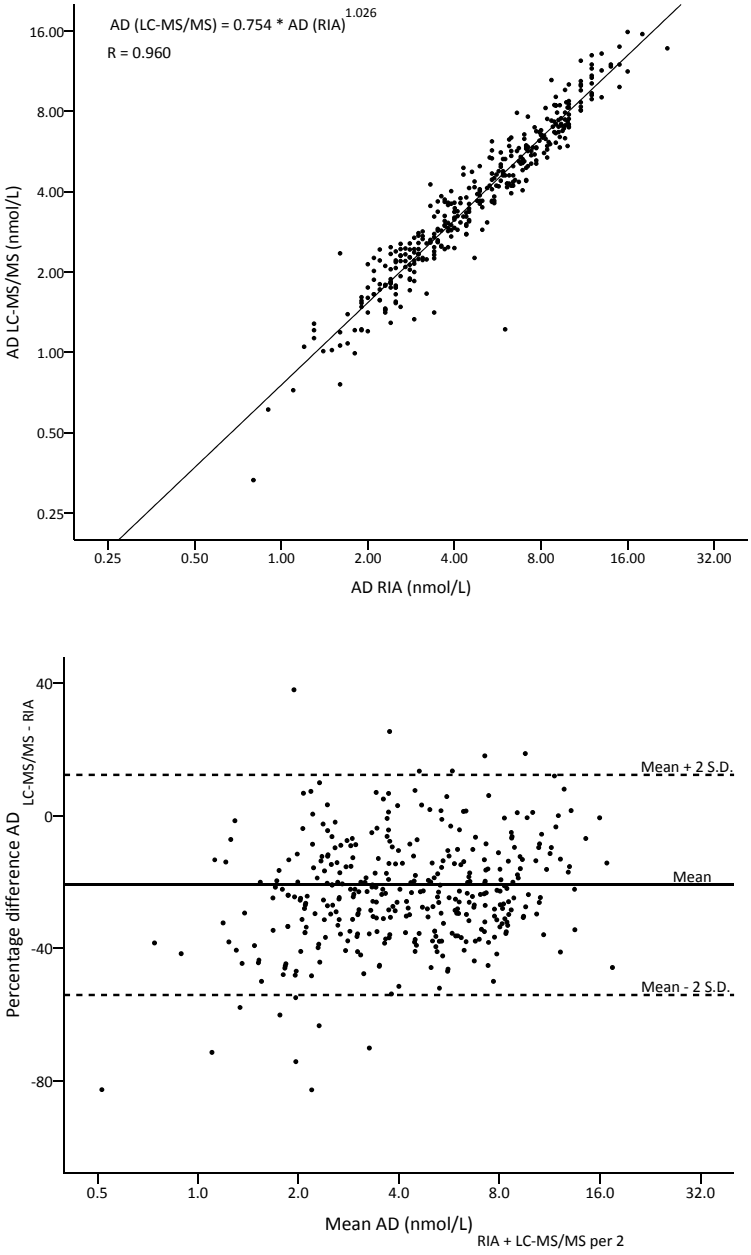

Figure 3. Scatter plot (upper panel) and Bland-Altman plot (bottom panel) to show the extent to which AD measurements agreed in both methods.
The solid line represents the mean percentage difference between the methods (mean bias) and the dashed lines 2 SD of the mean percentage difference (limits of agreement). Filled circles represent individual measurements.

were calculated to identify whether the extraction RIA or the LC-MS/MS assays showed a difference in these associations (*Supplementary Tables 1-3*). In general, most correlations were similar among the assays. The identification of some small significant differences indicates that the strength of the association increased when related steroids were measured employing the identical assay with which the original measurement was performed.

Discussion

In this study, the performances of LC-MS/MS and an in-house extraction RIA were compared for a broad range of androgen concentrations encountered in women of reproductive age, including the clinical conditions POI and PCOS. LC-MS/MS and extraction RIA demonstrated good agreement for the measurement of testosterone, DHEA and AD, although LC-MS/MS gave significantly lower concentrations when compared with extraction RIA. Furthermore, similar correlations were identified in the comparison with the association for each assay with other steroids. Therefore, LC-MS/MS, compared with a labor-intensive extraction RIA, shows good precision, sensitivity, and high accuracy for the measurement of androgens in women with PCOS, POI, and regularly cycling female controls.

Androgen concentrations for women with PCOS were significantly higher compared with female controls and women affected by POI, which confirms other recent studies applying LC-MS/MS (27,28). However, this is the first study to measure androgens with LC-MS/MS in women with POI. In this group, testosterone and DHEA concentrations were similar as female controls, while AD concentrations were significantly lower. In the existing literature, it is debated whether POI is associated with decreased androgen concentrations (14,30-34), and only one study presented with results similar to ours (35). As previous studies only applied direct RIA, chemiluminescence immunoassays, and extraction RIA, conflicting findings most probably resulted from lower sensitivity, lack of specificity, or lower accuracy of these assays (10). While decline of androgen synthesis may result from menopausal status, this has also been associated with increasing age (33,36). Therefore, the results of the current study support the concept that young women with POI encounter normal testosterone and DHEA concentrations, in contrast with women who experience menopause at a regular age. Currently, there is no explanation for the significantly lower AD concentrations encountered in women with POI. This finding needs further investigation.

MS after GC or LC is a relatively new technique for steroid sex hormone measurement and data on its applicability, particularly in the female sex hormone concentration range, are scarce. The LC-MS/MS assay in this study showed excellent precision and accuracy down to at least 0.173 nmol/L (precision -0.2%; accuracy 1.5% for authentic standards). This allows for a great deal of confidence

in the reliability of an assay, which is completely specific for testosterone or other nominated steroid analytes. A study comparing LC-MS/MS with direct and extraction RIAs in normal and hypogonadal male subjects by Bland-Altman plots showed systematically lower results by LC-MS/MS (37). Studies on female and pediatric samples confirmed this finding, while also identifying that LC-MS/MS showed greater accuracy in lower androgen concentrations than direct assays (38,39). This study included serum samples of women with PCOS, POI, and normally cyclic women. We identified good agreement between LC-MS/MS and the in-house extraction RIA (even for lower ranges encountered in POI) and slightly lower hormone concentrations reported after LC-MS/MS. Previous studies on the comparison of LC-MS with extraction RIA identified overestimation of extraction RIA between 20% and 80% in testosterone concentrations below 3.47 nmol/L (1 ng/dL), similar to our findings: between -0.2% and 63% (37). We are cautious to generalize the agreement between extracted RIA and LC-MS/MS in the current study, because only two centers participated in the current study. But the results are in accordance with previous studies (37,38,40), including a study in women with PCOS, which confirms that well-chosen RIA may offer equal precision and accuracy as LC-MS/MS (41).

When examining the performance of extraction RIA and LC-MS/MS, it is important to include a clinical validation of the androgens measured by each assay. Ideally, correlation analyses should be performed with clear clinical endpoints, such as degree of hirsutism or presence of the female androgen deficiency syndrome. However, until now, clear clinical endpoints related to androgen concentrations cannot irrefutably be identified (41-43). Therefore, the associations of androgens, measured by both assays, were compared with other physiologically related steroids (androgens and E_2). Our results indicate that for the two assays evaluated in this study, the correlations were similar and both assays showed equal performance.

The major strength of this study is the large sample size of well-phenotyped women with PCOS and POI, representing the broad spectrum of female androgen concentrations during reproductive life. Laboratory personnel at both sites were blinded for study design and identity of the samples. But this study may also have potential weaknesses. While all extraction RIA assessments were done in fresh samples, frozen samples were shipped to Australia before LC-MS/MS processing. However, previous studies on long-term stored samples identified that steroids are very stable after being isolated in serum (44). Furthermore, early follicular androgen concentrations in regularly cyclic controls may underrepresent androgen levels present during the middle third of the cycle (19). Finally, the group of female controls was relatively small, which may have concealed potential differences in androgen concentrations compared with those in women with POI. Although our results show that LC-MS/MS is a reliable alternative for measuring steroid sex hormones in women, its introduction into clinical or research practice

may depend on several factors: costs of the LC-MS equipment, LC-MS expertise in laboratories, and future RIA development. The LC-MS is now introduced in many laboratories, but the methods designed on this analyzer should meet the same requirements (defined by the FDA or European Directive) like the commercial immunoassays. This means that the validation of an LC-MS/MS procedure, like the validation of extraction RIAs, has to be much more extensive than for commercial immunoassays, because LC-MS assays are mostly in-house-developed methods unlike already validated kits. Furthermore, inappropriate sample preparation and analyte detection may greatly influence the performance of LC-MS/MS and extraction RIA procedures, thus emphasizing the importance of validation. Extraction RIA may gain increased specificity if more specific antibodies are selected, and the method for the displacement of bound testosterone from its binding proteins is optimized (37). If this occurs, new comparison studies with LC-MS/MS should be performed to assess whether the advantage of the high-throughput character of the latter assay is still preferable. Extraction RIAs may be highly reliable when appropriately validated, but the method is time consuming and costly (11). Commercially available direct testosterone assays, although simple and convenient, are known for the general overestimation of steroid concentrations in women (37). Both LC-MS/MS and extraction RIA lack this bias in the lower concentration range, although the extraction RIA needs to be recalibrated against proper standards. Proper validation will reduce the number of false positives and therefore reduce unnecessary medical examinations. LC-MS/MS is a promising new, accurate, sensitive, and high-throughput method for the measurement of sex hormone levels.

With the demonstration that LC-MS/MS, compared with a labor-intensive RIA, exhibits good precision, sensitivity, and high accuracy for measuring female androgen concentrations, a convenient assay has become available for clinical and research purposes. Reproducibility of the LC-MS/MS between different LC-MS/MS laboratories needs to be assessed to meet performance criteria as is customary for new assays, before this assay will be adopted universally. The next phase in the introduction of LC-MS/MS should aim at obtaining highly true and precise reference values. These reference values could then be applied using other assays with same accuracy for normo-, hypo-, and hyperandrogenic women.

Acknowledgements

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Supplementary materials

Table S1. Assessment of the correlation of T measured by extraction RIA and LC-MS/MS with other androgens and E_2

	Univariate Standardized Beta Coefficient		Difference of beta coefficients	95% CI of difference[b]
	T RIA (S.E.)[a]	*T LC-MS/MS (S.E.)*[a]		
E_2 RIA	0.40 (0.05)	0.40 (0.05)	0.0037	-0.02 - 0.03
SHBG RIA	-0.02 (0.05)	-0.01 (0.05)	0.0090	-0.02 - 0.04
DHEA RIA	0.48 (0.05)	0.42 (0.05)	-0.054	-0.08 - -0.03*
DHEA LC-MS/MS	0.50 (0.04)	0.47 (0.04)	-0.030	-0.05 - -0.01*
DHEAS RIA	0.36 (0.05)	0.33 (0.05)	-0.035	-0.07 - -0.001*
AD RIA	0.87 (0.03)	0.84 (0.03)	-0.030	-0.05 - -0.02*
AD LC-MS/MS	0.86 (0.02)	0.86 (0.02)	-0.0066	-0.02 - 0.006

Linear regression analyses were performed to assess correlations of T (RIA) and T (LC-MS/MS). Univariate standardized beta coefficients and associated standard errors are reported.
* Differences between the correlations of T (RIA) and T (LC-MS/MS) show a statistically significant difference.
a Larger univariate standardized β coefficients represent stronger associations with the steroids.
b Univariate standardized β coefficients of both T assays were compared using a bootstrapping procedure with 2000 replications.

Table S2. Assessment of the correlation of DHEA measured by extraction RIA and LC-MS/MS with other androgens and E_2

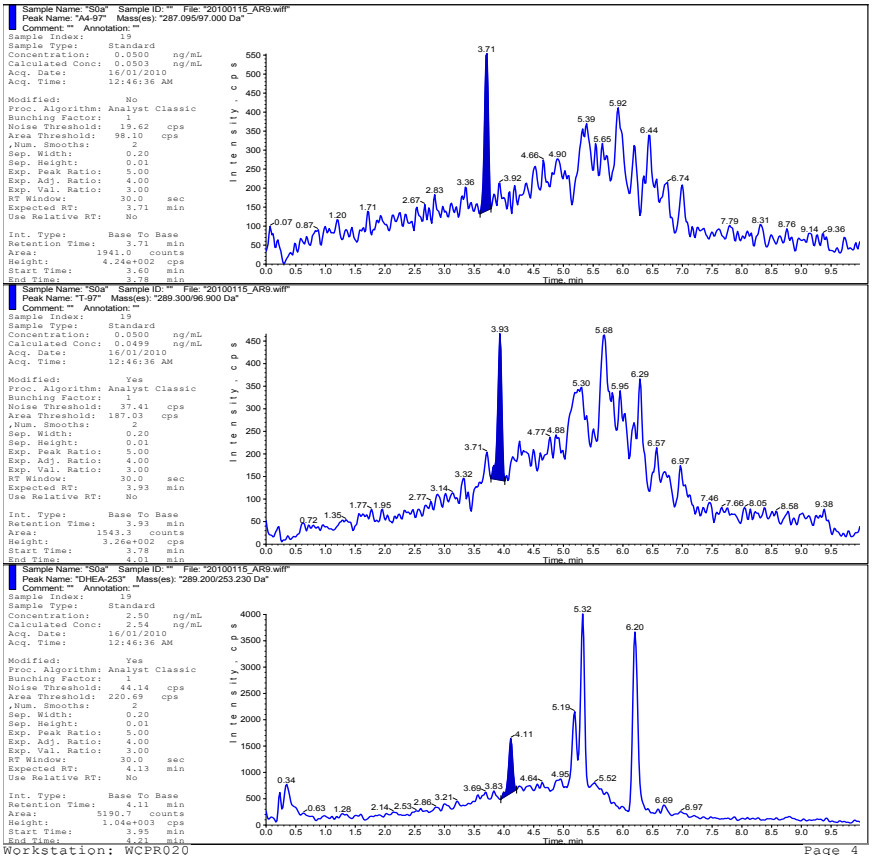
	Univariate Standardized Beta Coefficient		Difference of beta coefficients	95% CI of difference[b]
	DHEA RIA (S.E.)[a]	*DHEA LC-MS/MS (S.E.)*[a]		
E_2 RIA	0.04 (0.06)	0.03 (0.06)	-0.013	-0.099 - 0.069
SHBG RIA	-0.11 (0.05)	-0.17 (0.05)	-0.067	-0.148 - 0.006
T RIA	0.48 (0.05)	0.50 (0.04)	0.020	-0.037 - 0.010
T LC-MS/MS	0.42 (0.05)	0.47 (0.04)	0.044	0.004 - 0.085*
DHEAS RIA	0.72 (0.04)	0.67 (0.04)	-0.058	-0.11 - 0.008
AD RIA	0.57 (0.04)	0.54 (0.04)	-0.027	-0.081 - 0.026
AD LC-MS/MS	0.55 (0.04)	0.58 (0.04)	0.030	-0.029 - 0.091

Linear regression analyses were performed to assess correlations of DHEA (RIA) and DHEA (LC-MS/MS). Univariate standardized beta coefficients and associated standard errors are reported.
* Differences between the correlations of DHEA (RIA) and DHEA (LC-MS/MS) show a statistically significant difference.
a Larger univariate standardized β coefficients represent stronger associations with the steroids.
b Univariate standardized β coefficients of both DHEA assays were compared using a bootstrapping procedure with 2000 replications.

Table S3. Assessment of the correlation of AD measured by extraction RIA and LC-MS/MS with other androgens and E_2

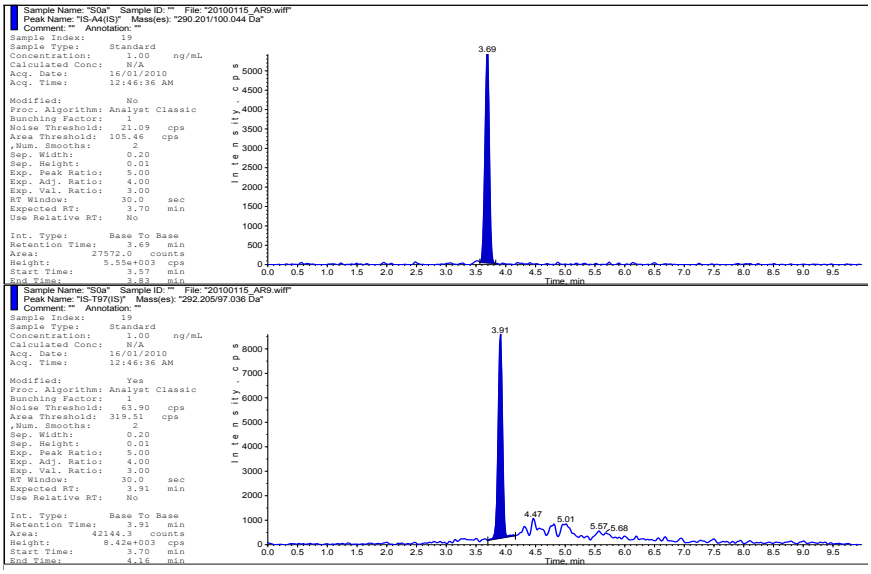
	Univariate Standardized Beta Coefficient		Difference of beta coefficients	95% CI of difference[b]
	AD RIA (S.E.)[a]	*AD LC-MS/MS (S.E.)*[a]		
E_2 RIA	0.34 (0.06)	0.37 (0.05)	0.029	-0.001 - 0.061
SHBG RIA	-0.16 (0.05)	-0.21 (0.05)	-0.041	-0.092 - 0.008
T RIA	0.87 (0.03)	0.86 (0.02)	-0.0025	-0.018 - 0.015
T LC-MS/MS	0.84 (0.03)	0.86 (0.02)	0.021	0.001 - 0.042
DHEAS RIA	0.44 (0.05)	0.43 (0.05)	-0.010	-0.038 - 0.018
DHEA RIA	0.57 (0.04)	0.55 (0.04)	-0.014	-0.037 - 0.010
DHEA LC-MS/MS	0.54 (0.04)	0.58 (0.04)	0.044	0.0043 - 0.085*

Linear regression analyses were performed to assess correlations of AD (RIA) and AD (LC-MS/MS). Univariate standardized beta coefficients and associated standard errors are reported.
* Differences between the correlations of AD (RIA) and AD (LC-MS/MS) show a statistically significant difference.
a Larger univariate standardized β coefficients represent stronger associations with the steroids.
b Univariate standardized β coefficients of both AD assays were compared using a bootstrapping procedure with 2000 replications.



Supplementary figure 1.

Chromatogram of LC-MS/MS for lower LOQ of AD: 0.0500 ng/mL (0.173 nmol/L), T : 0.0500 ng/mL (0.173 nmol/L), and DHEA: 2.50 ng/mL (8.68 nmol/L) (above). The panels on page 150 depict chromatograms for internal standards (19-d3-androstenedione (IS-A), and d3-testosterone (IS-T).



Supplementary figure 1.
Chromatogram of LC-MS/MS for lower LOQ of AD: 0.0500 ng/mL (0.173 nmol/L), T : 0.0500 ng/mL (0.173 nmol/L), and DHEA: 2.50 ng/mL (8.68 nmol/L) (on page 149). The panels above depict chromatograms for internal standards (19-d3-androstenedione (IS-A), and d3-testosterone (IS-T).

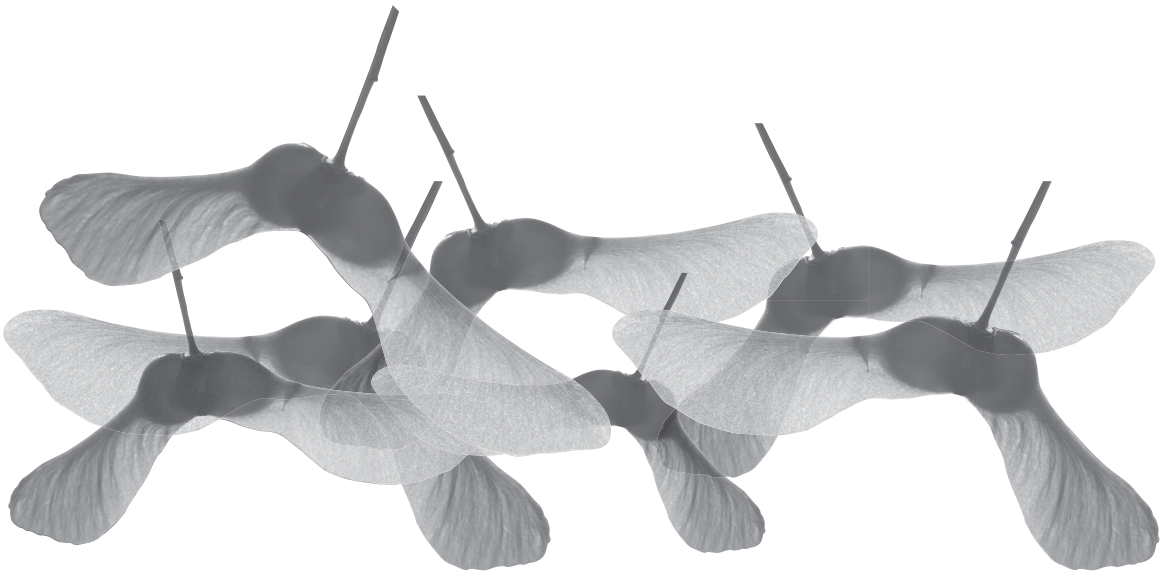
References

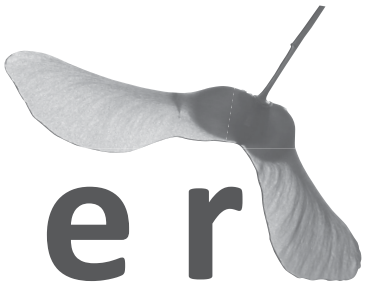
1. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab 2007;92:405-413.
2. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 1999;84:3666-3672.
3. Herold DA, Fitzgerald RL. Immunoassays for testosterone in women: better than a guess? Clin Chem 2003;49:1250-1251.
4. Longcope C. Adrenal and gonadal androgen secretion in normal females. Clin Endocrinol Metab 1986;15:213-228.
5. Burger HG. Androgen production in women. Fertil Steril 2002;77:3-5.
6. Dunn JF, Nisula BC, Rodbard D. Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab 1981;53:58-68.
7. Stanczyk FZ, Cho MM, Endres DB, Morrison JL, Patel S, Paulson RJ. Limitations of direct estradiol and testosterone immunoassay kits. Steroids 2003;68:1173-1178.
8. Taieb J, Mathian B, Millot F, Patricot MC, Mathieu E, Queyrel N, Lacroix I, Somma-Delpero C, Boudou P. Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. Clin Chem 2003;49:1381-1395.
9. Boots LR, Potter S, Potter D, Azziz R. Measurement of total serum testosterone levels using commercially available kits: high degree of between-kit variability. Fertil Steril 1998;69:286-292.
10. Rosner W, Vesper H. Toward excellence in testosterone testing: a consensus statement. J Clin Endocrinol Metab 2010;95:4542-4548.
11. Stanczyk FZ. Measurement of Androgens in Women. Semin Reprod Med 2006;24:78-85.
12. Vesper HW, Botelho JC, Shacklady C, Smith A, Myers GL. CDC project on standardizing steroid hormone measurements. Steroids 2008;73:1286-1292.
13. Shifren JL. Androgen deficiency in the oophorectomized woman. Fertil Steril 2002;77:60-62.
14. van der Stege JG, Groen H, van Zadelhoff SJ, Lambalk CB, Braat DD, van Kasteren YM, van Santbrink EJ, Apperloo MJ, Weijmar Schultz WC, Hoek A. Decreased androgen concentrations and diminished general and sexual well-being in women with premature ovarian failure. Menopause 2008;15:23-31.
15. Davison SL, Bell R, Donath S, Montalto JG, Davis SR. Androgen Levels in Adult Females: Changes with Age, Menopause, and Oophorectomy. J Clin Endocrinol Metab 2005;90:3847-3853.
16. Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 2004;19:41-47.

17. Chang WY, Knochenhauer ES, Bartolucci AA, Azziz R. Phenotypic spectrum of polycystic ovary syndrome: clinical and biochemical characterization of the three major clinical subgroups. Fertil Steril 2005;83:1717-1723.
18. Danforth KN, Eliassen AH, Tworoger SS, Missmer SA, Barbieri RL, Rosner BA, Colditz GA, Hankinson SE. The association of plasma androgen levels with breast, ovarian and endometrial cancer risk factors among postmenopausal women. Int J Cancer 2010;126:199-207.
19. Bachmann G, Bancroft J, Braunstein G, Burger H, Davis S, Dennerstein L, Goldstein I, Guay A, Leiblum S, Lobo R, Notelovitz M, Rosen R, Sarrel P, Sherwin B, Simon J, Simpson E, Shifren J, Spark R, Traish A. Female androgen insufficiency: the princeton consensus statement on definition, classification, and assessment. Fertil Steril 2002;77:660-665.
20. Goverde AJ, van Koert AJ, Eijkemans MJ, Knauff EA, Westerveld HE, Fauser BC, Broekmans FJ. Indicators for metabolic disturbances in anovulatory women with polycystic ovary syndrome diagnosed according to the Rotterdam consensus criteria. Hum Reprod 2009;24:710-717.
21. Janse F, Knauff EA, Niermeijer MF, Eijkemans MJ, Laven JS, Lambalk CB, Fauser BC, Goverde AJ. Similar phenotype characteristics comparing familial and sporadic premature ovarian failure. Menopause 2010;17:758-765.
22. Coulam CB, Adamson SC, Annegers JF. Incidence of premature ovarian failure. Obstet Gynecol 1986;67:604-606.
23. Knauff EAH, Eijkemans MJC, Lambalk CB, ten Kate-Booij MJ, Hoek A, Beerendonk CCM, Laven JSE, Goverde AJ, Broekmans FJM, Themmen APN, de Jong FH, Fauser BCJM, on behalf of the Dutch Premature Ovarian Failure Consortium. Anti-mullerian hormone, inhibin B, and antral follicle count in young women with ovarian failure. J Clin Endocrinol Metab 2009;94:786-792.
24. Thienpont LM, van Uytfanghe K, Blincko S, Ramsay CS, Xie H, Doss RC, Keevil BG, Owen LJ, Rockwood AL, Kushnir MM, Chun KY, Chandler DW, Field HP, Sluss PM. State-of-the-art of serum testosterone measurement by isotope dilution-liquid chromatography-tandem mass spectrometry. Clin Chem 2008;54:1290-1297.
25. Carter GD, Holland SM, Alaghband-Zadeh J, Rayman G, Dorrington-Ward P, Wise PH. Investigation of hirsutism: testosterone is not enough. Ann Clin Biochem 1983;20:262-263.
26. Shrout PE, Fleiss JL. Intraclass correlations: uses in assessing rater reliability. Psychol Bull 1979;86:420-428.
27. Stener-Victorin E, Holm G, Labrie F, Nilsson L, Janson PO, Ohlsson C. Are there any sensitive and specific sex steroid markers for polycystic ovary syndrome? J Clin Endocrinol Metab 2010;95:810-819.
28. Barth JH, Field HP, Yasmin E, Balen AH. Defining hyperandrogenism in polycystic ovary syndrome: measurement of testosterone and androstenedione by liquid chromatography-tandem mass spectrometry and analysis by receiver operator characteristic plots. Eur J Endocrinol 2010;162:611-615.

29. Helsel DR. Nondetects and data analysis: statistics for censored environmental data. Edn 1. Eds M Scott & V Barnett. Hoboken, NJ:John Wiley & Sons, Inc., 2005.
30. Duignan NM, Shaw RW, Glass MR, Butt WR, Edwards RL. Sex hormone levels and gonadotrophin release in premature ovarian failure. Br J Obstet Gynaecol 1978;85:862-867.
31. Bernardi F, Hartmann B, Casarosa E, Luisi S, Stomati M, Fadalti M, Florio P, Santuz M, Luisi M, Petraglia F, Genazzani AR. High levels of serum allopregnanolone in women with premature ovarian failure. Gynecol Endocrinol 1998;12:339-345.
32. Falsetti L, Scalchi S, Villani MT, Bugari G. Premature ovarian failure. Gynecol Endocrinol 1999;13:189-195.
33. Benetti-Pinto CL, Bedone AJ, Magna LA. Evaluation of serum androgen levels in women with premature ovarian failure. Fertil Steril 2005;83:508-510.
34. Kalantaridou SN, Calis KA, Vanderhoof VH, Bakalov VK, Corrigan EC, Troendle JF, Nelson LM. Testosterone deficiency in young women with 46,XX spontaneous premature ovarian failure. Fertil Steril 2006;86:1475-1482.
35. Elias AN, Pandian MR, Rojas FJ. Serum levels of androstenedione, testosterone and dehydroepiandrosterone sulfate in patients with premature ovarian failure to age-matched menstruating controls. Gynecol Obstet Invest 1997;43:47-48.
36. Parker CR Jr., Slayden SM, Azziz R, Crabbe SL, Hines GA, Boots LR, Bae S. Effects of Aging on Adrenal Function in the Human: Responsiveness and Sensitivity of Adrenal Androgens and Cortisol to Adrenocorticotropin in Premenopausal and Postmenopausal Women. J Clin Endocrinol Metab 2000;85:48-54.
37. Wang C, Catlin DH, Demers LM, Starcevic B, Swerdloff RS. Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 2004;89:534-543.
38. Moal V, Mathieu E, Reynier P, Malthiery Y, Gallois Y. Low serum testosterone assayed by liquid chromatography-tandem mass spectrometry. Comparison with five immunoassay techniques. Clin Chim Acta 2007;386:12-19.
39. Owen WE, Rawlins ML, Roberts WL. Selected performance characteristics of the Roche Elecsys testosterone II assay on the Modular analytics E 170 analyzer. Clin Chim Acta 2010;411:1073-1079.
40. Dorgan JF, Fears TR, McMahon RP, Aronson FL, Patterson BH, Greenhut SF. Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry. Steroids 2002;67:151-158.
41. Legro RS, Schlaff WD, Diamond MP, Coutifaris C, Casson PR, Brzyski RG, Christman GM, Trussell JC, Krawetz SA, Snyder PJ, Ohl D, Carson SA, Steinkampf MP, Carr BR, McGovern PG, Cataldo NA, Gosman GG, Nestler JE, Myers ER, Santoro N, Eisenberg E, Zhang M, Zhang H. Total Testosterone Assays in Women with Polycystic Ovary Syndrome: Precision and Correlation with Hirsutism. J Clin Endocrinol Metab 2010;95:5305-5313.

42. Rivera-Woll LM, Papalia M, Davis SR, Burger HG. Androgen insufficiency in women: diagnostic and therapeutic implications. Hum Reprod Update 2004;10:421-432.
43. Mueller A, Cupisti S, Binder H, Hoffmann I, Kiesewetter F, Beckmann MW, Dittrich R. Endocrinological markers for assessment of hyperandrogenemia in hirsute women. Horm Res 2007;67:35-41.
44. Bolelli G, Muti P, Micheli A, Sciajno R, Franceschetti F, Krogh V, Pisani P, Berrino F. Validity for epidemiological studies of long-term cryoconservation of steroid and protein hormones in serum and plasma. Cancer Epidemiol Biomarkers Prev 1995;4:509-513.





Chapter 7

Limited value of ovarian function markers following orthotopic transplantation of ovarian tissue after gonadotoxic treatment

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Abstract

Context

In young women, some treatments for cancer or other conditions (such as sickle cell anemia) may give rise to primary ovarian insufficiency. Ovarian transplantation is one of the available options for fertility preservation, with highly variable pregnancy rates.

Objective

The objective of this study was to investigate markers of ovarian reserve and ovarian function in women up to 7 years after orthotopic ovarian transplantation. Secondary objectives were to assess the relationship between markers of ovarian reserve and pregnancy rate along with the duration of ovarian function.

Design

This was a prospective cohort study in 10 women, with mean follow-up of 2.5 years.

Setting

This study was conducted at a university hospital in Brussels, Belgium.

Patients

Patients included 10 women who were about to receive or had previously received gonadotoxic treatment. In seven women cryopreservation of ovarian tissue was performed before starting treatment. Subsequently autografts were orthotopically transplanted in these women. Three women, who had already developed primary ovarian insufficiency due to treatment, underwent orthotopic transplantation of ovarian allograft tissue originating from their human leukocyte antigen-compatible sisters.

Main Outcome Measures

Serum concentrations of FSH, LH, estradiol, inhibin B and anti-müllerian hormone (AMH) were measured.

Results

On average, first menses took place after 4.7 months. Duration of graft functioning varied from 2 to more than 60 months. FSH concentrations remained elevated, whereas estradiol levels normalized and AMH was low to undetectable. Inhibin B varied among women and was not associated with the duration of ovarian function (hazard ratio 0.966, 95% CI 0.881 - 1.059). Two spontaneous pregnancies occurred. Endocrine characteristics were not significantly different in these women.

Conclusions

Low AMH and inhibin B concentrations may suggest decreased ovarian reserve in women after ovarian transplantation. AMH and inhibin B levels may not be associated with the duration of ovarian graft function, or probability to achieve a pregnancy.

Introduction

Advances in the diagnosis and treatment of childhood, adolescent, and adult cancer have greatly increased the life expectancy of young women with cancer. Approximately 82% of patients diagnosed before the age of 19 years survive the disease for at least five years (1). Long-term effects of treatment strategies applied for childhood cancer have become more apparent, especially the high incidence of primary ovarian insufficiency and associated infertility in these women (2-4). Alkylating agents, such as cyclophosphamide and procarbazine, and irradiation are particularly toxic to the ovaries (5,6).

Several options to preserve fertility in cancer patients are currently available enabling women to conceive when they have overcome their disease. These options include embryo or oocyte cryopreservation and ovarian tissue cryostorage. Although freezing of oocytes and subsequent in vitro maturation may represent an alternative in the future, cryopreservation of ovarian tissue is currently the only option for prepubertal girls and for women who cannot delay the start of chemotherapy (7). The main aim of this strategy is to reimplant ovarian cortical tissue into the pelvic cavity (orthotopic site) or a heterotopic site once treatment is completed and the patient is disease-free (8).

In 2004 the first live birth after orthotopic transplantation of cryopreserved ovarian tissue was reported (9). Up until now around 13 live births after orthotopic transplantation of (cryopreserved) ovarian tissue have been described (10-17). Despite these encouraging results, the restoration of fertility after ovarian cryopreservation and subsequent transplantation is highly variable and seems limited in duration (18). The impression arises that only successful ovarian autotransplantation reports have been published, and no data on the actual success rate of the procedure are known.

Finally, little information is currently available regarding the possibility for markers of ovarian reserve to predict the extent of the restoration of ovarian function and the associated chances for conception. Only a single case report describing anti-müllerian hormone (AMH) and Inhibin B returning to normal concentrations and the subsequent occurrence of a pregnancy has been published to date (19). This underlines the need for the further evaluation of ovarian reserve in women undergoing ovarian transplantation. Here we describe markers of ovarian function and reserve in a series of patients who underwent orthotopic ovarian transplantation.

Materials and methods

Patients

Since 1997, ovarian tissue cryopreservation is proposed in Brussels for fertility preservation to all patients presenting a high risk for iatrogenic ovarian failure. Patient characteristics are described in *Table 1*.

Seven patients (patients 1-7) were referred for ovarian cryopreservation before starting on primary ovarian insufficiency-inducing treatments and have been described in previously published papers (8,20). Patients 3 and 4 had received pre-treatment with chemotherapeutic agents before cryopreservation was performed. In addition, for patients 3 and 5, a second reimplantation of ovarian tissue was carried out after exhaustion of the first graft.

Furthermore, transplantation with fresh ovarian allografts was performed in three women (patients 8-10), as recently reported (21). These patients presented with primary ovarian insufficiency after treatments for β-thalassemia, acute myeloid leukemia and sickle cell anemia. In all three women, allograft ovarian tissue originated from their human leukocyte antigen-compatible sisters. The donors were 32, 36, and 34 years of age at the time of reimplantation, respectively. Sisters of patients 9 and 10 both have two children.

After receiving gonadotoxic treatment, all women experienced amenorrhea for at least two months, along with elevated FSH concentrations, before the start of hormone replacement therapy and after iatrogenic primary ovarian insufficiency had been diagnosed. After discontinuation of hormone replacement therapy, amenorrhea persisted in all patients and FSH and estradiol concentrations returned to menopausal levels (respectively, >45 IU/L and >10 pg/mL).

The time line between cessation of chemotherapy and ovarian transplantation was 6-7 years for patients 1-4 and 6. For patient 5, there were only 2 years between the cessation of chemotherapy and transplantation. Indeed, she received oral cyclophosphamide from 2001 to April 2006 and transplantation went ahead in March 2008. Patient 7 did not receive any chemotherapy as she underwent castrative surgery, and her ovarian tissue was frozen at the time of the surgery. Patients 8-10 were treated by chemotherapy before ovarian tissue cryopreservation was proposed (from 1988 to 1992). All three were transplanted with fresh ovarian tissue from their sisters who already donated bone marrow before and after genetic diagnosis of complete chimerism.

After ovarian transplantation, patients were followed up for a mean of 2.5 ± 2.0 years until pregnancy had occurred, until cessation of transplanted ovarian tissue function, or until the time of writing this report.

Table 1. Patient characteristics for 10 patients undergoing ovarian transplantation

Patient	Diagnosis	Treatment	Auto- / Allo-transplantation	Age at treatment (years)[a]	Chemotherapy before cryopreservation	Age at transplantation (years)
1	Hodgkin's lymphoma	MOPP-ABV hybrid, radiotherapy	Auto	25	No	31
2	Sickle cell anemia	Busulfan, Cyclophosphamide, BMT	Auto	27	No	27
3	Hodgkin's lymphoma	ABVD-MOPP, BEAM, stem cell, transplantation, pelvic irradiation	Auto	23	Yes[b]	31 (1) 33 (2)
4	Non-Hodgkin lymphoma	ACVBP, Cyclophosphamide, VP16, Novantrone, BCNU	Auto	28	Yes[c]	34
5	Wegener's granulomatosis	Cyclophosphamide	Auto	22	No	27 (1) 29 (2)
6	Cerebral tumor (PNET)	Surgery, radiotherapy VIDE, stem cell transplantation	Auto	17	No	24
7	Tubo-ovarian abscess and endometriosis	Castrating surgery	Auto	29	No	33
8	Major β-thalassemia	TBI, BMT, Busulfan, Cyclophosphamide	Allo	20	NA	35
9	Acute myeloid leukemia	TBI, BMT	Allo	12	NA	33
10	Sickle cell anemia	TBI, BMT, Busulfan, Cyclophosphamide	Allo	15	NA	31

(1), First transplantation; (2), second transplantation; MOPP, Mustargen oncovin procarbazine prednisone; ABV, adriamycin bleomycin vinblastine; BMT, bone marrow transplantation; ABVD, adriamycin bleomycin vinblastine dacarbazine; BEAM, BCNU etoposide Ara-C melphalan; ACVBP, doxorubicin cyclophosphamide vindesine bleomycin prednisone; VP16, etoposide; VIDE, vincristine ifosfamide doxorubicin etoposide; TBI, total body irradiation; NA, not applicable.
a Age at treatment corresponds to age at which cryopreservation was performed.
b Eight cycles ABVD at age 22 years.
c Three cycles ACVBP at age 28 years.

Cryostorage, thawing procedure and transplantation

Freezing and thawing of ovarian tissue were carried out according to the protocols described earlier (9,20,22). After biopsy, samples were immersed in Leibovitz L-15 medium supplemented with Glutamax (GIBCO, Paisley, UK) and immediately taken to the laboratory, in which remaining stromal tissue was gently removed. The cortical samples were cut into small strips (±10 x 3 mm) or cubes (±2 x 2 mm). These ovarian tissue fragments were suspended in the cryoprotective medium and placed into precooled 2-mL cryogenic vials (Simport, Québec, Canada), filled with L-15 medium supplemented with 0.4 mg/mL of human serum albumin (Red Cross, Brussels, Belgium) and 1.5 mmol/L of dimethylsulfoxide (Sigma, St. Louis, MO). The cryotubes were cooled in a programmable freezer (Kryo 10, Series III; Planer, Sunbury-on-Thames, UK) according to the following program: cooling from 0 to -8 °C at -2 °C/min; seeding manually by touching the cryotubes with forceps prechilled in liquid nitrogen; cooling to -40 °C at -0.3 °C/min; cooling to -150 °C at -30 °C/min; and immediate transfer to liquid nitrogen (-196 °C) for storage.

The cryogenic vials were thawed at room temperature (21-23 °C) for 2 minutes and immersed in a water bath at 37 °C for another 2 minutes. Ovarian tissue was immediately transferred from the vials to tissue culture dishes (Becton Dickinson, Hancock, NY) in L-15 medium (Gibco). Subsequently tissue was washed three times with fresh medium to remove cryoprotectant. Thawed ovarian cortical tissue was then placed in sterile medium and immediately transferred to the operating theatre.

As previously described, orthotopic transplantation of the cryopreserved ovarian tissue was performed after a mean of 6.0 years (SD 0.89 years) when chemotherapy and hormone replacement therapy was discontinued, and there was a wish to conceive (8,20). Thawed ovarian tissue was reimplanted to either a peritoneal window created near the ovary (patient 1) or on decorticated medulla of atrophic ovaries. The strips or cubes were sutured (patient 1) or covered with Interceed (Johnson and Johnson, New Brunswick, NJ) (20).

In allograft recipients, both atrophic ovaries were decorticated. When the ovaries were ready to receive donor ovarian cortex, either one large biopsy (2 x 2 cm) was taken from the one donor ovary or two smaller biopsies (10 x 6 mm) were taken from both donor ovaries. Larger biopsies were cut into two parts, and in each recipient two ovarian tissue fragments were sutured to the decorticated ovarian medulla. Time between cortex removal and the start of suturing was less than one minute, and both sutures were achieved within 30 minutes of excision from the donor.

All patients, with the exception of patient 1, received GnRH antagonists or agonists and estroprogestogens from 20 days preoperatively for a total of two months to decrease FSH and LH concentrations before reimplantation, in line with previous

recommendations (20). Immunosuppressive treatment was not indicated for allograft recipients because complete human leukocyte antigen compatibility was confirmed by the short tandem repeat technique (21).

Hormonal measurements

In Brussels, measurement of the following hormones was performed every two weeks following transplantation (irrespective of their menstrual cycle status): FSH, LH and estradiol (E_2). FSH and LH levels were measured using a chemiluminescent immunoassay (Access human FSH and Acces human LH 2000; Beckman Coulter, Inc., Brea, CA). The detection limits of the assays were 0.2 IU/L, inter- and intraassay coefficients of variation were less than 10%. Estradiol was measured by the Architect-i2000 chemiluminescent immunoassay (Abbott Laboratories, Abbott Park, IL). Within- and between-run imprecisions were below 10%, and the detection limit was 25 pg/mL.

For each patient inhibin B and AMH concentrations were also assessed at least twice after transplantation; once when E_2 concentration was high and another measurement when E_2 was low. Inhibin B was measured with immunoenzymometric assay (Oxford BioInnovation, Oxford, UK, now marketed by Beckman Coulter, Brea, CA). Intra- and interassay coefficient of variation were: less than 5% and less than 10%, and sensitivity was 5 ng/L. An in-house immunoenzymometric assay was used to measure AMH, using the antibodies also present in the AMH assay Diagnostic Systems Laboratories (Webster, TX), which is now also marketed by Beckman Coulter. Results should be divided by 2.145 to be comparable with those of the original Beckman Coulter assay (23). Intra- and interassay coefficients of variation were less than 5% and less than 10%. Sensitivity was 0.026 µg/L. Selected samples were reanalyzed for AMH in the laboratory of one of the investigators (E.A.). Serum AMH (by E.A.) was determined by the Immunotech AMH enzyme immunoassay (Beckman Coulter, Marseilles, France). The intra-assay and inter-assay coefficients of variation were less than 9.5% (3.3 µg/L). Functional sensitivity of the assay was 0.35 µg/L. Basal AMH (by E.A.) values in 21- to 37-year-old normo-ovulatory women defined as normal responders in an in vitro fertilization (IVF) cycle were 1.7-8.2 µg/L (n = 345).

Statistical analysis

The distributions of FSH and E_2 for all women receiving ovarian transplantation after cryopreservation are depicted as box-and-whisker plots. A Kaplan-Meier survival curve was constructed for the duration of graft survival grouped on detectable vs. undetectable AMH concentrations. Data were censored when the graft was still functioning at time of writing of this manuscript. The log rank test was performed to identify statistically significant differences in the duration of

graft function according to AMH. Cox regression tests were performed for AMH and inhibin B in comparison with duration of tissue functioning, and reported as hazard ratios with associated 95% confidence intervals. For all patients, the first inhibin B measurement after transplantation was chosen except when using GnRH (ant-)agonists, in which case the second inhibin B measurement was included. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL).

Results

Characteristics of menstrual cycle, duration of graft functioning and pregnancies are reported for all patients in *Table 2*. In all women except for patient 7, first menses took place at mean 4.7 (range 3.5-7.0) months after transplantation. Ovarian function of these women was monitored for a mean of 2.5 years. Duration of cryopreserved graft functioning varied from 9 to 86 months, with three of seven grafts still functioning in July 2010. Two of three fresh allografts still functioned in July 2010, and the duration varied from 17 to 28 months. Two pregnancies were achieved, of which the first has been previously reported (9). In patient 7, menstrual cyclicity did not return. At the time of thawing and grafting, two small fragments were sent for histological investigation and no follicles were found in serial sections.

Furthermore, AMH and inhibin B measurements are described along with simultaneously measured FSH and E_2 concentrations in *Table 2*. AMH concentrations were detectable above lower assay limits in five out of 27 measurements only and were low. AMH does not seem to correspond well with pregnancy chances because AMH was undetectable in the two patients who conceived. Although measurements by the additional AMH assay showed some higher AMH concentrations, many were still below the detection limit and do not correspond well with ovarian transplant function (data not shown).

Inhibin B concentrations varied widely among patients and increased as menstrual cycles returned. High Inhibin B concentrations were found in patients 6, 9, and 10, but only one of these three women conceived. In contrast, Inhibin B remained only 30 ng/L in patient 1, who also achieved pregnancy.

Figure 1 presents box-and-whisker plots for FSH and E_2 concentrations for all patients receiving ovarian transplantation after cryopreservation (n = 7) with regard to time since transplantation. As shown, FSH levels tend to remain relatively high, even when menstrual cycle patterns returned after a mean of 4.7 months. E_2 concentrations increase over time when ovarian function resumes. Individual FSH and E_2 concentrations for three women receiving fresh allograft ovarian transplantations are depicted in *Figure 2*. In patient 8 high FSH and low E_2 concentrations are found. In both patient 9 and 10, FSH remains below 20 IU/L

Table 2. Ovarian function and endocrinology after ovarian transplantation

Patient	First menses	Graft function[a]	Hormone measurement	Hormone assay					Pregnancy
					FSH	*E_2*	*AMH*	*Inhibin B*	
	(Time in months from transplantation)			*Cycle day*	*(IU/L)*	*(pg/mL)*	*(µg/L)*	*(ng/L)*	
1	5	>86	3	6	31	25	<0.025	23	Yes[b]
			6	24	21	58	<0.025	30	
2	4	43	0[c]				<0.025		No
			7	NA	17	44	<0.025	15	
			31	32	47	89	0-0.01	55	
3 (1)	5	30	0[c]				<0.025		No
			5	NA	42	194	<0.025	27	
			14	25	5.5	126	<0.025	12	
3 (2)	5	>32	5	NA	27	128	<0.025	13	No
4	4.5	42	0[c]				<0.025		No
			4	NA	70	13	<0.025	6	
			8	6	18	70	<0.025	8	
5 (1)	6.5	24	1[c]				<0.025		No
			4	5	80	26	<0.025	19	
			7	96	10	69	<0.025	63	
5 (2)	6	18	0[c]				0.0-0.01		No
			5	16	6.8	186	<0.025	55	
6	3.5	>9	0[c]				<0.025		Yes[d]
			5	43	11	83	<0.025	99	
			8	8	6.8	62	<0.025	100	

Table 2. Continued

Patient	First menses	Graft function[a]	Hormone measurement	Hormone assay					Pregnancy
					FSH	E_2	*AMH*	*Inhibin B*	
	(Time in months from transplantation)			*Cycle day*	*(IU/L)*	*(pg/mL)*	*(µg/L)*	*(ng/L)*	
7		0	2[c]				<0.025		No
			5	NA	64	12	<0.025	10	
8	7	28	0[c]				<0.025		No
			20	120	4	167		21	
9	3.5	>19	1	NA	40	19	<0.025	28	No
			3	9	6.7	104	0.33	83	
10	3.5	>17	1[c]				0.09		No
			5	8	14	196	0.09	97	

(1), First transplantation; (2), second transplantation; NA, not appropriate, when the blood sample was taken before the first menstruation after grafting.
a Assessed by cessation of menses (often coinciding with elevated FSH).
b Pregnancy spontaneously achieved 11 months after transplantation.
c Only AMH concentrations are reported because samples were drawn at or shortly after transplantation during administration of GnRH analogs or combined oral contraceptives.
d Pregnancy spontaneously achieved 9 months after transplantation.

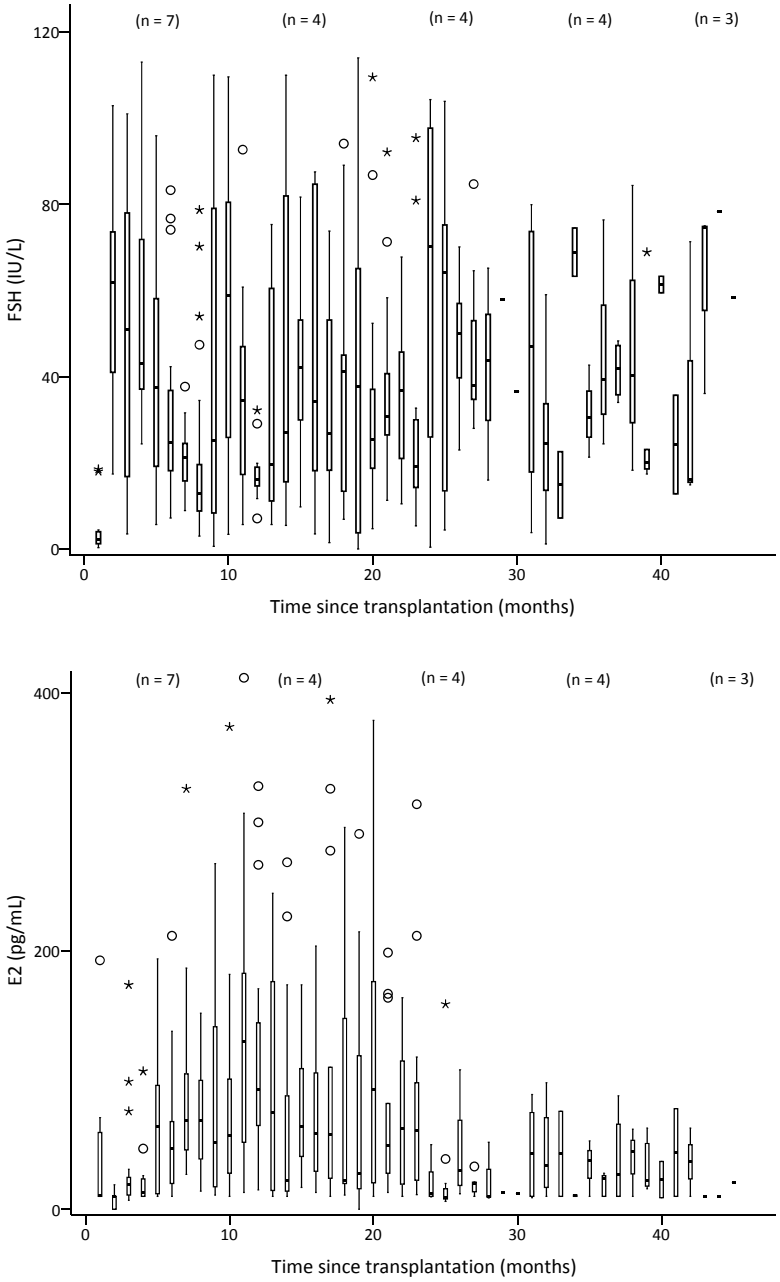

Figure 1. Box-and-whisker plots for FSH (upper panel) and E_2 (bottom panel) concentrations following cryopreserved ovarian tissue transplantation (n = 7).
Legend: Blood withdrawal took place every other week, irrespective of cycle phase or length. Boxes represent the interquartile ranges (p25 and p75), the lines within the boxes are the median values, and the whiskers depict p5 and p95. ○ and *, outliers.

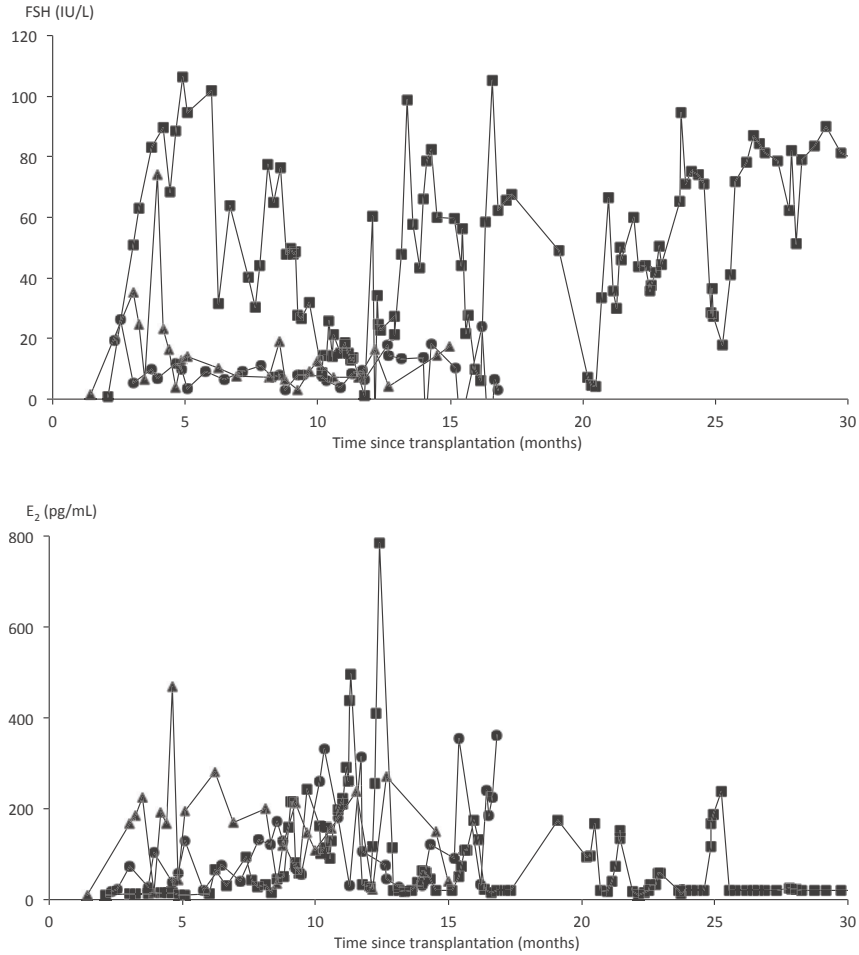

Figure 2. Individual FSH (upper panel) and E_2 (bottom panel) concentrations after fresh ovarian tissue transplantation (n = 3).
Legend: ■, Patient 8; ●, Patient 9; ▲, Patient 10.

and E_2 returns to normal values after three months, and first menses took place shortly afterwards.

A comparison of detectable vs. undetectable AMH concentrations with regard to duration of tissue function was performed for which a Kaplan-Meier curve was constructed (*Figure 3*). The curves for detectable and undetectable AMH concentrations are similar. Cox regression analysis for this relationship was not performed because only one of the grafts for which AMH was detectable had stopped functioning at the time of this writing. The log rank test for the Kaplan-Meier curve was not statistically significant (P = 0.99). Cox regression analysis for inhibin B concentrations compared with duration of tissue functioning resulted in a hazard ratio of 0.966 (95% confidence interval 0.881-1.059), indicating similar duration of graft function, regardless of inhibin B concentrations.

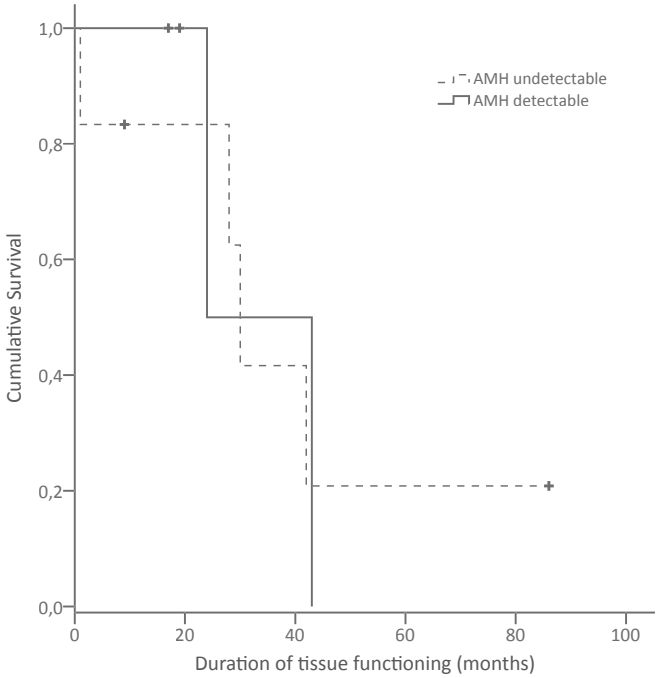

Figure 3. Kaplan-Meier curve showing the survival of the ovarian tissue graft in relation to AMH concentrations.
Legend: Solid line, undetectable AMH (n = 6); broken line, detectable AMH (n = 4); +, censored data (graft still functioning in July 2010).

Discussion

To our knowledge, this is the first study to report comprehensive follow-up data on ovarian function and markers of ovarian reserve after transplantation of ovarian tissue in ten women. Despite the return of the menstrual cycle and the increase of E_2 concentrations within five months after transplantation, FSH tended to stay relatively high. Surprisingly, AMH remained low or undetectable in most cases, whereas inhibin B increase varied widely among patients.

The only safe established option for fertility preservation is embryo cryopreservation after IVF. However, this is not an option for patients without a partner or those not wanting to use a sperm donor, for prepubertal girls, in case chemotherapy has to be initiated immediately or when stimulation is contraindicated due to the type of cancer (24). The use of ovarian transplantation techniques to circumvent the need for hormone substitution therapy is still experimental.

In the existing literature, 13 live births after orthotopic transplantation of cryopreserved ovarian tissue have been described to date. A recent study reported a high incidence of pregnancies after fresh allograft transplantation (18). In the current study, two women spontaneously conceived after cryopreservation and reimplantation of ovarian tissue; neither of them had received chemotherapy before cryopreservation. It appears that both pregnancies occurred as a result of an oocyte ovulating from the graft (confirmed by ultrasound imaging in patient 1 and the fact that both ovaries had been decorticated in patient 6). Indeed, in patient 1, we observed follicular growth outside of the right ovary at the site of reimplantation (in the peritoneal window) and ten days later concentrations of progesterone as high as 37 ng/L concomitant with the presence of a corpus luteum at the reimplantation site, which was confirmed by ultrasound. Moreover, serial sections of biopsy samples taken from both atrophic ovaries of patient 1 and from removed ovarian cortex of patient six failed to demonstrate the presence of any follicles.

The duration of ovarian function after reimplantation of cryopreserved ovarian tissue was nine to at least 86 months (end of study). This is not essentially different from the recently published life span of six to over 54 months in women under similar conditions (25). It has been reported that the duration of ovarian function after allograft transplantation is three years or more (18). Patient 8 became amenorrheic after 28 months; this could be due to the nonoptimal ovarian reserve of the donor that was identified on biopsy. The remaining two allograft recipients still experienced regular menstrual cycles after 17 and 19 months.

Cryopreservation of ovarian tissue seems effective for storing primordial follicles but not for preserving the small number of growing follicles present in the tissue (26). The process of folliculogenesis, from the recruitment of primordial follicles

to ovulation, takes 4-6 months (27). In the current study, the first menses after reimplantation took place after 4.7-7.0 months, which is in line with the previously described extended duration of follicle maturation. Our findings in the human are also supported by data from similar studies in the sheep model and in a recent study in women undergoing cryopreserved ovarian transplantation (25,28). Although a longer interval until first cycle has been reported in women who had received chemotherapeutic treatments before cryopreservation (20), the present study does not support this for all women. Patient 7 did not experience any ovarian activity after transplantation and histological evaluation confirmed the absence of follicles. This could possibly be due to the endometriosis and the tuboovarian abscess present at the time of cryopreservation. This suggests that histological analysis (including follicle count) may be useful before transplantation.

E_2 concentrations returned to normal when menstrual cyclicity resumed, suggestive for dominant follicle development. Still, FSH levels remained above 20 IU/L during menses and the early follicular phase, in contrast to the fairly direct inverse correlation between E_2 and FSH under physiological conditions. Sheep studies confirm elevated FSH concentrations after autograft transplantation, supporting the hypothesis that some other inhibitory mechanisms, such as inhibin B, are significantly diminished in transplanted tissue (28,29). Inhibin B concentrations varied significantly among different subjects. The discrepancy between E_2 and inhibin B concentrations may indicate a declining ovarian follicle pool (30). Indeed, few patients reached normal inhibin B concentrations, and only patient 6 conceived after inhibin B concentrations reached 100 ng/L. Cox regression analysis showed similar duration of graft function irrespective of inhibin B levels.

AMH and inhibin B were measured to assess ovarian reserve in transplanted ovarian tissue. AMH is produced by granulosa cells of the developing follicles, and is produced until the antral stage (31). Inhibin B is produced by the cohort of developing preantral and early antral follicles. Inhibin B concentrations are maximal during early to midfollicular phase, whereas AMH is cycle independent (32). Both inhibin B and AMH decrease with increasing age due to diminishing ovarian reserve, and their clinical value is topic of extensive research. Currently, AMH is regarded as the superior marker in the prediction of ovarian response to ovarian stimulation or age at menopause (33,34).

Previously normalization of AMH was reported in a single patient after ovarian function was restored after reimplantation of cryopreserved ovarian tissue (19). Another case report described a minor increase of AMH from 0.6 to 1.8 µg/L after transplantation (17). These anecdotal findings, however, cannot be confirmed in the present study. Low or undetectable AMH concentrations were found in all women regardless of time since transplantation. It appears that AMH was more frequently detectable after transplantation of fresh ovarian tissue compared to

cryopreserved tissue. All AMH levels assessed in the two women who conceived were below detection limits. Repeated measurements of selected samples from the current study in yet another laboratory confirmed low AMH concentrations (data not shown). Therefore, the use of AMH in the prediction of duration of ovarian tissue graft functioning seems limited. The current sample size may not allow for any firm conclusion. However, another very recent report confirms the present findings by demonstrating AMH concentrations to be undetectable to low in five patients (including a woman who conceived), under comparable conditions (25). Therefore, the conclusion seems justified that the use of AMH in the prediction of duration of ovarian tissue graft functioning seems limited.

The low to undetectable concentrations of AMH in the current study suggest that the primordial follicle pool is low after transplantation. A recent report described a correlation between histologically determined primordial follicle numbers and ovarian reserve tests. After adjustment for age, significant correlations were found for antral follicle count and AMH (35). One of the aims of our study was to determine the relationship between markers of ovarian reserve and pregnancy rate along with ovarian function duration. Finding such low AMH values in all women does not help us distinguish between successful and unsuccessful transplantations of ovarian tissue. Therefore, AMH measurement does not appear to be useful in these circumstances. It is likely that diminished numbers of developing preantral and early antral follicles give rise to low to undetectable AMH concentrations, low inhibin B levels, and high FSH values, as recently described (33,35). Additional studies concerning AMH concentrations following various surgical transplantation techniques are needed.

Current observations are in line with the recently reported poor ovarian response to stimulation for IVF in patients following ovarian transplantation (36). This could be due to several factors that are specific to the transplantation, such as damage due to cryopreservation or hypoxia during the revascularization time (37). Oocytes could be damaged by freezing and thawing procedures, leading to a higher rate of empty follicles or oocyte alteration (38). A recent report found a 58% decrease of human oocytes after cryopreservation compared with fresh ovarian tissue grafts (18). Experimental studies have indicated that the fall in number of primordial follicles in grafted tissue is mostly due to hypoxia and the delay of revascularization (2-4 days) and is estimated to be 50% to 60% (37,39). Overall, the exact cause of a decreased cohort size of ovarian follicles along with its possible association with a reduced duration of ovarian function and poor response to stimulation remains open for speculation.

In conclusion, both AMH and inhibin B do not seem to be useful as markers of ovarian reserve or for prediction of pregnancy under the conditions of ovarian transplantation, despite the fact that AMH has been shown to be very a useful ovarian reserve marker in many other clinical conditions.

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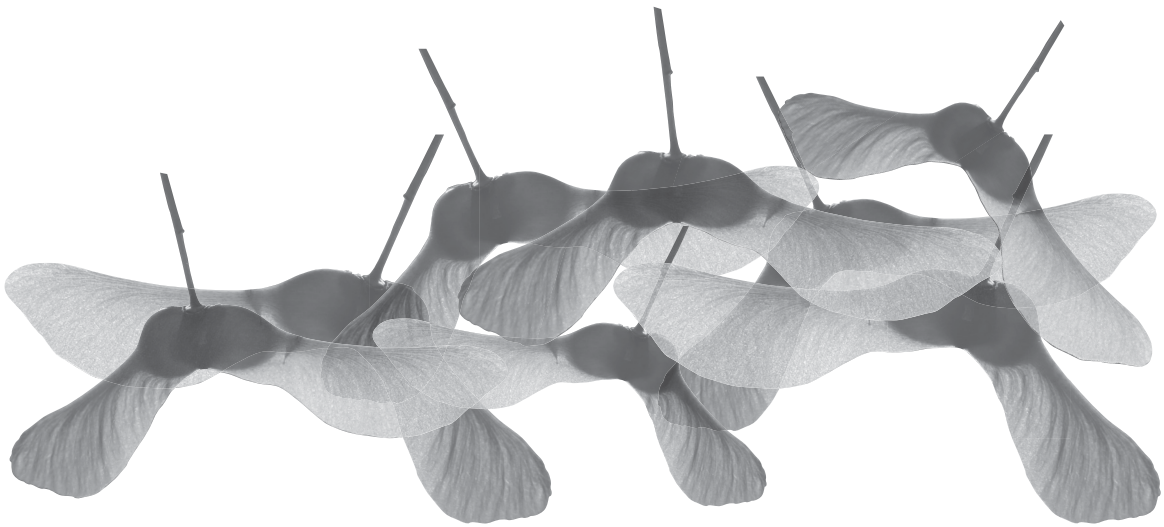
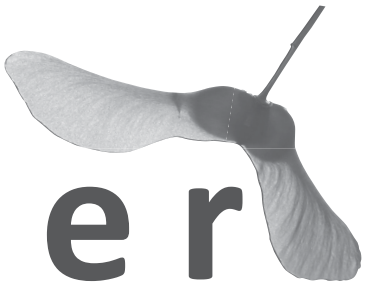
References

1. Altekruse SF, Kosary CL, Krapcho M, Neyman N, Aminou R, Waldron W, Ruhl J, Howlader N, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Cronin K, Chen HS, Feuer EJ, Stinchcomb DG, Edwards BK. SEER Cancer Statistics Review, 1975-2007. Available at seer.cancer.gov/csr/1975_2007/ 2010.
2. Meirow D. Reproduction post-chemotherapy in young cancer patients. Mol Cell Endocrinol 2000;169:123-131.
3. Wallace WH, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Oncol 2005;6:209-218.
4. de Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. Lancet 2010;376:911-921.
5. Wallace WH, Thomson AB, Saran F, Kelsey TW. Predicting age of ovarian failure after radiation to a field that includes the ovaries. Int J Radiat Oncol Biol Phys 2005;62:738-744.
6. Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. Cancer 2007;110:2222-2229.
7. Borini A, Levi Setti PE, Anserini P, Luca RD, Santis LD, Porcu E, La Sala GB, Ferraretti A, Bartolotti T, Coticchio G, Scaravelli G. Multicenter observational study on slow-cooling oocyte cryopreservation: clinical outcome. Fertil Steril 2010;94:1662-1668.
8. Donnez J, Martinez-Madrid B, Jadoul P, van Langendonckt A, Demylle D, Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update 2006;12:519-535.
9. Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, van Langendonckt A. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 2004;364:1405-1410.
10. Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, Schiff E, Dor J. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. N Engl J Med 2005;353:318-321.
11. Demeestere I, Simon P, Emiliani S, Delbaere A, Englert Y. Fertility preservation: successful transplantation of cryopreserved ovarian tissue in a young patient previously treated for Hodgkin's disease. Oncologist 2007;12:1437-1442.
12. Andersen CY, Rosendahl M, Byskov AG, Loft A, Ottosen C, Dueholm M, Schmidt KL, Andersen AN, Ernst E. Two successful pregnancies following autotransplantation of frozen/thawed ovarian tissue. Hum Reprod 2008;23:2266-2272.
13. Silber SJ, DeRosa M, Pineda J, Lenahan K, Grenia D, Gorman K, Gosden RG. A series of monozygotic twins discordant for ovarian failure: ovary transplantation (cortical versus microvascular) and cryopreservation. Hum Reprod 2008;23:1531-1537.
14. Demeestere I, Simon P, Moffa F, Delbaere A, Englert Y. Birth of a second healthy girl more than 3 years after cryopreserved ovarian graft. Hum Reprod 2010;25:1590-1591.

15. Ernst E, Bergholdt S, Jorgensen JS, Andersen CY. The first woman to give birth to two children following transplantation of frozen/thawed ovarian tissue. Hum Reprod 2010;25:1280-1281.
16. Roux C, Amiot C, Agnani G, Aubard Y, Rohrlich PS, Piver P. Live birth after ovarian tissue autograft in a patient with sickle cell disease treated by allogeneic bone marrow transplantation. Fertil Steril 2010;93:2413-2419.
17. Sanchez-Serrano M, Crespo J, Mirabet V, Cobo AC, Escriba MJ, Simon C, Pellicer A. Twins born after transplantation of ovarian cortical tissue and oocyte vitrification. Fertil Steril 2010;93:268.e11-268.e13.
18. Silber S, Kagawa N, Kuwayama M, Gosden R. Duration of fertility after fresh and frozen ovary transplantation. Fertil Steril 2010;94:2191-2196.
19. Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Yemini Z, Dor J. Monitoring the ovaries after autotransplantation of cryopreserved ovarian tissue: endocrine studies, in vitro fertilization cycles, and live birth. Fertil Steril 2007;87:418-418.
20. Donnez J, Squifflet J, van Eyck AS, Demylle D, Jadoul P, van Langendonckt A, Dolmans MM. Restoration of ovarian function in orthotopically transplanted cryopreserved ovarian tissue: a pilot experience. Reprod Biomed Online 2008;16:694-704.
21. Donnez J, Squifflet J, Pirard C, Jadoul P, Dolmans MM. Restoration of ovarian function after allografting of ovarian cortex between genetically non-identical sisters. Hum Reprod 2010;25:2489-2495.
22. Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196 degrees C. Hum Reprod 1994;9:597-603.
23. Lie FS, Laven JS, Hakvoort-Cammel FG, Schipper I, Visser JA, Themmen AP, de Jong FH, van den Heuvel-Eibrink MM. Assessment of ovarian reserve in adult childhood cancer survivors using anti-Mullerian hormone. Hum Reprod 2009;24:982-990.
24. Rodriguez-Wallberg KA, Oktay K. Fertility preservation in women with breast cancer. Clin Obstet Gynecol 2010;53:753-762.
25. Schmidt KT, Rosendahl M, Ernst E, Loft A, Andersen AN, Dueholm M, Ottosen C, Andersen CY. Autotransplantation of cryopreserved ovarian tissue in 12 women with chemotherapy-induced premature ovarian failure: the Danish experience. Fertil Steril 2010;95:695-701.
26. Gook DA, Edgar DH, Stern C. Effect of cooling rate and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1, 2-propanediol. Hum Reprod 1999;14:2061-2068.
27. Goucheon A. Regulation of Ovarian Follicular Development in Primates: Facts and Hypotheses. Endocr Rev 1996;17:121-155.
28. Baird DT, Campbell B, de SC, Telfer E. Long-term ovarian function in sheep after ovariectomy and autotransplantation of cryopreserved cortical strips. Eur J Obstet Gynecol Reprod Biol 2004;113 Suppl 1:S55-S59.

29. Campbell BK, Telfer EE, Webb R, Baird DT. Ovarian autografts in sheep as a model for studying folliculogenesis. Mol Cell Endocrinol 2000;163:131-139.
30. van Rooij I, Broekmans FJ, Scheffer GJ, Looman CW, Habbema JD, de Jong FH, Fauser BJ, Themmen AP, Te Velde ER. Serum antimullerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: a longitudinal study. Fertil Steril 2005;83:979-987.
31. Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC, Themmen AP. Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. Mol Hum Reprod 2004;10:77-83.
32. Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather JP, McNeilly AS. Measurement of dimeric inhibin B throughout the human menstrual cycle. J Clin Endocrinol Metab 1996;81:1401-1405.
33. van Disseldorp J, Faddy MJ, Themmen AP, de Jong FH, Peeters PH, van der Schouw YT, Broekmans FJ. Relationship of serum antimullerian hormone concentration to age at menopause. J Clin Endocrinol Metab 2008;93:2129-2134.
34. Broekmans FJ, Visser JA, Laven JS, Broer SL, Themmen AP, Fauser BC. Anti-Mullerian hormone and ovarian dysfunction. Trends Endocrinol Metab 2008;19:340-347.
35. Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with histologically determined primordial follicle number. Fertil Steril 2010;95:170-175.
36. Dolmans MM, Donnez J, Camboni A, Demylle D, Amorim C, Van LA, Pirard C. IVF outcome in patients with orthotopically transplanted ovarian tissue. Hum Reprod 2009;24:2778-2787.
37. van Eyck AS, Jordan BF, Gallez B, Heilier JF, van Langendonckt A, Donnez J. Electron paramagnetic resonance as a tool to evaluate human ovarian tissue reoxygenation after xenografting. Fertil Steril 2009;92:374-381.
38. Camboni A, Martinez-Madrid B, Dolmans MM, Nottola S, Van Langendonckt A, Donnez J. Autotransplantation of frozen-thawed ovarian tissue in a young woman: ultrastructure and viability of grafted tissue. Fertil Steril 2008;90:1215-1218.
39. Nisolle M, Casanas-Roux F, Qu J, Motta P, Donnez J. Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. Fertil Steril 2000;74:122-129.





Chapter 8

General discussion

The Dutch POI Consortium

In 2004, the Dutch primary ovarian insufficiency (POI) consortium was established with the following aims: (i) to further describe the phenotype of women affected by POI, (ii) to extend knowledge concerning biochemical and endocrine characteristics (including AMH and testosterone), (iii) to unravel the genetic components of POI, (iv) to assess general health risks, and (v) to initiate proper longitudinal follow-up of women with POI. For these purposes, clinical, biochemical and DNA data are collected in a systematic and standardized way. Together, the 14 participating centers have currently included 530 women with POI (February 2012), and the Dutch POI consortium represents one of the largest cohorts of women with POI worldwide. This work presents results from the ongoing research activities based on the national POI collaboration.

From phenotype to genotype

Although POI is nowadays regarded as a complex genetic trait, researchers worldwide have also focused on the possibility that specific single gene mutations could explain considerate proportions of women with POI, especially in familial cases. One of these candidate genes is *NR5A1*, coding for steroidogenic factor 1. In Chapter 3, we sought to evaluate previous findings of a high mutation frequency of *NR5A1* in women with POI (1) by sequencing the entire coding region of *NR5A1*. We found that the overall mutation rate was only 1.4% and *NR5A1* is therefore not considered a major contributor to the phenotype of POI. Similarly, *FMR1* premutations and intermediate-sized CGG repeat alleles were previously found to contribute to POI in up to 13% of all women affected by POI (2). In contrast, we found that only 1.7% of women in our POI cohort were *FMR1* premutation carriers (Chapter 4). Moreover, measures of POI severity did not significantly depend on CGG repeat numbers, which emphasizes that normal and intermediate *FMR1* CGG repeat sizes do not seem to play a central role in POI.

While the candidate-gene approach has lead to the identification of numerous monogenic causes of POI, generally, these mutations explain only a small minority of POI etiology. A summary of these monogenic causes of POI is described in *Table 1*. The results of the candidate gene studies in this thesis emphasize the fact that candidate genes need to be fully sequenced to identify the true incidence and the exact genetic changes with the phenotype of POI. A prerequisite for performing candidate gene studies is a homogeneous, unselected population. By its nature, POI, defined by the arbitrary cut-off age of 40 years and FSH of 40 IU/L, is a heterogeneous condition. Standardized phenotyping and inclusion of all consecutive women visiting an outpatient clinic with POI, such as is protocol in the Dutch POI Consortium, are the only ways to create a reliable, unbiased cohort for candidate gene studies.

Another pitfall of candidate gene studies is that it concerns hypothesis-based testing, and genetic studies are performed in coding regions only. In contrast, complex genetic diseases probably arise from interplay of gene expression modulating variants, regulatory sequences and locus heterogeneity of coding alleles (3). In recent years, such nonhypothesis-based genetic studies (so-called genome-wide association studies (GWAS)) focused on disease-associated SNP and CNV detection, which was perceived as a very promising technique to elucidate genetic pathways. Through this common disease - common variant approach, important new genes and non-coding alleles involved in gene expression modulation have been identified in many diseases. Also in GWAS, it is of utmost importance to include a well-phenotyped, homogeneous population. In this light, it was necessary to identify whether a distinction should be made between familial POI (incidence of 29%) and sporadic POI, because a distinct phenotype could point at a different genetic make-up or background. We were able to demonstrate that women with familial POI share a similar proportion of genetic abnormalities and have no distinctive clinical presentation than women with sporadic POI (Chapter 2).

However, while a few GWAS have been performed in POI, results were rather disappointing, because suggestive loci could not always be replicated in validation cohorts (4,5). A major problem in relatively infrequent diseases such as POI is the creation of a large enough cohort. The Dutch POI consortium has now included over 500 women, but continuous efforts are needed to gather the minimum of 2,000 to 3,000 patients to gain sufficient power. Therefore, it is an absolute necessity to seek international collaboration to establish a large enough cohort.

Finally, it has become clear that only a small fraction of the heritability was identified through GWAS in many other complex genetic traits. This phenomenon is referred to as missing heritability (6). The issue of missing heritability suggests that, also in POI, we should not only focus on the common disease - common variant approach, but acknowledge that rare variants may underlie POI instead. The newly available whole genome sequencing (or exome sequencing) techniques are aimed at the identification of rare variants, but they also require large cohorts of patients. However, if combined with a linkage analysis approach, whole genome sequencing may offer a fruitful possibility to identify those rare variants in an effective manner (7). A start could be made with X chromosome sequencing in strong POI families, because at least two so-called “critical regions” and many candidate genes were previously identified on the X chromosome (8,9).

Table 1. Summary of monogenic causes of POI

Human gene	Full gene name	Cytogenetic location	Syndrome (OMIM)	Reported incidence in POI	Reference
GPR3	G protein-coupled receptor 3	1p36.1-p35	none	rare	(26)
LHX8	LIM homeobox 8	1p31.1	none	rare	(27)
HSD3B2	Hydroxy-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 2	1p13.1	none	unknown	(28)
LMNA	Lamin A/C	1q22	Malouf syndrome (212112)	rare	(29)
LHR	LH receptor	2p21	none	<1%	(30)
FSHR	FSH receptor	2p21-p16	none	<1%	(31)
FIGLA	Folliculogenesis specific basic helix-loop-helix	2p13.3	none	2%	(32)
EIF5B	Eukaryotic translation initiation factor 5B	2q11.2	none	rare	(33)
INHA	Inhibin alpha	2q33-q36	none	0-7%	(34,35)
FOXL2	Forkhead box L2	3q23	Blepharophimosis ptosis epicanthus inversus syndrome (110100)	2.8%	(36)
TP73L	Tumor protein p63	3q27	Rapp-Hodgkin syndrome (129400)	rare	(37)
BMPR1B	Bone morphogenetic protein receptor 1B	4q23-24	Demirhan syndrome (609441)	rare	(38)
ADAMTS19	ADAM metallopeptidase with thrombospondin type 1 motif, 19	5q31	none	Polymorphism (not mutation)	(4)
SIL1	S. Cerevisa homolog 1	5q31	Marinesco-Sjogren Syndrome (248800)	rare	(39)
GDF9	Growth-differentiation factor 9	5q31.1	none	1.6%	(40)
MSH5	MutS homolog 5	6p21.3	none	4.9%	(41)
FOXO3A	Forkhead box O3A	6q21	none	2.2%	(42)

Table 1. Continued I

Human gene	Full gene name	Cytogenetic location	Syndrome (OMIM)	Reported incidence in POI	Reference
ESR1	Estrogen receptor α	6q25.1	none	Polymorphism (not mutation)	(43)
NOBOX	Newborn ovary homeobox	7q35	none	0-1%	(44,45)
WRN	RecQ protein-like 2	8p12-p11.2	Werner syndrome (277700)	unknown	(46)
STAR	steroidogenic acute regulatory protein	8p11.2	Lipoid congenital adrenal hyperplasia (600617)	unknown	(47)
GALT	galactose-1-phosphate uridyltransferase	9p13	Galactosemia (230400)	unknown	(48)
NR5A1	Nuclear receptor subfamily 5, group A, member 1	9q33	none	1.4-8%	(1,49)
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	10q24.3	Congenital adrenal hyperplasia due to 17α-OH deficiency (202110)	unknown	(50)
FSHB	FSH β subunit	11p13	none	rare	(51)
ATM	Ataxia telangiectasia mutated	11q22.3	Ataxia telangiectasia (208900)	unknown	(52)
FOXO1A	Forkhead box O1A	13q14.1	none	1.1%	(42)
EIF2B2	Eukaryotic translation initiation factor 2B, subunit 2	14q24	Ovarian leukodystrophy (603896)	unknown	(33)
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	15q21.1	Aromatase deficiency (107910)	rare	(53)
POLG	DNA polymerase gamma	15q25	Progressive external ophtalmoplegia with mitochondrial DNA deletions (157640)	unknown	(54,55)
BLM	DNA helicase RecQ protein-like-3	15q26.1	Bloom syndrome (210900)	unknown	(46)
PMM2	Phosphomanno-mutase 2	16p13.3-13.2	Congenital disorder of glycosylation, type 1A (212065)	unknown	(56)
FA	Fanconi anemia complentation groups	16q24.3	Fanconi anemia (227650)	rare	(57)

Table 1. Continued II

Human gene	Full gene name	Cytogenetic location	Syndrome (OMIM)	Reported incidence in POI	Reference
Ybx2	Y box binding protein 2	17p11.2-13.1	none	unknown	(58)
NOG	Noggin	17q22	none	1%	(59)
NANOS3	Nanos homolog 3	19p13.12	none	rare	(60)
LHB	LH beta polypeptide	19q13.33	none	rare	(61)
AIRE	Autoimmune regulator	21q22.3	Autoimmune polyendocrine syndrome 1 (240300)	rare	(62)
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination	22q13.1	none	2.4%	(41)
BMP15	Bone morphogenic protein 15	Xp11.2	none	1.5-12%	(63,64)
AR	Androgen receptor	Xq11-12	none	4.5%	(65)
XIST	X-inactivation gene	Xq13.2	Familial skewed X inactivation (300087)	unknown	(66)
POF1B	Premature ovarian failure, 1B gene	Xq21.2	none	unknown	(67)
DACH2	Homolog of drosophilia dachshund gene	Xq21.2	none	2.7%	(68)
DIAPH2	Diaphanous homolog 2	Xq21.33	none	unknown	(69)
PGRMC1	Progesterone receptor membrane component 1	Xq22-q24	none	1.5%	(70)
BHLHB9	Basic helix-loop-helix domain containing, class B, 9	Xq25	none	unknown	(71)
FMR1	Fragile-X mental retardation gene 1	Xq27.3	Fragile-X syndrome (309550)	0.8-13%	(2)
FRAXE (FMR2)	Fragile-X mental retardation gene 2	Xq28	X-linked mental retardation (309548)	1.5%	(72)

Adapted from Persani et al. 2010 (73), de Vos et al. 2010 (24), and Simpson 2008 (74).
When multiple gene names exist, ENTREZ gene name was chosen (available at http://www.ncbi.nlm.nih.gov/gene).
OMIM, online medelian inheritance in man.

Androgens in POI

The premature loss of ovarian function in POI results in a wide spectrum of women's health issues, as was described in Chapter 1. While hypoestrogenemia in women with POI is well established and the associated effects, such as climacteric symptoms and osteoporosis, have been fairly well documented, the role of androgens and the effects on health remain to be determined. Circulating androgen concentrations decrease with increasing age (10). Whether the ovarian theca cells remain functional for androgen production after menopause is topic of extensive debate. Conflicting evidence on hypoandrogenism in postmenopausal women points either to a gradual decrease in both ovarian and adrenal production of androgens with increasing age (11), or to a further, more abrupt decrease of only ovarian androgen production during menopausal transition (12,13). Since up until now these issues have not been tackled in women with menopause at a regular age, research regarding androgen concentrations in women with POI is certainly challenging. In Chapter 5, a systematic review and meta-analysis identified that women with POI are indeed at risk for decreased total testosterone concentrations, both compared with regularly cycling controls and with women experiencing menopause at a regular age. The question remains why these androgen concentrations are lower in women with POI. One may speculate that POI should be regarded as a distinct phenotype from normal menopause, and that perhaps ovarian androgen production decreases as a result of ovarian theca cell disruption due to autoimmune causes, or as a consequence of vascular damage to the ovarian vessels.

A complicating factor in this matter is that androgen measurement in female samples is problematic due to trueness and precision problems (14). We identified that, compared with a labor-intensive extraction radioimmunoassay, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a reliable, fast alternative for measuring steroid sex hormones in women (Chapter 6). However, because LC-MS/MS techniques are mostly in-house developed methods, external validation is urgently needed. Fortunately, international efforts are made to create programs in which individual laboratories participate through a biphasic investigation of calibration and method bias assessment (15). The standardization of androgen assays will allow for the identification of reference standards for women of all ages, which are necessary to come to a final conclusion regarding androgen production in women with POI. We propose to measure androgens with a reliable assay in women with POI, age-matched female controls with a regular cycle, and in postmenopausal controls that should be matched for duration of amenorrhea. This will provide a definite answer to the question whether women with POI are truly in a hypoandrogenic state.

Managing expectations for fertility preservation strategies

Women about to undergo gonadotoxic treatment are increasingly seeking medical help to achieve fertility preservation. Up until now, the only reliable method is the cryopreservation of embryos, but ovarian hyperstimulation may not always be an option. Although considered experimental, approximately 15 children have now been born following ovarian cryopreservation and subsequent autotransplantation (16-18). The question remains whether this technique is sufficiently successful (with a possible publication bias towards successful reimplantations) and safe to offer to women about to undergo gonadotoxic treatment. The possibility of reintroducing malignant cells seems realistic in some cancer types, especially in hematologic cancers such as leukemia (19). Furthermore, we identified that ovarian reserve parameters such as AMH and inhibin B, are very low following this procedure, which indicates a low ovarian reserve status, probably explaining the poor pregnancy rates (Chapter 7). Therefore, we believe that other fertility preservation options, such as oocyte vitrification, may prove to be more successful in the long term. Similar to embryo cryopreservation, ovarian hyperstimulation is necessary for oocyte vitrification. Current studies estimate that approximately 20 vitrified oocytes are needed to achieve one pregnancy (20,21), and the success rates are quickly approaching standard IVF techniques using fresh oocytes. Besides women who are about to undergo gonadotoxic treatment, oocyte vitrification should also be offered to women who have a high chance of developing POI because of a familial tendency and when embryo freezing is not an option. The biggest challenge in this regard is to define which women are particularly at risk and when to proceed to fertility preservation, because neither ovarian reserve tests nor genetic tests are available for long-term prediction of fecundity yet. If we succeed to define and reliably predict women at high chance of developing POI, we should aim at reaching out to these young women before POI sets in.

Implications for clinical practice

POI etiology remains unknown in the majority of cases. While some of the studies in this thesis were aimed at unraveling genetic components of the disease (Chapters 2-4), this has not lead to the identification of major genetic contributors. Current Dutch guidelines (NVOG) advise the following diagnostic tests to be performed in newly diagnosed women with POI: karyotyping and *FMR1* premutation screening, and screening for autoantibodies against adrenal and thyroid glands. Indeed, cytogenetic abnormalities were identified in a considerable proportion in women included in the Dutch POI consortium (see Chapter 1, unpublished data). However, *FMR1* premutation screening should only be offered because of

the possibility to expand to a full mutation in their offspring and when a familial tendency of POI is suspected (case finding). Positive autoantibody titers against thyroid or adrenal glands may lead to future gland dysfunction with serious health implications, therefore testing for these autoantibodies is indicated (22,23).
While clinicians should be aware that women with POI are susceptible to sexual dysfunction and diminished emotional well-being, it remains to be elucidated whether these health effects are due to hypoandrogenemia. Current routine testosterone immunoassays are notoriously inaccurate and not sensitive enough to detect low testosterone concentrations in women, therefore clinicians should refrain from testosterone measurement. Consequently, there currently is no evidence to support androgen therapy in women with POI.
Furthermore, while not topic of the current thesis, clinicians should discuss the possible long-term health implications of POI, including potential risks for dyslipidemia and cardiovascular disease, osteoporosis, and impaired cognition (24).
Finally, women at high risk for POI development due to gonadotoxic treatment or a strong familial tendency should be counseled about the possibilities for fertility preservation and should be adequately informed about the possible benefits and disadvantages of all available options. To increase awareness and to decrease involuntary childlessness among women about to undergo menopause-inducing treatment, health care providers should be encouraged to provide information about the possibilities and arrange referral to reproductive specialists in an early stage (25). Most importantly, we advocate that clinicians from all over the field take on a central role at educating young women and their (future) partners about the age-related decline of ovarian reserve. This becomes increasingly important because couples are still postponing to start a family, and are frequently unaware that even artificial reproductive techniques may fail when ovarian reserve is diminished.

Proposed future research

The existing Dutch POI consortium offers a great opportunity to further explore pathophysiological mechanisms and endocrine characteristics of women with POI, such as the future genetic studies and androgen measurement proposals as stated above. The development of a predictive test for the development of POI would be an important endpoint, although important ethical issues need to be addressed in this perspective.
Furthermore, the extensive cross-sectional set-up provides a fertile background against which longitudinal, prospective research projects can be initiated. Such studies will enable us to identify which health risks actually are parts of the POI phenotype and what the developmental trajectories of such health effects are.

Moreover, future research should aim at clarification of the potentially protective roles or adverse effects of both endogenous and exogenous estrogens and/or androgens in bone density, cardiovascular health, and well-being issues.
And finally, through all these proposed studies, we may be able to answer the fundamental question: Should POI be seen as a continuum of normal menopause?

References

1. Lourenço D, Brauner R, Lin L, De Perdigo A, Weryha G, Muresan M, Boudjenah R, Guerra-Junior G, Maciel-Guerra AT, Achermann JC, McElreavey K, Bashamboo A. Mutations in *NR5A1* associated with ovarian insufficiency. N Engl J Med 2009;360:1200-1210.
2. Wittenberger MD, Hagerman RJ, Sherman SL, Conkie-Rosell A, Welt CK, Rebar RW, Corrigan EC, Simpson JL, Nelson LM. The *FMR1* premutation and reproduction. Fertil Steril 2007;87:456-465.
3. Cordell HJ, Clayton DG. Genetic association studies. Lancet 2005;366:1121-1131.
4. Knauff EA, Franke L, van Es MA, van den Berg LH, van der Schouw YT, Laven JS, Lambalk CB, Hoek A, Goverde AJ, Christin-Maitre S, Hsueh AJ, Wijmenga C, Fauser BC. Genome-wide association study in premature ovarian failure patients suggests *ADAMTS19* as a possible candidate gene. Hum Reprod 2009;24:2372-2378.
5. Qin Y, Zhao H, Xu J, Shi Y, Li Z, Qiao J, Liu J, Qin C, Ren C, Li J, Chen S, Cao Y, Simpson JL, Chen ZJ. Association of 8q22.3 locus in Chinese Han with idiopathic premature ovarian failure (POF). Hum Mol Genet 2012;21:430-436.
6. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, McCarthy MI, Ramos EM, Cardon LR, Chakravarti A, Cho JH, Guttmacher AE, Kong A, Kruglyak L, Mardis E, Rotimi CN, Slatkin M, Valle D, Whittemore AS, Boehnke M, Clark AG, Eichler EE, Gibson G, Haines JL, Mackay TF, McCarroll SA, Visscher PM. Finding the missing heritability of complex diseases. Nature 2009;461:747-753.
7. Bailey-Wilson JE, Wilson AF. Linkage analysis in the next-generation sequencing era. Hum Hered 2011;72:228-236.
8. Krauss CM, Turksoy RN, Atkins L, McLaughlin C, Brown LG, Page DC. Familial premature ovarian failure due to an interstitial deletion of the long arm of the X chromosome. N Engl J Med 1987;317:125-131.
9. Powell CM, Taggart RT, Drumheller TC, Wangsa D, Qian C, Nelson LM, White BJ. Molecular and cytogenetic studies of an X;autosome translocation in a patient with premature ovarian failure and review of the literature. Am J Med Genet 1994;52:19-26.
10. Haring R, Hannemann A, John U, Radke D, Nauck M, Wallaschofski H, Owen L, Adaway J, Keevil BG, Brabant G. Age-Specific Reference Ranges for Serum Testosterone and Androstenedione Concentrations in Women Measured by Liquid Chromatography-Tandem Mass Spectrometry. Journal of Clinical Endocrinology & Metabolism 2011; epub doi: 10.1210/jc.2011-213.
11. Davison SL, Bell R, Donath S, Montalto JG, Davis SR. Androgen Levels in Adult Females: Changes with Age, Menopause, and Oophorectomy. J Clin Endocrinol Metab 2005;90:3847-3853.
12. Rannevik G, Jeppsson S, Johnell O, Bjerre B, Laurell-Borulf Y, Svanberg L. A longitudinal study of the perimenopausal transition: altered profiles of steroid and pituitary hormones, SHBG and bone mineral density. Maturitas 1995;21:103-113.

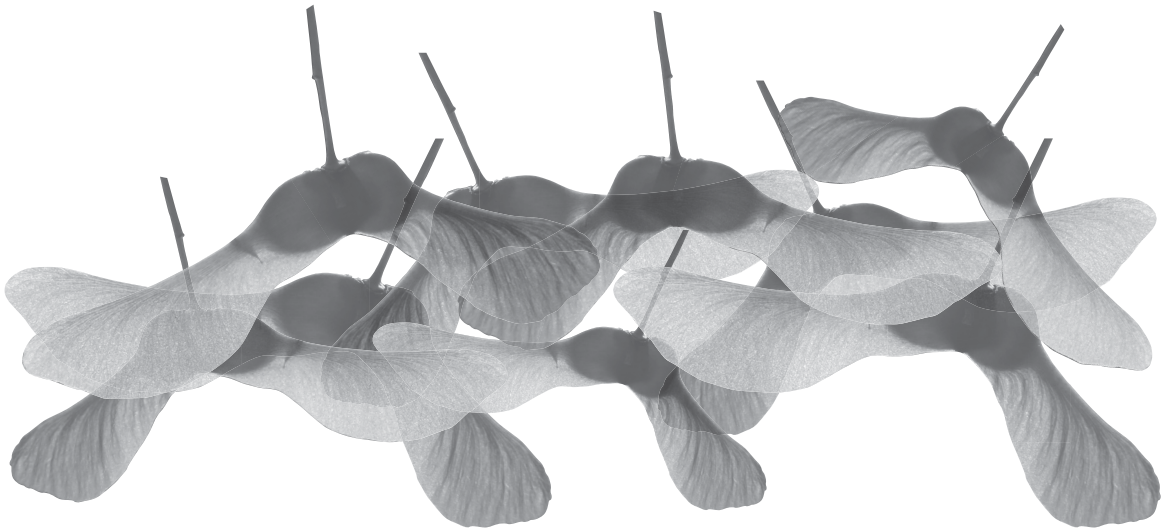
13. Couzinet B, Meduri G, Lecce MG, Young J, Brailly S, Loosfelt H, Milgrom E, Schaison G. The Postmenopausal Ovary Is Not a Major Androgen-Producing Gland. J Clin Endocrinol Metab 2001;86:5060-5066.
14. Herold DA, Fitzgerald RL. Immunoassays for testosterone in women: better than a guess? Clin Chem 2003;49:1250-1251.
15. Rosner W, Vesper H. Toward excellence in testosterone testing: a consensus statement. J Clin Endocrinol Metab 2010;95:4542-4548.
16. Silber S, Kagawa N, Kuwayama M, Gosden R. Duration of fertility after fresh and frozen ovary transplantation. Fertil Steril 2010;94:2191-2196.
17. Donnez J, Silber S, Andersen CY, Demeestere I, Piver P, Meirow D, Pellicer A, Dolmans MM. Children born after autotransplantation of cryopreserved ovarian tissue. a review of 13 live births. Ann Med 2011;43:437-450.
18. Revel A, Laufer N, Ben MA, Lebovich M, Mitrani E. Micro-organ ovarian transplantation enables pregnancy: a case report. Hum Reprod 2011;26:1097-1103.
19. Dolmans MM, Marinescu C, Saussoy P, van Langendonckt A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. Blood 2010;116:2908-2914.
20. Smith GD, Serafini PC, Fioravanti J, Yadid I, Coslovsky M, Hassun P, Alegretti JR, Motta EL. Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. Fertil Steril 2010;94:2088-2095.
21. van Landuyt L, Stoop D, Verheyen G, Verpoest W, Camus M, van de Velde H, Devroey P, van den Abbeel E. Outcome of closed blastocyst vitrification in relation to blastocyst quality: evaluation of 759 warming cycles in a single-embryo transfer policy. Hum Reprod 2011;26:527-534.
22. Kim TJ, Anasti JN, Flack MR, Kimzey LM, Defensor RA, Nelson LM. Routine endocrine screening for patients with karyotypically normal spontaneous premature ovarian failure. Obstet Gynecol 1997;89:777-779.
23. Coco G, Dal PC, Presotto F, Albergoni MP, Canova C, Pedini B, Zanchetta R, Chen S, Furmaniak J, Rees SB, Mantero F, Betterle C. Estimated risk for developing autoimmune Addison's disease in patients with adrenal cortex autoantibodies. J Clin Endocrinol Metab 2006;91:1637-1645.
24. de Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. Lancet 2010;376:911-921.
25. Quinn GP, Vadaparampil ST, Lee JH, Jacobsen PB, Bepler G, Lancaster J, Keefe DL, Albrecht TL. Physician referral for fertility preservation in oncology patients: a national study of practice behaviors. J Clin Oncol 2009;27:5952-5957.
26. Kovanci E, Simpson JL, Amato P, Rohozinski J, Heard MJ, Bishop CE, Carson SA. Oocyte-specific G-protein-coupled receptor 3 (GPR3): no perturbations found in 82 women with premature ovarian failure (first report). Fertil Steril 2008;90:1269-1271.
27. Qin Y, Zhao H, Kovanci E, Simpson JL, Chen ZJ, Rajkovic A. Analysis of LHX8 mutation in premature ovarian failure. Fertil Steril 2008;89:1012-1014.

28. Arif S, Underhill JA, Donaldson P, Conway GS, Peakman M. Human leukocyte antigen-DQB1* genotypes encoding aspartate at position 57 are associated with 3beta-hydroxysteroid dehydrogenase autoimmunity in premature ovarian failure. J Clin Endocrinol Metab 1999;84:1056-1060.
29. McPherson E, Turner L, Zador I, Reynolds K, Macgregor D, Giampietro PF. Ovarian failure and dilated cardiomyopathy due to a novel lamin mutation. Am J Med Genet A 2009;149A:567-572.
30. Latronico AC, Chai Y, Arnhold IJ, Liu X, Mendonca BB, Segaloff DL. A homozygous microdeletion in helix 7 of the luteinizing hormone receptor associated with familial testicular and ovarian resistance is due to both decreased cell surface expression and impaired effector activation by the cell surface receptor. Mol Endocrinol 1998;12:442-450.
31. Aittomaki K, Herva R, Stenman UH, Juntunen K, Ylostalo P, Hovatta O, de la Chapelle A. Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. J Clin Endocrinol Metab 1996;81:3722-3726.
32. Zhao H, Chen ZJ, Qin Y, Shi Y, Wang S, Choi Y, Simpson JL, Rajkovic A. Transcription factor FIGLA is mutated in patients with premature ovarian failure. Am J Hum Genet 2008;82:1342-1348.
33. Fogli A, Rodriguez D, Eymard-Pierre E, Bouhour F, Labauge P, Meaney BF, Zeesman S, Kaneski CR, Schiffmann R, Boespflug-Tanguy O. Ovarian failure related to eukaryotic initiation factor 2B mutations. Am J Hum Genet 2003;72:1544-1550.
34. Shelling AN, Burton KA, Chand AL, van Ee CC, France JT, Farquhar CM, Milsom SR, Love DR, Gersak K, Aittomaki K, Winship IM. Inhibin: a candidate gene for premature ovarian failure. Hum Reprod 2000;15:2644-2649.
35. Sundblad V, Chiauzzi VA, Andreone L, Campo S, Charreau EH, Dain L. Controversial role of inhibin alpha-subunit gene in the aetiology of premature ovarian failure. Hum Reprod 2006;21:1154-1160.
36. Harris SE, Chand AL, Winship IM, Gersak K, Aittomaki K, Shelling AN. Identification of novel mutations in *FOXL2* associated with premature ovarian failure. Mol Hum Reprod 2002;8:729-733.
37. Holder-Espinasse M, Martin-Coignard D, Escande F, Manouvrier-Hanu S. A new mutation in TP63 is associated with age-related pathology. Eur J Hum Genet 2007;15:1115-1120.
38. Demirhan O, Turkmen S, Schwabe GC, Soyupak S, Akgul E, Tastemir D, Karahan D, Mundlos S, Lehmann K. A homozygous BMPR1B mutation causes a new subtype of acromesomelic chondrodysplasia with genital anomalies. J Med Genet 2005;42:314-317.
39. Anttonen AK, Mahjneh I, Hamalainen RH, Lagier-Tourenne C, Kopra O, Waris L, Anttonen M, Joensuu T, Kalimo H, Paetau A, Tranebjaerg L, Chaigne D, Koenig M, Eeg-Olofsson O, Udd B, Somer M, Somer H, Lehesjoki AE. The gene disrupted in Marinesco-Sjogren syndrome encodes SIL1, an HSPA5 cochaperone. Nat Genet 2005;37:1309-1311.
40. Kovanci E, Rohozinski J, Simpson JL, Heard MJ, Bishop CE, Carson SA. Growth differentiating factor-9 mutations may be associated with premature ovarian failure. Fertil Steril 2007;87:143-146.

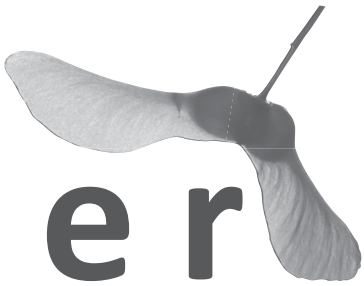
41. Mandon-Pepin B, Touraine P, Kuttenn F, Derbois C, Rouxel A, Matsuda F, Nicolas A, Cotinot C, Fellous M. Genetic investigation of four meiotic genes in women with premature ovarian failure. Eur J Endocrinol 2008;158:107-115.
42. Watkins WJ, Umbers AJ, Woad KJ, Harris SE, Winship IM, Gersak K, Shelling AN. Mutational screening of FOXO3A and FOXO1A in women with premature ovarian failure. Fertil Steril 2006;86:1518-1521.
43. Yoon SH, Choi YM, Hong MA, Lee GH, Kim JJ, Im HJ, Min EG, Kang BM, Yoon BK, Moon SY. Estrogen receptor {alpha} gene polymorphisms in patients with idiopathic premature ovarian failure. Hum Reprod 2010;25:283-287.
44. Zhao XX, Suzumori N, Yamaguchi M, Suzumori K. Mutational analysis of the homeobox region of the human *NOBOX* gene in Japanese women who exhibit premature ovarian failure. Fertil Steril 2005;83:1843-1844.
45. Qin Y, Choi Y, Zhao H, Simpson JL, Chen ZJ, Rajkovic A. *NOBOX* homeobox mutation causes premature ovarian failure. Am J Hum Genet 2007;81:576-581.
46. Du X, Shen J, Kugan N, Furth EE, Lombard DB, Cheung C, Pak S, Luo G, Pignolo RJ, DePinho RA, Guarente L, Johnson FB. Telomere shortening exposes functions for the mouse Werner and Bloom syndrome genes. Mol Cell Biol 2004;24:8437-8446.
47. Nakae J, Tajima T, Sugawara T, Arakane F, Hanaki K, Hotsubo T, Igarashi N, Igarashi Y, Ishii T, Koda N, Kondo T, Kohno H, Nakagawa Y, Tachibana K, Takeshima Y, Tsubouchi K, Strauss JF, III, Fujieda K. Analysis of the steroidogenic acute regulatory protein (StAR) gene in Japanese patients with congenital lipoid adrenal hyperplasia. Hum Mol Genet 1997;6:571-576.
48. Knauff EA, Richardus R, Eijkemans MJ, Broekmans FJ, de Jong FJ, Fauser BC, Bosch AM. Heterozygosity for the classical galactosemia mutation does not affect ovarian reserve and menopausal age. Reprod Sci 2007;14:780-785.
49. Janse F, de With LM, Duran KJ, Kloosterman WP, Goverde AJ, Lambalk CB, Laven JS, Fauser BC, Giltay JC. Limited contribution of *NR5A1* (SF-1) mutations in women with primary ovarian insufficiency (POI). Fertil Steril 2012;97:141-146.
50. Miura K, Yasuda K, Yanase T, Yamakita N, Sasano H, Nawata H, Inoue M, Fukaya T, Shizuta Y. Mutation of cytochrome P-45017 alpha gene (CYP17) in a Japanese patient previously reported as having glucocorticoid-responsive hyperaldosteronism: with a review of Japanese patients with mutations of CYP17. J Clin Endocrinol Metab 1996;81:3797-3801.
51. Matthews CH, Borgato S, Beck-Peccoz P, Adams M, Tone Y, Gambino G, Casagrande S, Tedeschini G, Benedetti A, Chatterjee VK. Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone. Nat Genet 1993;5:83-86.
52. Miller ME, Chatten J. Ovarian changes in ataxia telangiectasia. Acta Paediatr Scand 1967;56:559-561.
53. Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K. Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 1995;80:3689-3698.

54. Luoma P, Melberg A, Rinne JO, Kaukonen JA, Nupponen NN, Chalmers RM, Oldfors A, Rautakorpi I, Peltonen L, Majamaa K, Somer H, Suomalainen A. Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. Lancet 2004;364:875-882.
55. Pagnamenta AT, Taanman JW, Wilson CJ, Anderson NE, Marotta R, Duncan AJ, Bitner-Glindzicz M, Taylor RW, Laskowski A, Thorburn DR, Rahman S. Dominant inheritance of premature ovarian failure associated with mutant mitochondrial DNA polymerase gamma. Hum Reprod 2006;21:2467-2473.
56. Matthijs G, Schollen E, Pardon E, Veiga-Da-Cunha M, Jaeken J, Cassiman JJ, van Schaftingen E. Mutations in PMM2, a phosphomannomutase gene on chromosome 16p13, in carbohydrate-deficient glycoprotein type I syndrome (Jaeken syndrome). Nat Genet 1997;16:88-92.
57. Joenje H, Patel KJ. The emerging genetic and molecular basis of Fanconi anaemia. Nat Rev Genet 2001;2:446-457.
58. Yang J, Medvedev S, Yu J, Tang LC, Agno JE, Matzuk MM, Schultz RM, Hecht NB. Absence of the DNA-/RNA-binding protein MSY2 results in male and female infertility. Proc Natl Acad Sci U S A 2005;102:5755-5760.
59. Laissue P, Christin-Maitre S, Bouchard P, Fellous M, Veitia RA. Mutations in the NOG gene are not a common cause of nonsyndromic premature ovarian failure. Clin Endocrinol (Oxf) 2007;66:900.
60. Qin Y, Zhao H, Kovanci E, Simpson JL, Chen ZJ, Rajkovic A. Mutation analysis of NANOS3 in 80 Chinese and 88 Caucasian women with premature ovarian failure. Fertil Steril 2007;88:1465-1467.
61. Takahashi K, Ozaki T, Okada M, Kurioka H, Kanasaki H, Miyazaki K. Increased prevalence of luteinizing hormone beta-subunit variant in patients with premature ovarian failure. Fertil Steril 1999;71:96-101.
62. Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Horm Metab Res 1996;28:353-356.
63. Di Pasquale E, Beck-Peccoz P, Persani L. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (*BMP15*) gene. Am J Hum Genet 2004;75:106-111.
64. Rossetti R, Di Pasquale E, Marozzi A, Bione S, Toniolo D, Grammatico P, Nelson LM, Beck-Peccoz P, Persani L. *BMP15* mutations associated with primary ovarian insufficiency cause a defective production of bioactive protein. Hum Mutat 2009;30:804-810.
65. Panda B, Rao L, Tosh D, Dixit H, Padmalatha V, Kanakavalli M, Raseswari T, Deenadayal M, Gupta N, Chakrabarty B, Nallari P, Singh L. Germline study of AR gene of Indian women with ovarian failure. Gynecol Endocrinol 2011;27:572-578.
66. Yoon SH, Choi YM, Hong MA, Kang BM, Kim JJ, Min EG, Kim JG, Moon SY. X chromosome inactivation patterns in patients with idiopathic premature ovarian failure. Hum Reprod 2008;23:688-692.

67. Lacombe A, Lee H, Zahed L, Choucair M, Muller JM, Nelson SF, Salameh W, Vilain E. Disruption of POF1B binding to nonmuscle actin filaments is associated with premature ovarian failure. Am J Hum Genet 2006;79:113-119.
68. Bione S, Rizzolio F, Sala C, Ricotti R, Goegan M, Manzini MC, Battaglia R, Marozzi A, Vegetti W, Dalpra L, Crosignani PG, Ginelli E, Nappi R, Bernabini S, Bruni V, Torricelli F, Zuffardi O, Toniolo D. Mutation analysis of two candidate genes for premature ovarian failure, DACH2 and POF1B. Hum Reprod 2004;19:2759-2766.
69. Bione S, Sala C, Manzini C, Arrigo G, Zuffardi O, Banfi S, Borsani G, Jonveaux P, Philippe C, Zuccotti M, Ballabio A, Toniolo D. A human homologue of the Drosophila melanogaster diaphanous gene is disrupted in a patient with premature ovarian failure: evidence for conserved function in oogenesis and implications for human sterility. Am J Hum Genet 1998;62:533-541.
70. Mansouri MR, Schuster J, Badhai J, Stattin EL, Losel R, Wehling M, Carlsson B, Hovatta O, Karlstrom PO, Golovleva I, Toniolo D, Bione S, Peluso J, Dahl N. Alterations in the expression, structure and function of progesterone receptor membrane component-1 (PGRMC1) in premature ovarian failure. Hum Mol Genet 2008;17:3776-3783.
71. Prueitt RL, Ross JL, Zinn AR. Physical mapping of nine Xq translocation breakpoints and identification of XPNPEP2 as a premature ovarian failure candidate gene. Cytogenet Cell Genet 2000;89:44-50.
72. Murray A, Webb J, Dennis N, Conway G, Morton N. Microdeletions in FMR2 may be a significant cause of premature ovarian failure. J Med Genet 1999;36:767-770.
73. Persani L, Rossetti R, Cacciatore C. Genes involved in human premature ovarian failure. J Mol Endocrinol 2010;45:257-279.
74. Simpson JL. Genetic and phenotypic heterogeneity in ovarian failure: overview of selected candidate genes. Ann N Y Acad Sci 2008;1135:146-154.



Chapter 9



Summary

Nederlandse samenvatting

In 2004, the Dutch primary ovarian insufficiency (POI) consortium was established. This national collaboration is aimed at unraveling the genetic components of POI and to further describe the phenotype of women affected by POI. This thesis presents results from the ongoing research activities based on the national POI collaboration.

Chapter 1

In the introduction of this thesis, we describe the phenotype of primary ovarian insufficiency along with the varying definitions that are used to describe this phenomenon of amenorrhea and estradiol deficiency in association with postmenopausal follicle-stimulating hormone (FSH) levels in young women. Current knowledge regarding genetic and other etiologic factors of POI is reviewed, and data derived of the Dutch POI Consortium cohort is interpreted. Furthermore, we provide an extensive overview of the impact of this diagnosis on fertility and other short- and long-term general health aspects of POI.

Chapter 2

POI is regarded as a complex genetic disease in which multiple genetic variants and environmental factors contribute. A familial form of POI is present in a considerable proportion of women with POI. We assumed that if women with familial POI would represent a distinct phenotype regarding POI presentation and POI severity, their genetic make-up or background would differ from that in women without any family member with POI (sporadic POI). Based on a detailed 3-generation family history, all women who were consecutively included in the Dutch POI consortium were identified as having either familial (defined as having at least one relative with POI) (n = 58) or sporadic POI (n = 142).
We demonstrated that women with familial POI have no distinctive clinical presentation and share a similar proportion of genetic abnormalities compared with women with sporadic POI. The only exception was the lower age at menopause in mothers of women with familial POI, which we consider as a direct consequence of the definition used for familial POI. These findings imply that no distinction between the two phenotypes of POI is necessary when high-throughput sequencing methods using genomic variants are applied to identify genetic risk loci in POI.

Chapter 3

The nuclear receptor subfamily 5, group A, member 1 (*NR5A1*) gene, coding for steroidogenic factor 1, plays an important role in the pathogenesis of primary adrenal insufficiency and sexual development disorders, including male

infertility. Earlier research suggested that as much as 8% of women with POI carry mutations in the *NR5A1* gene. We undertook a study to evaluate the contribution of *NR5A1* mutations in women with POI, and to seek and describe any new *NR5A1* mutations associated with POI. In total, 386 well-phenotyped women with secondary amenorrhea due to POI, including 77 women with familial POI, were included. The entire coding region and splice sites of the *NR5A1* gene were PCR-amplified and sequenced. Thereafter pathogenicity of identified mutations was predicted in silico.

In total, 9 mutations were identified in 10 patients. Five of these mutations concerned novel nonconservative mutations occurring in 5 patients. Prediction of effect on protein function showed low to intermediate pathogenicity for all nonconservative mutations. The overall *NR5A1* gene mutation rate was only 1.4%. Therefore, there seems limited value in the inclusion of *NR5A1* mutation screening in the diagnostic workup of women affected by POI. POI remains unexplained in the great majority of patients and continued efforts are needed to elucidate its underlying genetic factors.

Chapter 4

The most frequent monogenic cause of POI is the premutation of the fragile-X mental retardation gene 1 (*FMR1*) with an incidence reported between 0.8% and 13%. Several small sample size studies investigated the role of intermediate CGG repeat size in POI. Some previous studies found an increased frequency of intermediate alleles in (occult) POI, but such findings could not be confirmed in a larger sample size study. We initiated a study to assess the incidence of normal, intermediate and premutation *FMR1* alleles in 144 well-phenotyped women with POI and 34 women with occult POI (defined as elevated FSH concentrations in women with regular or irregular menstrual cycles). In addition, the association of number of *FMR1* CGG repeats with POI severity was investigated. A PCR amplification of the CGG-repeat region of the *FMR1* gene was performed and subsequently, fragment length determined.

A total of 3 (2%) premutations were identified, which occurred only in women with overt POI. Overall, 6 (3%) intermediate alleles were found; 2 (6%) in occult POI and 4 (3%) in overt POI. No significant linear association between CGG repeat size and age at POI or FSH levels could be demonstrated (P = 0.93 and P = 0.09, respectively). Therefore, the evaluation of normal- and intermediate *FMR1* repeat size seems of limited value in the diagnostic work-up of women affected by POI, or in women at risk for developing POI. The biological pathway and the clinical relevance of intermediate *FMR1* CGG repeat alleles remain to be elucidated.

Chapter 5

Increasing age and postmenopausal status are associated with decreasing androgen concentrations in women. Women with premature loss of ovarian function - such as POI or iatrogenic menopause - may be at increased risk for diminished testosterone levels at a relatively young age. The meta-analysis presented in Chapter 5 was conducted to evaluate current literature reporting serum total testosterone concentrations under these conditions. The electronic databases of Pubmed, Embase and the Cochrane Library were systematically searched for comparative studies on total testosterone concentrations in women with spontaneous POI or iatrogenic menopause, compared to controls. Stratification for assay type was included in the analyses, because androgen assay problems such as lack of trueness, precision, and sensitivity are well known.
262 Articles for spontaneous POI and 1,358 for iatrogenic menopause were reviewed, of which 9 and 17 papers, respectively, were selected for final analysis. In POI and iatrogenic menopause total testosterone concentrations were significantly lower compared with controls (weighted mean difference (95% CI) -0.38 (-0.55 to -0.22) nmol/L, and -0.29 (-0.39 to -0.18) nmol/L, respectively), yet with substantial between-study heterogeneity. Subgroup analysis for assay type, comparing direct vs. indirect testosterone assays, was statistically significant in spontaneous POI only. In conclusion, total testosterone concentrations were demonstrated to be decreased in women with spontaneous POI or iatrogenic menopause. The potential implications of hypoandrogenism in these women remain to be elucidated.

Chapter 6

The measurement of serum testosterone in women is challenging due to lack of trueness, precision, and sensitivity of various available testosterone assays. Accurate assessment of testosterone in women is crucial especially in conditions associated with alleged over- or underproduction of testosterone, such as in polycystic ovary syndrome (PCOS) and POI, respectively. The aim of this chapter was to measure and compare androgen concentrations in women with PCOS (n = 200), POI (n = 208), and female controls (n = 45) and to evaluate the performance of extraction radioimmunoassay (RIA) and liquid chromatography- tandem mass spectrometry (LC-MS/MS) in these women. Androgen concentrations were measured using both extraction RIA and LC-MS/MS, and subsequently, a method comparison analysis was performed.
All androgen levels were significantly elevated in women with PCOS compared with POI patients ($P < 0.05$) and controls ($P < 0.05$). Women with POI presented with similar androgen concentrations as controls, except for androstenedione.

Compared to measurements by extraction RIA, LC-MS/MS measurements were systematically lower. However, both assay measurements were shown to have good agreement as assessed by Bland-Altman analysis and intraclass correlation coefficient. We concluded that LC-MS/MS, compared with a labor-intensive extraction RIA, shows good precision, sensitivity, and high accuracy for measuring female androgens concentrations under various clinical conditions. LC-MS/MS represents a convenient and reliable assay for both clinical and research purposes.

Chapter 7

Ovarian transplantation is one of the available options for fertility preservation in women about to undergo gonadotoxic treatment, but highly variable pregnancy rates have been reported. The objective of this study was to investigate markers of ovarian reserve and ovarian function in women after orthotopic ovarian transplantation. Ten women, who were about to receive or had previously received gonadotoxic treatment, were followed for a mean of 2.5 years. In seven, cryopreservation of ovarian tissue was performed before starting treatment, and, subsequently, autografts were orthotopically transplanted. Three women, who had already developed iatrogenic POI, underwent orthotopic transplantation of ovarian allograft tissue originating from their HLA-compatible sisters.
On average, first menses took place after 4.7 months. Duration of graft functioning varied from two to more than 60 months. FSH concentrations remained elevated and AMH was low to undetectable, whereas estradiol levels normalized. Inhibin B varied among women and was not associated with the duration of ovarian function (hazard ratio 0.966, 95% CI 0.881-1.059). Two spontaneous pregnancies occurred. Endocrine characteristics were not significantly different in these women. The identified low AMH and inhibin B concentrations are suggestive for decreased ovarian reserve in women after ovarian transplantation. AMH and inhibin B levels are probably not associated with the duration of ovarian graft function, or the probability to achieve a pregnancy.

Chapter 8

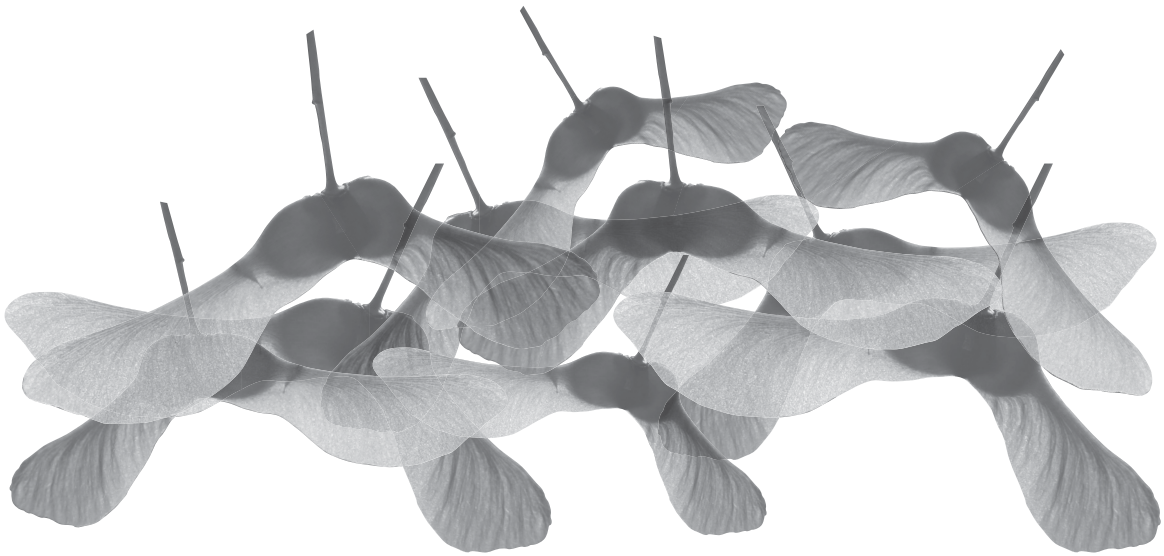
In the final chapter of this thesis, the conclusions that can be drawn from the research contributing to this thesis, and the implications for current practice and future research are discussed.
Disappointing results from candidate gene studies and GWAS point out that it remains crucial to create a very large, well-phenotyped, and unbiased cohort through international collaboration to gain sufficient power for studies aimed to identify POI pathophysiology. Candidate gene studies may only offer a valuable contribution to this search when the entire gene is sequenced and if performed

in well-phenotyped groups. Future genetic studies should focus on rare variants, and whole genome sequencing combined with linkage analysis may prove a promising new approach to identify etiologic factors in POI.

To further describe the endocrine implications of POI, it is necessary to have access to trustworthy, precise and sensitive assays for androgens, estrogens and other steroids. At the moment, the extent to which androgens are decreased and the possible implications for general health aspects in women with POI remain to be determined. Collaborative efforts for standardization of assays along with the development of reference values for women of all ages are essential to enable future research efforts in well-phenotyped women with POI.

Ovarian cryopreservation and subsequent reimplantation may not be a highly successful fertility preservation technique, because ovarian reserve is severely compromised. Oocyte vitrification seems a promising approach instead, which should be offered to women about to undergo gonadotoxic treatment or those at risk for developing POI due to a familial tendency.

Future research is proposed, in which both the cross-sectional Dutch POI consortium and a proposed prospective follow-up of these already included women will be of great value and enable us to study both short- and long term health effects of POI, and investigate potential risk-modifying factors such as hormone therapy.



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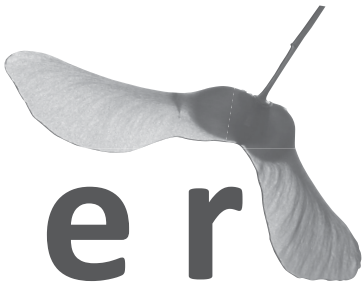


Summary

Nederlandse samenvatting

Primaire ovariële insufficiëntie (POI) kenmerkt zich door het uitblijven van menstruaties voor het 40e levensjaar gedurende tenminste vier maanden, met daarbij een verhoogde serum FSH-concentratie van meer dan 40 EH/L. Ongeveer één op de 100 vrouwen bereikt de menopauze voor het 40e levensjaar, en één op de 1.000 vrouwen ontwikkelt POI voordat zij 30 jaar is. POI leidt tot infertiliteit, gaat gepaard met karakteristieke overgangsklachten, en kan op korte en lange termijn leiden tot algemene gezondheidsproblemen bij de vrouw.
In 2004 is het Nederlandse POI consortium opgericht. Dit nationale onderzoeksverband is erop gericht om factoren die ten grondslag liggen aan POI, zoals genetische variaties, verder te ontrafelen. Daarnaast heeft het Nederlandse POI consortium het doel om het fenotype van vrouwen met POI beter te beschrijven. In dit proefschrift wordt een aantal studies beschreven die voortgekomen zijn uit de lopende activiteiten van het POI consortium.

Hoofdstuk 1

Hoofdstuk 1 vormt de introductie van dit proefschrift. POI wordt gekenmerkt door amenorroe en oestrogeendeficiëntie in combinatie met postmenopauzale FSH-concentraties bij jonge vrouwen. Dit hoofdstuk begint met een opsomming van de verschillende definities die worden gebruikt om dit klinische beeld te beschrijven. Daarna volgt een samenvatting van de huidige kennis ten aanzien van het ontstaan van POI, waarbij aandacht besteed wordt aan genetische factoren, iatrogene oorzaken, en auto-immuniteit. Het hoofdstuk wordt afgesloten met een uitgebreid overzicht van de gevolgen van de diagnose POI op fertiliteit, welzijn en de algemene gezondheid van de vrouw op zowel korte als lange termijn.

Hoofdstuk 2

Hoewel er veelvuldig wordt gezocht naar monogene oorzaken van POI en er een aantal belangrijke genen is ontdekt, blijken mutaties in (de meeste van) deze genen slechts in een klein gedeelte van alle vrouwen een directe verklaring te zijn voor het ontstaan van POI. Naast genen hebben ook omgevingsfactoren zoals roken invloed op de leeftijd waarop een vrouw de menopauze bereikt. Het is daarom aannemelijk dat POI gezien moet worden als het resultaat van een samenspel tussen multipele genetische varianten en omgevingsfactoren. Dit samenspel wordt een complex genetische ziekte genoemd. Verder presenteert POI zich in een aanzienlijk deel van de aangedane vrouwen in een familiaire vorm. Wij veronderstelden dat vrouwen met een familiaire vorm van POI mogelijk een ander fenotype zouden hebben dan vrouwen met sporadische POI (i.e. zonder familieleden met POI), bijvoorbeeld in de presentatie en leeftijd waarop POI wordt vastgesteld. Als deze gedachte juist zou zijn, zou dat kunnen betekenen dat vrouwen met familiaire POI een andere genetische achtergrond

of andere omgevingsfactoren hebben dan vrouwen met sporadische POI. Om dit te onderzoeken werden familiestambomen van drie generaties opgesteld van alle vrouwen met POI die zich bij een van de deelnemende centra van het POI consortium meldden. Op basis hiervan werd bepaald of een vrouw familiaire POI had (tenminste één familielid met POI) (n = 58), of dat er sprake was van sporadische POI (n = 142).
Dit onderzoek toonde aan dat de klinische presentatie van vrouwen met familiaire POI niet onderscheidend is ten opzichte van vrouwen met sporadische POI. Bovendien werden er evenveel genetische afwijkingen en andere etiologische factoren gevonden in beide groepen. Het enige significante verschil was het feit dat de moeders van vrouwen met familiaire POI op jongere leeftijd postmenopauzaal werden; dit verschil is echter terug te voeren op de door ons gestelde definitie van familiaire POI. Deze bevindingen steunen de gedachte dat er geen onderscheid gemaakt dient te worden tussen familiaire en sporadische POI fenotypes in genetische studies op basis van high-throughput sequencing technieken met genomische varianten om risicoloci voor POI te identificeren.

Hoofdstuk 3

Een eerdere studie toonde aan dat 2 van de 25 (8%) onderzochte vrouwen met POI een mutatie had in het *NR5A1* gen. Het nucleaire receptor subfamilie 5, groep A, lid 1 (*NR5A1*) gen codeert voor steroïdogene factor 1 en mutaties in dit gen spelen een belangrijke rol in de pathogenese van primaire bijnierinsufficiëntie en seksuele ontwikkelingsstoornissen, zoals mannelijke infertiliteit. Wij hebben een studie geïnitieerd om te onderzoeken wat het aandeel van *NR5A1* mutaties is in een grotere groep vrouwen met POI. Tevens had deze studie tot doel om eventuele nieuwe *NR5A1* mutaties te zoeken en verder te beschrijven. In totaal werden 386 goed gefenotypeerde vrouwen met POI geïncludeerd, onder wie 77 vrouwen met familiaire POI. Alle coderende regionen en de splice sites van het *NR5A1* gen werden middels PCR geamplificeerd en gesequenced. Daarna werd een voorspelling gemaakt van de pathogeniciteit van de gevonden mutaties.
In totaal werden 9 verschillende mutaties gevonden bij 10 patiënten. Vijf van deze mutaties waren nieuwe, nog niet eerder beschreven mutaties van het *NR5A1* gen, die invloed hadden op de eiwitstructuur (i.e. niet-conservatieve mutaties). Echter, voor al deze niet-conservatieve mutaties bleek dat het voorspelde effect op eiwitfunctie slechts tot lage of middelmatige pathogeniciteit leidde. Bovendien was de gemiddelde mutatiefrequentie van het *NR5A1* gen in dit grote cohort vrouwen slechts 1,4%. Het is daarom aannemelijk dat er weinig toegevoegde waarde is voor het verrichten van screening naar *NR5A1* genmutaties als onderdeel van de standaard diagnostiek van vrouwen met POI. De oorzaak van POI blijft onverklaard in de meerderheid van alle vrouwen met POI en nieuwe studies naar oorzakelijke (genetische) factoren blijven daarom noodzakelijk.

Hoofdstuk 4

De belangrijkste monogene oorzaak van POI betreft de premutatie van het fragiele X mentale retardatie gen 1 (*FMR1*). Deze premutatie komt voor bij 0,8% tot 13% van alle vrouwen met POI. Een aantal kleine studies heeft gekeken naar de rol van zogenaamde intermediaire *FMR1* allelen bij vrouwen met POI. Hoewel sommige studies een verhoogde frequentie van deze intermediaire *FMR1* allelen vond bij vrouwen met POI of met dreigende POI, konden deze bevindingen niet bevestigd worden in een veel grotere studie. In hoofdstuk 4 beschrijven wij onze studie naar het voorkomen van normale, intermediaire en premutatie *FMR1* allelen in 144 goed gefenotypeerde vrouwen met POI en in 34 vrouwen met dreigende POI. Dreigende POI werd gedefinieerd als een verhoogde serum FSH-concentratie bij vrouwen met een regelmatige of onregelmatige menstruatiecyclus. Daarnaast werd er gekeken naar de relatie tussen het aantal *FMR1* CGG repeats en de ernst van de POI, uitgedrukt als zowel de leeftijd waarop POI werd gediagnosticeerd alsook de hoogte van de FSH concentratie. De CGG-repeat regio van het *FMR1* gen werd middels PCR geamplificeerd en vervolgens werd de lengte van het fragment bepaald.
Er werden 3 (2%) premutaties gevonden in de totale onderzoeksgroep, welke alleen voorkwamen bij vrouwen met POI. In totaal werden 6 (3%) intermediaire allelen geïdentificeerd: 2 (6%) bij vrouwen met dreigende POI en 4 (3%) bij vrouwen met POI. Er kon geen significante lineaire associatie tussen CGG repeat lengte en ernst van de POI worden vastgesteld; P = 0,93 voor leeftijd waarop POI werd gediagnosticeerd en P = 0,09 voor FSH concentratie. Samenvattend lijkt er weinig toegevoegde waarde te zijn voor het onderzoeken van normale en intermediaire *FMR1* repeat lengtes bij vrouwen met POI, noch bij vrouwen die risico lopen op het ontwikkelen van POI. De biologische cascade en de klinische relevantie van intermediaire *FMR1* CGG repeat allelen moet nog verder onderzocht worden.

Hoofdstuk 5

Het wordt algemeen aangenomen dat androgeenconcentraties bij vrouwen dalen met het toenemen van de leeftijd en bij een postmenopauzale status. Het is mogelijk dat vrouwen die op zeer jonge leeftijd de menopauze bereiken, zoals vrouwen met POI of met iatrogene menopauze, risico hebben op lage testosteronconcentraties (hypoandrogenisme) op relatief jonge leeftijd. Hypoandrogenisme is mogelijk geassocieerd met verminderd welzijn, seksuele disfunctie, dislipidaemie en coronaire hartziekte. In hoofdstuk 5 wordt een systematische review en een meta-analyse beschreven van alle beschikbare literatuur over serum testosteronconcentraties bij vrouwen met POI en bij

vrouwen met iatrogene menopauze. De elektronische databases Pubmed, Embase en the Cochrane Libary werden systematisch doorzocht naar studies waarin een vergelijking werd gemaakt tussen testosteronconcentraties bij vrouwen met POI of met iatrogene menopauze ten opzichte van gezonde vrouwen (normale menopauzeleeftijd, of normale ovariële functie). Aangezien het duidelijk is dat androgeenbepalingen bij vrouwen ernstig gecompliceerd kunnen worden door problemen met de assay, zoals matige precisie en sensitiviteit, hebben we ook subgroepanalyses gedaan voor de verschillende assays die gebruikt werden in de studies die geïncludeerd werden in onze meta-analyse.
Er werden 262 artikelen gevonden voor spontane POI, waarvan er 9 werden geselecteerd voor de uiteindelijke analyse. Voor iatrogene menopauze werden 1.358 artikelen bestudeerd, waarvan uiteindelijk 17 artikelen geschikt bleken voor meta-analyse. Uit de meta-analyes kwam naar voren dat totaal testosteronconcentratie bij zowel vrouwen met spontane POI als vrouwen met iatrogene menopauze significant lager is dan bij controles (gewogen gemiddeld verschil (95% betrouwbaarheidsinterval) respectievelijk -0,38 (-0,55 tot -0,22) nmol/L en -0,29 (-0,39 tot -0,18) nmol/L). In de subgroepanalyses voor type testosteron assay werd alleen significantie bereikt voor de groep vrouwen met spontane POI. Wij concluderen dan ook dat totaal testosteronconcentraties bij vrouwen met spontane POI of met iatrogene menopauze verlaagd zijn. Voor een inschatting van de mogelijke implicaties van hypoandrogenisme bij deze vrouwen is verder onderzoek noodzakelijk.

Hoofdstuk 6

Omdat bestaande testosteronassays een gebrek aan precisie en sensitiviteit kennen voor de relatief lage waarden bij vrouwen, is het tot op heden een grote uitdaging gebleken om deze vrouwelijke serum testosteronspiegels betrouwbaar te meten. Het nauwkeurig en betrouwbaar meten van serum testosteronspiegels is echter van groot belang bij vrouwen waarbij een vermoeden van hyper- of hypoandrogenisme bestaat, zoals bij vrouwen met het polycysteuze ovarium syndroom (PCOS) of bij vrouwen met POI. Het onderzoek in hoofdstuk 6 had tot doel om androgeenconcentraties van vrouwen met PCOS (n = 200), POI (n = 208) en gezonde vrouwelijke controles (n = 45) te meten en onderling te vergelijken. Bovendien wilden we de prestaties van twee assays: extractie radioimmunoassay (RIA) en liquid chromatography- tandem mass spectrometry (LC-MS/MS), bij deze groepen vrouwen onderling vergelijken. De androgeenconcentraties werden met beide assays gemeten en vervolgens vergeleken middels een zogenaamde method comparison analyse.
Vrouwen met PCOS hadden significant hogere androgeenspiegels dan vrouwen met POI ($P < 0,05$) en controles ($P < 0,05$). Maar de androgeenspiegels van vrouwen

met POI waren vergelijkbaar met die van de controles, met uitzondering van androsteendion. Metingen die verricht werden met LC-MS/MS waren systematisch lager dan metingen die verricht waren met de extractie RIA. Echter, Bland-Altman analyse en de intraclass correlatiecoëfficiënt lieten een goede overeenkomst tussen beide methoden zien. Wij concludeerden daarom dat LC-MS/MS een goede precisie, sensitiviteit en accuratesse heeft voor metingen van vrouwelijke androgeenspiegels in uiteenlopende klinische omstandigheden. Vergeleken met de arbeidsintensieve extractie RIA is LC-MS/MS een gebruiksvriendelijke en betrouwbare assay voor zowel klinische als wetenschappelijke doeleinden.

Hoofdstuk 7

Wanneer een vrouw vervroegd in de overgang dreigt te raken, meestal als gevolg van iatrogene oorzaken, dan bestaat er een aantal mogelijkheden om de fertiliteit te bewaren. Een van deze opties is transplantatie van het ovarium, waarbij een deel van het ovarium gecryopreserveerd wordt voordat met de gonadotoxische therapie wordt begonnen. Na afloop van de iatrogene therapie en bij een vastgestelde POI, wordt het ontdooide ovariumweefsel teruggeplaatst. Hoewel het merendeel van de vrouwen bij wie een dergelijke procedure wordt gevolgd weer zal gaan menstrueren, is de variatie wat betreft zwangerschapskansen erg groot. Het doel van deze studie was het onderzoeken van de waarde van ovariële reserve testen voor het voorspellen van ovariële reserve en ovariële functie bij vrouwen die eerder een orthotopische ovariumtransplantatie hadden ondergaan. Hiertoe werden tien vrouwen gevolgd gedurende gemiddeld 2,5 jaar. Zeven van hen ondergingen de ovariumtransplantatie met eigen weefsel (autotransplantatie). Echter, drie vrouwen hadden al iatrogene POI ontwikkeld, en ondergingen daarom orthotopische transplantatie van ovarieel allograft weefsel afkomstig van hun HLA-compatibele zussen.
De eerste menstruatie trad gemiddeld 4,7 maanden na de transplantatie op. De levensduur van de graft varieerde echter tussen de 2 en meer dan 60 maanden. Hoewel oestradiol spiegels normaliseerden, bleef het FSH hoog en AMH varieerde van laag tot onmeetbaar laag. Inhibine B concentraties wisselden sterk tussen vrouwen en waren niet significant geassocieerd met levensduur van de graft (hazard ratio 0,966; 95% betrouwbaarheidsinterval 0,881-1,059). Twee vrouwen raakten spontaan zwanger, maar de endocriene eigenschappen van deze vrouwen waren niet significant anders dan van de vrouwen die niet zwanger raakten. De lage AMH en inhibine B concentraties zijn suggestief voor verminderde ovariële reserve bij deze vrouwen nadat zij ovariumtransplantaties ondergingen. AMH en inhibine B zijn waarschijnlijk niet geassocieerd met de levensduur van de ovarium graft, noch met de waarschijnlijkheid dat zwangerschap wordt bereikt.

Hoofdstuk 8

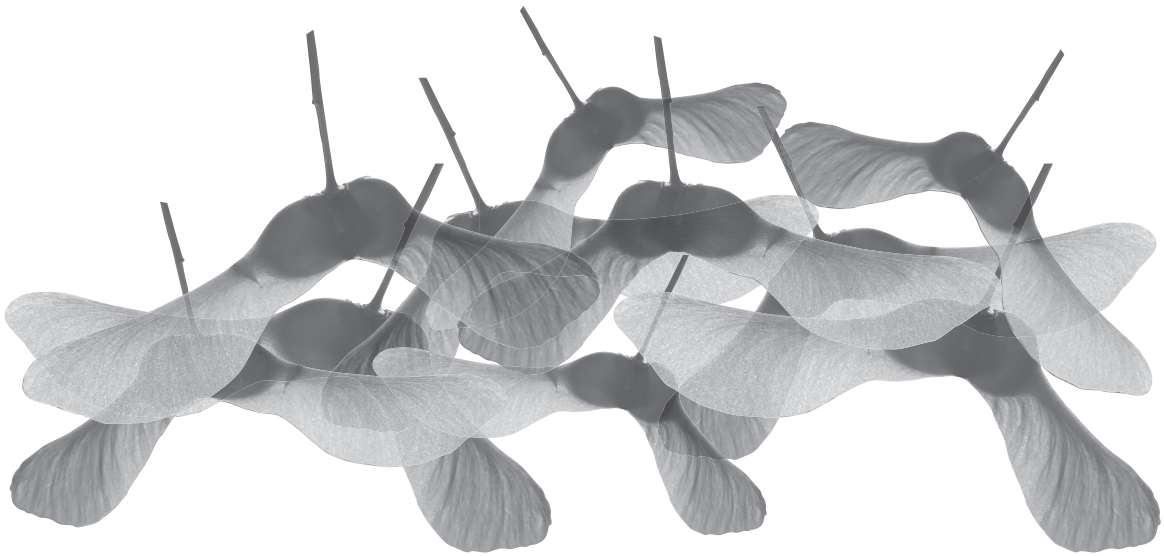
Het laatste hoofdstuk van dit proefschrift beschrijft de conclusies die getrokken kunnen worden uit het verrichte onderzoek en de implicaties die deze conclusies hebben voor de huidige praktijk en voor verder onderzoek.

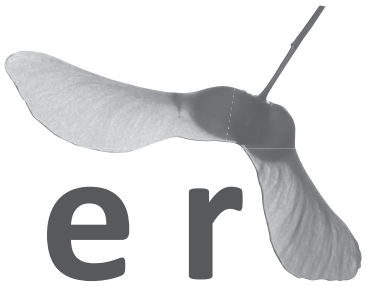
De teleurstellende resultaten van kandidaatgenstudies en genoomwijde associatiestudies benadrukken opnieuw dat het van groot belang blijft om een groot cohort van goed gefenotypeerde vrouwen met POI te verzamelen. Hierbij is internationale samenwerking onmisbaar. Voor kandidaatgenstudies geldt dat deze alleen zinvol en betrouwbaar zijn wanneer het gehele gen wordt gesequenced en indien deze worden verricht in een goed gefenotypeerde groep patiënten. In toekomstige genetische studies zou er meer aandacht geschonken moeten worden aan zeldzame varianten, waarbij een combinatie van whole genome sequencing met linkage analyse een mogelijk vruchtbare benadering kan vormen in de zoektocht naar de etiologie van POI.

Voordat het mogelijk is om eventuele endocriene implicaties van POI verder te onderzoeken en te beschrijven, is het noodzakelijk dat er betrouwbare, precieze, en sensitieve assays voor androgenen, oestrogenen en andere steroïden ontwikkeld en gebruikt worden. Op dit moment kunnen zowel de mate van hypoandrogenisme als de mogelijke gevolgen voor de algemene gezondheid bij vrouwen met POI nog niet goed worden onderzocht. Internationale samenwerking voor zowel standaardisatie van assays als het betrouwbaar ontwikkelen van referentiewaarden voor vrouwen van alle leeftijden is noodzakelijk om in de toekomst onderzoek te doen bij vrouwen met POI.

In hoofdstuk 7 werd beschreven dat cryopreservatie van het ovarium en vervolgens reïmplantatie van deze graft waarschijnlijk geen erg kansrijke fertiliteitsbehandeling is, omdat de ovariële reserve ernstig is aangetast. Daar staat tegenover dat eicelvitrificatie waarschijnlijk een succesvollere methode voor fertiliteitspreservatie is, welke dan ook moet worden aangeboden aan vrouwen die op het punt staan gonadotoxische behandeling te ondergaan of aan hen die zeer hoog risico op POI hebben op basis van een familiaire belasting.

Tenslotte wordt er een voorstel gedaan voor toekomstig wetenschappelijk onderzoek, waarbij zowel het cross-sectionele Nederlandse POI consortium als het voorstel om deze vrouwen prospectief te vervolgen een grote rol zal spelen. Deze benaderingen zullen het mogelijk maken om zowel korte- als langetermijn gezondheidseffecten van POI te onderzoeken, en om te onderzoeken welke factoren deze gezondheidsrisico's mogelijk moduleren.





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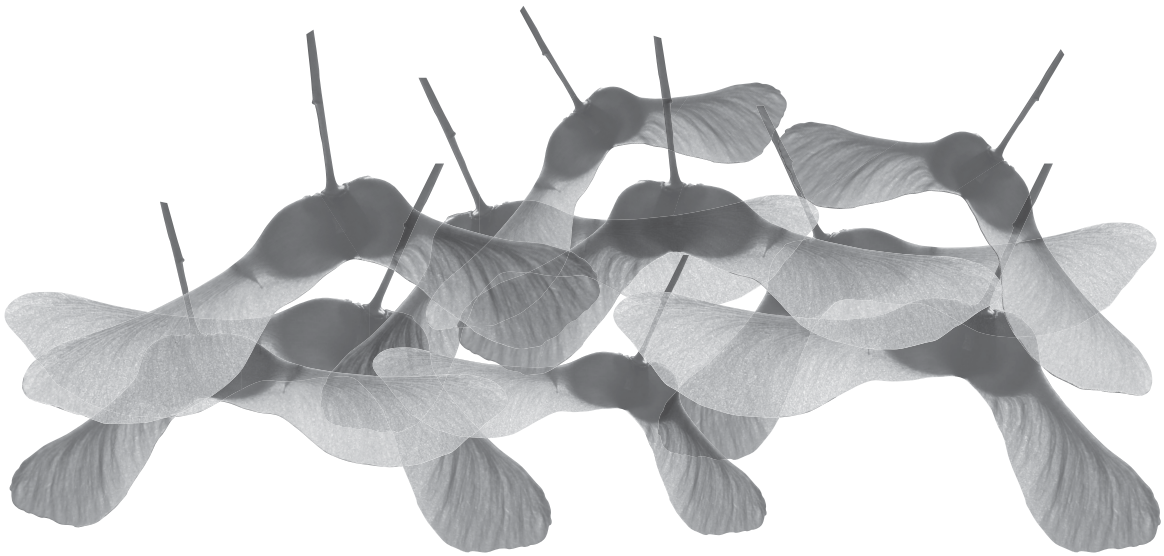

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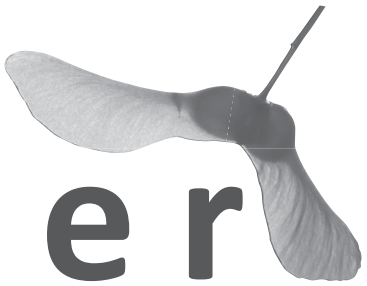

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Het is ongelooflijk: mijn boekje is af!
Ook al is een promotietraject een vrij individueel proces, dit werk kon alleen van de grond komen doordat heel veel mensen mij hebben geholpen. Waarvoor dank!

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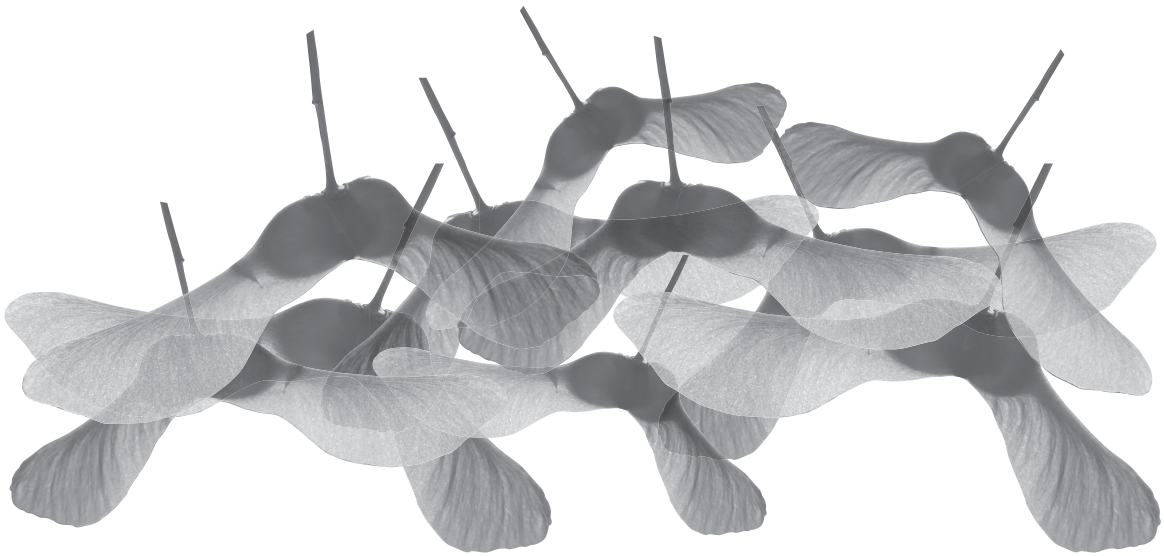

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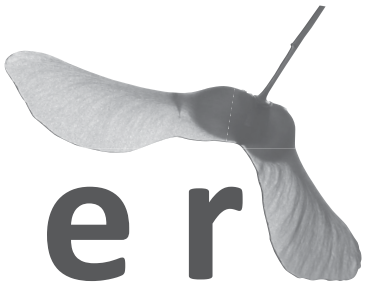

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Femi Janse was born on June 18th 1983 in Leiderdorp as the eldest of two children. She grew up in Leiden with her parents and younger sister Sophie. She graduated from the gymnasium at the Rijnlands Lyceum in Oegstgeest in 2001. Following graduation, she lived abroad and attended Beloit College, a small liberal arts college in Beloit, WI, USA. Upon return, she enrolled into medical school at the University of Utrecht in 2002. Her interest for Obstetrics and Gynecology awoke during a four-week extracurricular study abroad program at the Hospital Universitario San Cecilio in Granada, Spain. Since 2006 she has participated in several research activities in the department of reproductive medicine at the University Medical Center Utrecht. In 2007 she spent three months as a dermatology and forensic pathology intern at the Tygerberg Hospital, University of Stellenbosch, South Africa. Having returned, she followed an additional internship in the department obstetrics at the Wilhelmina Children's Hospital (WKZ) and was supervised by Prof.dr. H.W. Bruinse. After she obtained her medical degree in 2009, she continued her research project "Primary Ovarian Insufficiency: genes, hormones, and beyond" as a PhD student under supervision of Prof.dr. B.C.J.M. Fauser. She has started her residency in Obstetrics and Gynecology under Dr. K.M. Paarlberg's auspices at the Gelre Hospital in Apeldoorn in May 2012. Femi lives happily together with Matthijs van den Heuvel in Amsterdam, with whom she will marry on June 29th 2012.