Epidemiology and etiology of primary ovarian insuffciency

Elinor Chelsom Vogt

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

This work was conducted between March 2018 - May 2022 at the Department of Clinical Science, University of Bergen and at the Department of Medicine, Haukeland University Hospital. The research group of Endocrine Medicine is led by professor Eystein Husebye.

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Main supervisor was Marianne Øksnes. Co-supervisors were Eystein Husebye and Sigridur Bjørnsdottir.

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Elinor

Abbreviations

AAD	Autoimmune Addison's disease
AD	Addison's disease
AHR	Aryl hydrocarbon receptor
AIRE	Autoimmune regulator gene
AMH	Anti-Muller hormone
APS-1	Autoimmune polyendocrine syndrome-1
AR	Androgen receptors
B cell	Bone marrow-derived lymphocyte
BPA	Bisphenols
BPES	Blepharophimosis-ptosis-epichantus-inversus syndrome
cAMP	Cyclic adenosine monophosphate
DDT	Dichlorodiphnyltrichloroethane
DES	Diethylstilbesterol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
DM	Diabetes mellitus
ECRHS	European Community Respiratory Health Survey
EDC	Endocrine disrupting chemicals
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
FMR1	Fragil X Mental Retardation-1 gene
FSH	Follicle stimulating hormone
GAD	Glutamic acid decarboxylase
GnRH	Gonadotropin releasing hormone
GWAS	Genome-wide association studies
HIV	Human immunodeficiency virus
HRT	Hormone replacement therapy
Ig	Immunoglobulin
IGFs	Insulin-Like Growth Factors
IL	Interleukins

INF	Interferon
LH	Luteinizing hormone
MHT	Menopausal hormone therapy
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NALP5	NACHT leucine-rich-repeat protein 5
NGS	Next-generation sequencing
OC	Oral contraceptives
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PFAS	Polyfluoralkylsubstances
PI3K	Phosphatidylinositol 3-OH-kinase
POI	Premature/Primary ovarian insufficiency
PR	Progesterone receptor
RANK	Receptor activator of nuclear factor kappa-B ligand
RAS	Renin-angiotensin-aldosteron system
RHINESSA	The Respiratory Health in Northern Europe, Spain and Australia generation study
RIA	Radio-Ligand Binding Assay
ROAS	National Registry of Organ-Specific Autoimmune Diseases
RNA	Ribonucleic acid
SCC	Side-chain cleavage enzyme
SLE	Systemic lupus erythematosus
T cell	Thymus derived lymphocyte
TGFs	Transforming Growth Factors
TNFs	Tumor Necrosis Factors
WES	Whole exome sequencing
3β-HSD	3β-hydroxysteroid dehydrogenase
11β-OH	11β-hydroxylase
17β-HSD	17β-hydroxysteroid dehydrogenase
17-OH	17-α hydroxylase/17,20 lyase
21-OH	21-hydroxylase

Abstract

Background: Primary ovarian insufficiency (POI) is caused by inadequate ovarian activity and results in premature menopause (age <40 years). The diagnosis of POI has life-changing consequences due to infertility and increased risk of complications related to premature estrogen deficiency. Clinical presentations and pathogenic mechanisms are heterogeneous and include immunological, genetic, and environmental factors. However, in 70-90% of cases the underlying cause of POI remains unknown. It is essential to improve diagnostic precision in women with POI in order to tailor future treatments.

Methods: We used three different cohorts of women to investigate clinical, immunological, and genetic markers of POI in order to explore the prevalence and distribution of underlying etiologies.

Results: We found that POI affects 1.1% of women in the general population and 10.2% of women with Addison's disease. Nulliparity is the variable most strongly associated with POI. Autoimmune mechanisms account for 3-4.5% of POI cases, judged by positive steroidogenic autoantibodies. Autoantibodies against 21-hydroxylase (21-OH) are the most useful screening tool in the general population, while autoantibodies against Side-chain cleavage enzyme (SCC) are the most specific markers in Addison's disease. Genetic susceptibility for POI was found in one third (34%) of patients using Next Generation sequencing techniques (NGS) and we also recognized genetic variants of interest (FOXL2, PNO1, and DDX4) in women with Addison's disease and POI.

Conclusion: Identifying the underlying cause of POI remains challenging. No specific clinical markers predicted the etiology of POI, actualizing the discussion of which tests should be a part of extended diagnostic screening in clinical practice. By adding specific immunoprecipitating steroidogenic autoantibody assays and a POI-related NGS gene panel to the recommended diagnostic investigations, we were able to increase the identification of an etiological diagnosis from 11% to 45%.

What was already known	What these studies add
Paper 1 POI is more common among women with Addison's disease, with large variation in reported prevalence (6-20%) (1-4). Frequency of ovarian associated autoantibodies is high (60-100%), but unstandardized, unspecific methods have been used (3, 5). No data exists regarding potential genetic factors as basis of POI in Addison women.	In a large well characterized cohort of Addison women, we find that 1 in 10 women develop POI. Contrary to previous studies, we demonstrate that POI also can emerge years after Addisons disease. Our study confirms a high frequency of autoantibodies Addison women with POI, SCC is the most sensitive autoantibody for autoimmune POI in these patients. We identified three genetic variants of nominal significance in FOXL2, PNO1, and DDX4
Paper 2 There are several causes of premature menopause (<40 years), including non-ovarian (secondary) and primary ovarian insufficiency. Numerous lifestyle-related factors are associated with the timing of menopause. The prevalence of POI varies in general populations (1-3%) (6-9). The reported autoimmune etiology for POI evaluated by unstandardized tests varies greatly (0-30%) (10-14).	In a general population the prevalence of idiopathic POI was 1.1%. Autoimmune POI accounts for 4.5% of POI cases, judged by the presence of autoantibodies towards 21-OH +/- SCC. Women with premature menopause had a higher frequency of nulliparity, obesity, osteoporosis, use of HRT, and never smokers, compared to menopause ≥40 years of age. Nulliparity is the variable most strongly associated with POI, but no determinant could distinguish autoimmune POI from other causes.
Paper 3 In the majority of POI women the cause is unknown (70-90%) (15). The reported autoimmune etiology of POI evaluated by unstandardized tests varies greatly (0-30%) (10-14) New genetic analysis reveal underlying cause in more women (10-75%) (16).	In women with idiopathic POI, we expanded recommended diagnostics to include a POI-gene panel and steroid cell autoantibodies. We were able to increase the identification of underlying causes of POI from 11% to 45%. Autoimmune POI accounts for 3% and genetic abnormalities account for 42% of POI cases.

Sammendrag

Bakgrunn: Primær ovarialinsuffisiens (POI) er forårsaket av utilstrekkelig ovarieaktivitet og resulterer i for tidlig overgangsalder (alder <40 år). Diagnosen POI har livsendrende konsekvenser på grunn av infertilitet og økt risiko for komplikasjoner knyttet til for tidlig østrogenmangel. Kliniske presentasjoner og patogene mekanismer er heterogene og inkluderer immunologiske, genetiske og miljømessige faktorer. I 70-90% av tilfellene forblir imidlertid den underliggende årsaken til POI ukjent. Det er viktig å forbedre diagnostisk presisjon hos kvinner med POI for å kunne skreddersy fremtidige behandlinger.

Metoder: Vi brukte tre forskjellige kohorter av kvinner og undersøkte kliniske, immunologiske og genetiske markører for å utforske prevalensen og distribusjonen av underliggende årsaker til POI.

Resultater: Vi fant at POI oppstår hos 1,1 % av kvinner i den generelle befolkningen og 10,2 % av kvinner med Addisons sykdom. Nulliparitet er den variabelen som er sterkest assosiert med POI. Autoimmune mekanismer er involvert i 3-4,5 % av POI-tilfellene, bedømt ved positive steroidogene autoantistoffer. Autoantistoffer mot 21-hydroxylase (21-OH) er det mest nyttige screeningsverktøyet i den generelle befolkningen, mens autoantistoffer mot Side-chain cleavage enzyme (SCC) er den mest spesifikke markøren ved Addisons sykdom. Genetiske varianter assosiert med POI ble funnet hos en tredjedel (34%) av pasientene ved hjelp av neste generasjons sekvenseringsteknikker (NGS). Vi avdekket også genetiske varianter av interesse (FOXL2, PNO1 og DDX4) hos kvinner med Addisons sykdom og POI.

Konklusjon: Det er fortsatt utfordrende å identifisere den underliggende årsaken til POI. Ingen spesifikke kliniske markører predikerer POI etiologi, noe som aktualiserer diskusjonen om hvilke tester som bør være del av den utvidede diagnostisk screening i klinisk praksis. Ved å legge til spesifikke steroidogene autoantistoffanalyser og et POI-relatert NGS-genpanel til standard anbefalt utredning, var vi i stand til å øke identifiseringen av underliggende årsak til POI fra 11 % til 45 %.

List of Publications

Paper I

Vogt, Elinor C et al. “Primary Ovarian Insufficiency in Women With Addison's Disease.” *The Journal of clinical endocrinology and metabolism* vol. 106,7 (2021): e2656-e2663. doi:10.1210/clinem/dgab140

Paper II

Vogt, Elinor et al. “Premature menopause and autoimmune primary ovarian insufficiency in two international multi-center cohorts.” *Endocrine connections*, EC-22-0024. 1 Apr. 2022, doi:10.1530/EC-22-0024

Paper III

Vogt, Elinor et al. “Improving diagnostic precision in Primary Ovarian Insufficiency using comprehensive genetic and autoantibody testing”. (Submitted, September 2022)

Related publications

Vogt, E. C., Russell, H., Øksnes, M., & Lund, A. (2022). Prematur ovarialinsuffisiens. *Tidsskrift for den Norske laegeforening: tidsskrift for praktisk medicin, ny raekke*, *142*(11), 10.4045/tidsskr.21.0675. doi:10.4045/tidsskr.21.0675

Vogt, Elinor Chelsom et al. “Feminizing adrenal tumor identified by plasma steroid profiling.” *Endocrinology, diabetes & metabolism case reports*, vol. 2021 21-0104. 1 Nov. 2021, doi:10.1530/EDM-21-0104

Co-author:

Laakso, Saila et al. “Pregnancy Outcome in Women With APECED (APS-1): A Multicenter Study on 43 Females With 83 Pregnancies.” *The Journal of clinical endocrinology and metabolism* vol. 107,2 (2022): e528-e537. doi:10.1210/clinem/dgab705

Aranda-Guillén, Maribel et al. "A polygenic risk score for autoimmune Addison’s disease estimates individual risk and identifies patients with monogenic adrenal insufficiency". Nature Communications. (Accepted, September 2022)

Contents

2. Introduction

2.1 Normal physiology of the ovary

The functions of the ovaries are to produce oocytes for potential fertilization and sex hormones for development and adherence of biological sexual characteristics and function. Beyond their role in reproductive health, sex hormones are important in the health of multiple organ systems throughout the body. The follicles are the basic functional units of the ovary. Each follicle consisting of an oocyte surrounded by hormone producing granulosa and theca cells as well as stromal cells. (Figure 1).

In humans, the ovarian reserve is established during fetal life and is thereafter gradually depleted through the next 50-some-years, until exhaustion at menopause. Through fetal life, childhood and the reproductive period the oocytes develop and undergo maturation in stages of folliculogenesis. This process is tightly regulated by endocrine, paracrine and autocrine factors commencing with the recruitment of the primordial follicles and resulting in either ovulation or follicle atresia.

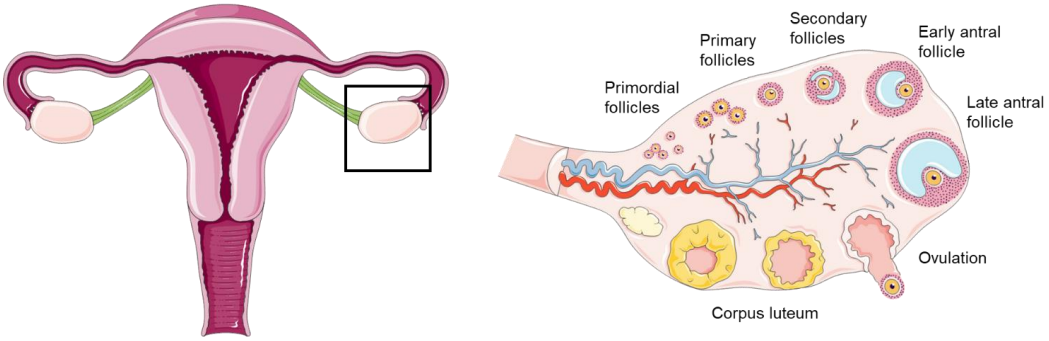

Figure 1. Uterus, ovary and follicles. The ovaries are located at each side of the uterus in the upper lateral pelvis suspended with the uteroovarian-, the mesovarium- and the infundibulopelvic-ligaments (containing blood vessels, lymphatics, and nerves) at the hilum. The average adult ovary is approximately 2.5-5 cm x 2.5 cm x 1 cm in size and weighs 3-8 g. The ovaries are organized in a well vascularized inner medulla with an outer stromal cortex containing the ovarian follicles of different stages. The figure was generated using Servier Medical Art.

2.1.1 Oogenesis

In the first trimester of pregnancy the common embryonic precursor of steroidogenic cells, the adrenogonadal primordium, separates into two distinct cell populations that differentiate into the adrenal cortex and the gonads (17). In female embryos the gonadal primordium encompass the germ cells and develop into ovaries (18, 19). Germ cells proliferate by mitosis, reaching a maximum of 6-7 million oocytes around 20 weeks of gestation. The process of oocyte maturation is initiated through the onset of meiotic division followed by years of meiotic arrest. In the second half of pregnancy a massive loss of oocytes take place through atresia resulting in 1-2 million oocytes remaining at time of birth. During childhood the gradual progressive decline in ovarian reserve continues and 4-500 000 oocytes remain at puberty, of which approximately 400-500 will resume meiosis, mature and ovulate during the menstruation cycles. The process of oogenesis, folliculogenesis, atresia and ovulation continues throughout a woman's reproductive years until approximately 1000 follicles remain, at which time menopause occurs, usually in her early 50s (20-22). If the oocyte pool is insufficient or the folliculogenesis is dysfunctional, reproductive senescence and menopause may take place earlier.

2.1.2 Folliculogenesis

Folliculogenesis is the process of oocyte maturation in the ovarian follicle. It starts with primordial follicle recruitment and results in either atresia or ovulation. Folliculogenesis (Figure 2) can be divided in two stages: a) The gonadotropin-independent phase, which is initiated in fetal life and continues throughout childhood, puberty and the reproductive period, and b) The gonadotropin-dependent phase, starting at puberty and continuing until menopause (27).).

Gonadotropin independent phase

The initial stages of folliculogenesis are largely controlled by intraovarian factors (12). A single layer of granulosa cells surrounds the oocyte of the **primordial follicles**. As the follicles grow in size, the oocyte is encapsuled by the zona pellucida and the granulosa cells undergo a morphological change from squamous to cuboidal in shape, forming the **primary follicles**. During the final stages of initial recruitment adjacent

stromal cells differentiate and become theca cells surrounding the granulosa cells, only separated by a basement membrane (23, 24). The follicles have not yet gained independent blood supply and this phase of initial recruitment relies on paracrine and autocrine regulatory mechanisms (25). The major signaling pathways in human ovarian follicle cells are phosphatidylinositol 3-OH-kinase (PI3K), the mammalian target of rapamycin (mTOR) and the Hippo signaling pathway (26, 27). A myriad of stimulatory and inhibitory factors coordinate folliculogenesis through these pathways, including Insulin-Like Growth Factors (IGFs) and Transforming Growth Factors (TGFs) (27). Various immune factors especially cytokines (IL-1, IL-6) and Tumor Necrosis Factors (TNFs) also play important roles as immunomodulators of follicle development (28). Increased understanding of these and other putative intraovarian regulators is gradually illuminating the intricate regulation of early follicle recruitment and pointing at potential therapeutic targets for ovarian stimulation in cases of ovarian failure and infertility (29, 30).

Dormant primordial follicles enter the growing follicle pool in a continuous manner from fetal life until menopause. A cohort of primordial follicles will be recruited, start to grow and mature at any point in time, but without gonadotropin stimulation they will eventually undergo atresia. The first stage of folliculogenesis is gonadotropin independent as supported by evidence that early follicles are formed also in complete absence of gonadotropins, i.e. in Kallman syndrome and anencephaly (26). In contrast, the final stages of follicle maturation start when the theca and granulosa cells acquire gonadotropin receptors.

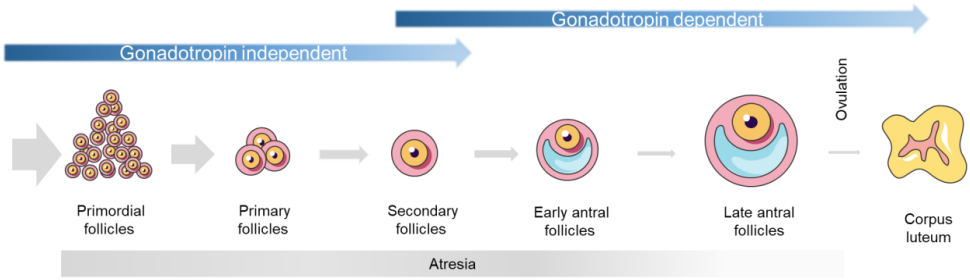

Figure 2. Folliculogenesis. Initial recruitment is gonadotropin independent and involves growth and differentiation of primordial, to primary and preantral follicles, the majority of which undergo atresia. The gonadotropin dependent cyclic recruitment starts at puberty and involves the maturation of antral follicles through ovulation and subsequent corpus luteum formation or atresia. This process continues throughout a females reproductive lifespan until the promordial follice pool is depleted. The Figure was generated using Servier Medical Art.

Gonadotropin dependent phase

In puberty the pituitary gland starts producing gonadotropins, inducing the cyclic maturation of follicles of the menstruation cycle (31). This gonadotropin-dependent phase is tightly controlled by complex feedback mechanisms between the hypothalamus, pituitary gland and gonad/ovary (HPG axis). Briefly, the hypothalamus releases Gonadotropin releasing hormone (GnRH) by pulsatile secretion into the portal vessels stimulating the gonadotropin-producing cells of the anterior pituitary to release gonadotropins: Follicle stimulating hormone (FSH) and Luteinizing hormone (LH). The secretion of gonadotropins varies in amplitude and frequency across the menstruation cycle (32). The gonadotropins then trigger steroidogenesis in the theca and granulosa cells promoting oocyte maturation and ultimately ovulation as well as systemic endocrine effects. Concurrently, the ovarian hormones feed back to the hypothalamus and pituitary, regulating the secretion of gonadotropins (23). (Figure 3).

A cohort of primary follicles will respond to gonadotropin stimulation at the beginning of the monthly cycle and continue to mature forming **secondary follicles** and thereafter **preantral-antral follicles**. Continued cell proliferation, increasing follicle size and

formation of antrum or fluid collections in the granulosa cell layer surrounding the oocyte appear as the follicle enters the **antral stage**. The theca cell layer becomes more vascularized exposing the granulosa cells to gonadotropin stimulation. The follicle with the largest number of FSH receptors continues to grow and becomes the dominant follicle, while the other follicles undergo atresia. At the middle of the menstrual cycle, LH sharply increases and just before ovulation the first meiotic division is completed. After ovulation the dominant follicle forms the corpus luteum responsible for estrogen and progesterone production for maintenance of the endometrium during early pregnancy (23).

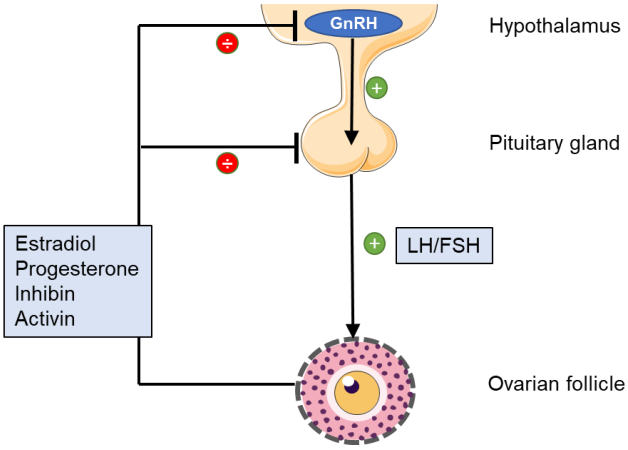

Figure 3. A simplyfied diagram of hypothalamic-pituitary-ovarian axis (HPG axis). Stimulated by gonadotropins, ovarian follicles secrete steroid hormones (estradiol and progesterone) and peptide hormones (inhibins and activins).Through complex feedback mechanisms to the hypothalamus and the pituitary gland, the secretion of gonadotropins is regulated. If the follicle reserve is depleted, lack of negative feedback will generate a high production of gonadotropins. The Figure was generated using Servier Medical Art.

The final stages of gonadotropin dependent folliculogenesis are also influenced by autocrine and paracrine factors derived from cells within the follicles (26, 33, 34). The granulosa cells produce peptides that are members of the TGF-β family, among them Inhibins, Activins and Follistatins. In addition to the endocrine function in regulating

pituitary secretion of gonadotropins through feedback mechanisms, these peptides regulate steroidogenesis directly by modulating theca and granulosa cell response to gonadotropins within the ovarian follicle (26, 35). Inhibins exist in two isoforms: Inhibin A and B. Inhibin A secretion is correlated to follicle maturation and luteinization whereas Inhibin B is produced by the small developing follicles (35, 36). Inhibins have been shown to enhance LH-stimulated androgen release by thecal cells and are probably important in selection of the dominant follicle. Activins exert an antagonistic effect to Inhibins by upregulating FSH receptors and steroidogenic enzyme gene expression in granulosa cells, while Follistatins plays a modulatory role in these autocrine and paracrine mechanisms (35). Decrease in Inhibin B levels are associated with diminished granulosa cell activity and have been proposed as a marker of follicular reserve. However, the normal reference range remains unclear (37-39).

Anti-Muller hormone (AMH) is another member of the TGF-β family expressed by the granulosa cells. It acts as a major suppressor counteracting follicle maturation. Growing follicles secrete AMH, with the highest levels at the early follicular stages (40, 41). In vitro treatment with AMH has demonstrated increased numbers of growing pre-antral follicles and decreased numbers of mature pre-ovulatory follicles, consistent with an inhibitory effect on differentiation of follicles (42). It has also been postulated that AMH has endocrine properties in organs other than the ovaries, in particular in regulation of GnRH pulsatility in the hypothalamus (43).

Clinically, serum AMH levels are used to assess the ovarian reserve and fertility (44, 45). During a woman´s lifetime, the serum AMH levels peak in her early 20s and gradually decline with age until menopause (46). AMH levels reflect the number of preantral and small antral follicles and are positively correlated with ultrasound antral follicle count. A low serum AMH concentration is associated with a low ovarian reserve, but due to large inter-individual differences, prediction of menopausal timing is challenging (47).

2.1.3 Steroidogenesis

The major steroid hormone-producing organs in females are the adrenal glands, the ovaries and the placenta. Steroid hormones are synthesized from cholesterol by the function of six enzymes of the cytochrome P450 and two enzymes of the hydroxysteroid dehydrogenase family: Side-chain cleavage enzyme (SCC), 17-alpha hydroxylase/17,20 lyase (17-OH), 21 hydroxylase (21-OH), 11-β hydroxylase (11β-OH), aromatase, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). The signaling molecules cyclic adenosine monophosphate (cAMP), SF1 and StAR are central in regulating the expression of genes encoding these enzymes. Steroidogenesis follows the same script in all steroid hormone producing cells, but the end-products differ depending on the cell-specific expression of steroidogenic enzymes. The main steroid hormones produced in the ovary are the sex hormones: estrogens, progesterones and androgens (48, 49). (Figure 4).

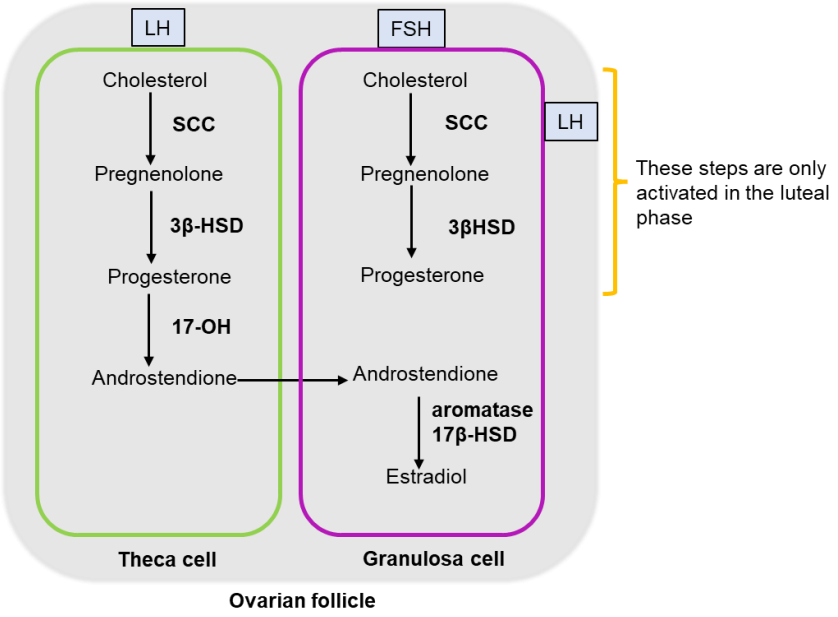

Figure 4. Two-cell hypothesis. Ovarian steroidogenesis is compartmentalized.

Two-cell hypotheses of ovarian steoidogenesis

The ovarian production of steroid hormones is synchronized through complex regulatory mechanisms resulting in cyclic patterns of hormone release. *The two-cell hypothesis* explains the ovarian steroidogenesis as a coordinated process between theca and granulosa cells: Theca cells will under LH stimulation convert cholesterol to androgens. They do, however, lack the enzyme aromatase and are therefore unable to synthesize estrogens. Granulosa cells on the other hand will respond to FSH stimulation by expressing the enzyme aromatase necessary for conversion of androgens to estrogens, but they do not express the enzyme CYP17A1, making them unable to produce androgenic precursors. Androgens from the theca cells are therefore used as substrate for estrogen production in the granulosa cells. In each cell type the expression of the various enzymes depends on the stage of follicular maturation. In the late stages of folliculogenesis after ovulation the corpus luteum is established and granulosa cells develop LH receptors, inducing expression of the enzymes necessary for quantitative progesterone synthesis (50, 51).

Sex hormones and their effects

In the female the sex hormones are responsible for the development and maintenance of reproductive tissues including the uterus, vagina, vulva and breasts. In addition to the immediate effects on the reproductive organs and functions, sex hormones exhibit endocrine properties that are critical to a wide range of biological processes. The somatic effects of all steroid hormones are mainly mediated through intracellular receptors regulating of transcription of target genes. Steroid hormone receptors are found in almost all cells in the body (52).

The main sex hormones are estrogens, progesterone and androgens. In addition, the ovary produces pregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone and estrone. These steroid hormones have weaker endocrine potency and for the most part act as precursor hormones through conversion in target tissue as well as in peripheral tissue with steroidogenic enzymatic activities (adipose tissue, skin) (49). (Figure 5) (49).

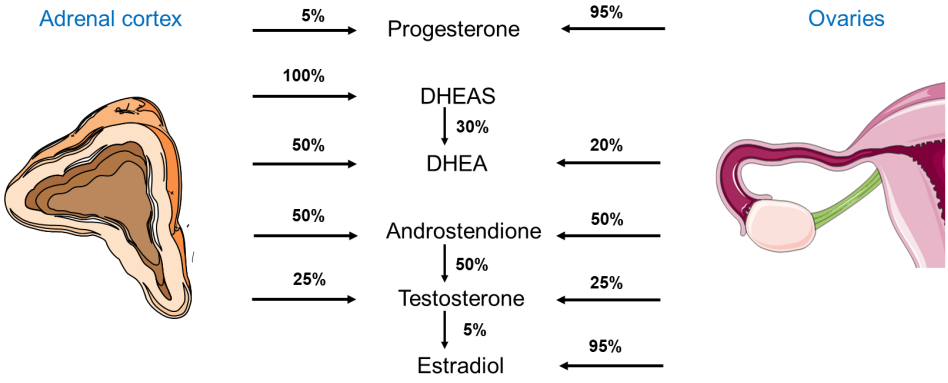

Figure 5. Steroid hormone production in premenopausal females. Estimations of approximate quantitative contribution. The Figure was generated using Servier Medical Art.

Estrogens

Estrogens include estrone, estradiol, and estriol. Estradiol, the predominant type during the reproductive years, is complemented by estriol during pregnancy and replaced by estrone as the dominant form after menopause. More than 95% of the most biologically potent estrogen, 17β-estradiol—is secreted by granulosa cells of the ovaries. Approximately one-half of the circulating estrone is secreted from the ovary, while the remaining is derived from peripheral conversion from androstenedione (by aromatase), or from estrone (by 17β-HSD). Estriol produced during pregnancy is secreted from the placenta (53).

With estrogen receptors (ERs) present in the cells of every organ system and tissue, the magnitude of estrogen's influence on female physiology make the specific contributions of estrogen difficult to catalogue. In addition to a crucial role in fertility, estrogens actions influence the cardiovascular system, bone, nerve system, liver, colon, skin, mucous membranes and immune function, among others (54). A comprehensive understanding of estrogens roles in female health is still incomplete. Often the function of a hormone becomes apparent in the face of deviant secretion rates. In cases of estrogen insensitivity due to genetic ER anomalies, disruption of the HPA feedback

system results in dysregulated folliculogenesis. Pathogenic ER variants are associated with anovulation and POI (55, 56).

Progesterones

Progesterones are metabolic intermediates in production of other steroid hormones but also act directly on nuclear progesterone receptors (PR) conveying crucial endocrine effect on the reproductive system through regulation of the menstrual cycle and differentiation of endometrial cells. Progesterone found in the circulation is produced mainly by theca and the luteinized granulosa cells in the ovaries. Besides the essential role it has in maintenance of early pregnancy, progesterone effects include development of breast tissue, bone metabolism, extracellular fluid regulation, and neuroprotective properties, among others (57, 58). It is also well known that brain regions involved in the modulation of mood and behavior, including the prefrontal cortex, hippocampus and thalamus, are influenced by fluctuations in progesterone levels associated with reproductive events (e.g., premenstrual, postpartum, menopausal transition) (59-61).

Androgens

In women of reproductive age the androgens originate from the theca cells of the ovaries and the zona reticularis of the adrenal cortex. The most abundant androgen precursor, dehydroepiandrosterone sulfate (DHEAS) is solely produced in the adrenal cortex while DHEA is derived from adrenal cortex (50%), ovary (20%) and from circulating DHEAS (30%). Equal amounts of androstenedione and testosterone are produced in the ovary and the adrenal cortex (62).

In women, the bioactive androgens are testosterone and dihydrotestosterone (DHT). These are mainly produced by peripheral conversion of androgen precursors secreted from both the ovary and adrenal gland. Androgens are precursors for estrogens, but they also exert autocrine and paracrine intra-ovarian effects as well as endocrine effects through the intracellular androgen receptor (AR) in somatic cells (63).

The clinical consequences of androgen depletion in females of reproductive age are still uncertain (64). Low libido has been linked to reduced androgen levels, but clinical

trials have shown inconsistent correlation between serum androgen levels and sexual dysfunction. This could be due to the complexity of sexuality or to unreliable methods for measuring androgens in women (65, 66).

2.2 The menopausal transition

Women undergo three major endocrine transition periods in life: puberty, pregnancy and menopause. These events are marked by major shifts in the HPG axis and exert a significant change in the body's hormonal milieu. Growing evidence suggest that the timing of these transitions is related to various health outcomes e.g., bone mineral density, cardiovascular events, autoimmune conditions, metabolism, cancer and mental health (67-69).

Menopause is the permanent cessation of the reproductive stage of a woman's life due to ovarian follicle depletion (20). Clinically menopause is defined at 12 months after the final menstrual period. In Norway, the average age of menopause is 52.9 years (± 2.1, range 40.3-58.7) (70). The timing is relatively normally distributed with approximately 5 percent of women undergoing menopause later than 55 years and 5 percent before 45 years (71-73). The term *Climacterium* encompasses the transition from the reproductive to the non-reproductive stage including the perimenopause which begins on average four years prior to the final menstrual period and is characterized by menstrual irregularity, vasomotor symptoms such as hot flashes and reduced fertility (74). Large hormonal fluctuations in serum estradiol and subsequently raised FSH and LH levels are characteristic in this period. Serum Inhibin B and AMH concentrations decline more gradually as the follicle numbers decrease in the years preceding menopause (39, 46).

Several factors are linked with a woman's age at natural menopause, including sociodemographic, lifestyle, and health related factors. Use of oral contraceptives (OCs), age at menarche ≥13 and multi-parity are associated with later menopause (75). Higher education is also associated with later age at menopause, while lower occupational levels increases the risk for earlier age at menopause (OR 0.64, 95% CI

0.26, 1.02) (76). The age at menopause is also reduced by approximately two years in women who smoke or are underweight (77-80).

It has long been observed that there is a correlation between mothers and daughters age at menopause and emerging evidence suggests that timing of this event is a complex genetic inherited trait (80, 81). Genome-wide association studies (GWAS) have identified a number of genetic variants associated with age at menopause among them genes regulating DNA repair, estrogen receptor and immune function (82-84).

2.2.1 Premature menopause

Menopause before the age of 40 years is considered premature. There are multiple causes for premature menopause as a defect in any part of the HPO axis can disturb ovarian functions, resulting in menstrual disorders and hypoestrogenism. If the deficiency is located in the ovary it is referred to as primary or premature ovarian insufficiency (POI) while a deficiency affecting the hypothalamic-pituitary function is called non-ovarian, central or secondary ovarian insufficiency. POI is characterized by hypergonadotropic hypogonadism with low levels of estradiol resulting in lack of negative feedback to the pituitary subsequently causing high levels of FSH. Biochemically, the pattern of hypergonadotropic hypogonadism separates POI from the hormone profile of secondary ovarian insufficiency which is characterized by hypogonadotropic hypogonadism with low levels of estrogen and low or sub-normal serum concentrations of FSH (85, 86).

2.3 POI

Fuller Albright, a Harvard endocrinologist, was in 1947 the first to describe a syndrome of amenorrhea, estrogen deficiency and high FSH levels. He called it primary ovarian insufficiency (87). Different terminology has later been used, but in recent times the term premature ovarian insufficiency is increasingly preferred because it includes the diagnostic age limit as well as describes the intermittent ovarian function that can be observed (88).

2.3.1 Diagnosis

A woman under 40 years of age with amenorrhea for 4 months or more and an FSH level in the postmenopausal reference range fulfills the diagnostic criteria for POI. A high FSH concentration in the presence of amenorrhea is a reliable indicator of ovarian follicular depletion or ovarian failure. Because the serum levels of hormones can fluctuate and because the clinical implications of the diagnosis are serious, measurements should be repeated at least twice, minimum 4 weeks apart (88).

2.3.2 Epidemiology

The prevalence of POI is reported to be 1-3 % (6-9). The incidence of POI is lower in younger women (89). However lately there are reports of increasing incidence of POI in younger age groups, possibly as result of prolonged survival after gonadotoxic treatments and/or altered exposure to environmental pollutants (15, 90). Population differences in the frequency of POI have been reported in some studies with higher prevalence in women of African and Hispanic ethnicity and women from in medium and low Human Development Index countries (6, 91). Thus, the frequency as well as the underlying cause of POI may have regional differences influenced by ethnicity and environmental factors (90). Different diagnostic criteria of POI as well as varying study designs may also influence the number of reported POI cases. The prevalence tends to be higher in cross-sectional than cohort studies (92).

2.3.3 Etiology

POI occurs as a result of follicle depletion or follicle dysfunction. A depletion of the follicle pool can be the result of a constitutive low number of ovarian follicles or acquired because of increased atresia of existing follicles. A defective follicle function can be caused by failure of normal maturation (85). There are several known causes of POI, often distinguishing between iatrogenic etiology related to treatment (chemotherapy, radiation and ovarian surgery) and spontaneous etiology which includes genetic, autoimmune and infectious causes as well as environmental factors. However, it can be challenging to identify the underlying mechanisms and, in the majority of cases, the cause of POI remains unknown (15, 88).

According to current guidelines, there is consensus for the following diagnostic investigations in the initial evaluation of women with newly diagnosed spontaneous POI (15, 88, 93):

- Karyotype testing
- Fragile-X premutation testing
- Screening for 21-OH autoantibodies (or alternatively adrenocortical autoantibodies (ACA) and thyroid autoantibodies

Increased understanding of the pathogenesis behind POI as well as new technological opportunities, allows us to revisit the background for the high frequency idiopathic POI and explore new diagnostic possibilities.

Genetic factors

Approximately 15-30% of women with POI have family members who are also affected, pointing to an underlying genetic component (15, 94, 95). Numerical and structural abnormalities on the X chromosome as well as dysfunction in several genes that regulate ovarian development and function are strongly associated with the condition (15).

Numerical and structural X chromosome anomalies

Turner syndrome (45,X) is caused by the complete or partial loss of one X chromosome and occurs with an incidence of approximately 1 in 2000-2500 live births (96, 97). Haploinsufficiency, when one copy of the X chromosome is missing, results in the lack of required dosage of particular X-linked gene products causing accelerated oocyte apoptosis, follicle depletion, and subsequently POI (98). Other characteristic phenotypic features of Turner syndrome include short stature, lymphedema, webbed neck, shield chest, wide-spaced nipples, cubitus valgus as well as cardiac anomalies (coarctation or aortic anomalies) and streak gonads (97). The diagnosis is usually set in childhood, however in women with mosaic Turner's syndrome (45,X/46,XX), spontaneous menarche can occur, and pregnancies have been reported (99).

Other X chromosome abnormalities are also recognized as a cause of POI, including triple X syndrome, and structural defects of the long Xq arm. Especially deletions,

inversions, isochromosomes and translocations in the critical regions Xq13-21 and Xq23-27 are associated with reduced ovarian function (96).

FMR1 premutation

The Fragil X Mental Retardation-1 gene (FMR1) is located on the X chromosome. Premutations of the FMR1 gene are carried by 1 of 250 women, and one in five of these women will develop POI, making it the most commonly known cause of heritable POI (100). When CGG trinucleotide repeats of the FMR1 gene are duplicated to 55-200 repeats the premutation becomes unstable, resulting in an insufficiency of proteins necessary for oocyte maturation and survival (96). Genetic screening of family members is recommended, not only for fertility assessment of female relatives but also because FMR1 premutations can expand to a full mutation (>200 repeats) when transmitted to the next generation, causing fragile X syndrome in male offspring, which is the most common inherited form of mental disability and autism in males (88, 101).

Other genes involved in ovarian function

Currently autosomal genetic testing is only done in cases suggestive of a specific genetic variant (88). With newer genetic techniques such as GWAS and Next-generation sequencing (NGS), a plethora of deviant genetic variants associated with POI have been identified (102, 103). Previously genetic POI was associated as a part of complex syndromes such as Blepharophimosis-ptosis-epichantus-inversus syndrome (BPES) or Galactosemia, but today most of the genetic variants are found in sporadic and non-syndromic POI cases (104). The inheritance pattern can be autosomal dominant or autosomal recessive. Recently there has been focus on oligogenic inheritance with possible synergistic effect explaining the variance in phenotype seen (105).

The majority of the POI associated genetic variants are related to dysfunction in the regulatory proteins involved in signaling pathways (PI3K, mTOR and the Hippo signaling pathway) and key biological processes, i.e. ovarian development (oogenesis and folliculogenesis), meiosis and DNA repair, hormonal signaling, metabolism and immune function (82, 96, 106).

Genes involved in the fetal development of the gonads and initial follicle formation are crucial for normal ovarian function and include among others: *BMP15, BMPR1B, EIF4ENIF1, FANCM, FOXL2, IL17TH, LMNA, NANOS3, NOBOX, NOG, POU5F1, SOHLH1, SOHLH2, SOX2, SOX8, SOX10, SYCP2L*. Dysfunction in these genes can result in POI.

Oocytes are the oldest cells in the female body, stored for decades before complete maturation and potential fertilization. During the massive mitotic activity of the oogenesis and the long process of meiosis, cell division can lead to DNA damage. The most common DNA damage repair genes associated with POI are*: BUB1B, C14ORF39, CUL4B, HFM1, MCM8, MCM9, MSH5, MSH4, NUP107, POF1B, PSMC3IP, SGO2, STAG3, SYCE14, ZSWIM7.* The majority of follicles undergo apoptosis and in a normal menstruation cycle, only one dominant follicle will mature and ovulate. Therefore, mutations in genes involved in apoptosis may also cause defective ovarian function and POI.

Production, development, and maturation of oocytes require autocrine, paracrine and endocrine follicular mechanisms. Several POI-associated genetic variants involving regulatory proteins, growth factors, hormones and immune function have been identified*: CYP17A1, CYP19A1, DACH2, EIF2B4, EIF2B5, ESR1, FANCM, FMR1, FSHB, FSHR, GDF9, HSD17B4, LHCGR, NR0B1, PGRMC1, PMM2, RCBTB1, STAR, AARS2, AIRE, CLPP, DDX4, HARS2, LARS2, PNO1, POLG, TWNK.*

The introduction of NGS technology has given us new insight into the underlying molecular mechanisms of POI. The number of POI associated genetic variants are constantly expanding, dramatically altering the possibilities to find underlying causes of POI.

Recent studies have estimated genetic causation in 10-75% of POI cases (16, 107). Improvements in sequencing techniques and interpretive approaches may hopefully allow a more precise determination of the mechanisms underlying ovarian dysfunction in the years to come.

Autoimmunity and immunological factors

In contrast to the testicles the ovaries do not constitute an immunologically privileged site as immune mechanisms have important functions in regulation of the ovarian physiology and menstruation cycle. Macrophages, mast cells, monocytes and lymphocytes are all in situ modulators of follicle function through secretion of regulatory molecules such as cytokines and growth factors (28, 108).

Autoimmune disease is characterized by immune cells reacting to autoantigens and the presence of autoantibodies (109). In systemic autoimmunity the immune responses are directed against autoantigens with ubiquitous expression patterns throughout the body, like cell nuclear components such as DNA, RNA and others. Examples of systemic autoimmune diseases are Systemic lupus erythematosus (SLE) and Sjøgren syndrome. In organ specific autoimmunity the autoimmune response is directed against specific autoantigens present in a certain tissue. Addison's disease , type 1 diabetes and autoimmune thyroiditis are typical examples of organ specific autoimmune diseases affecting endocrine organs (110). In Addison's disease an autoimmune destruction of the steroid hormone producing cells of the adrenal cortex takes place, the enzyme 21-OH being a target antigen (111). The ovary has also shown to be a target of autoimmune attack manifested by endocrine and reproductive dysfunction in POI (112).

Autoimmune oophoritis

Histopathological analysis of a subgroup of women with POI have demonstrated autoimmune oophoritis with mononuclear infiltrates of the theca cells in growing follicles, initially sparing the primordial and primary follicles (14, 113, 114). Recent immunohistochemical studies have revealed that the immune infiltrate contains both B- and T cells as well as polyclonal plasma cells, suggesting a complex immune system interplay (115-118). Women with autoimmune oophoritis present with higher serum inhibin B and AMH levels compared to women with other reasons for POI, reflecting the presence of functional intact granulosa cells within the quiescent follicles (119-121). On ultrasound, the ovaries can be of normal size or enlarged and follicles may have a cystic appearance due to gonadotropin stimulation (85, 114, 122).

Diagnostic biopsies of the ovaries are not recommended as a routine investigation partly because of the general inaccessibility of the ovaries but also because studies have shown good correlation between steroidogenic autoantibodies and biopsy findings confirming autoimmune POI (5, 14, 123).

Autoantibodies

To establish an autoimmune pathogenesis it is common practice to evaluate the presence of identified disease-specific autoantibody markers. Using methods of indirect immunofluorescence, anti-ovarian autoantibodies (AOA), were discovered already in the late 1960s (124). Multiple specific ovarian autoantigens have later been identified as targets for AOAs, including the oocyte, gonadotropin receptors, β-subunit of FSH, zona pellucida, corpus luteum, heat shock proteins, alpha-enolase, beta-actin and NACHT leucine-rich-repeat protein 5 (NALP5) (125-130).

Despite biopsy-confirmed autoimmune oophoritis being coherent with AOA in 100% of cases, the diagnostic significance of AOAs is questionable as up to 2/3 of all POI women are positive. In addition, AOAs have been demonstrated in up to 1/3 of women with infertility of unknown cause (30%) (10, 11, 131).

Most studies of autoimmune oophoritis are based on indirect immunofluorescence detecting serum Steroid-cell autoantibodies (SCA) in reaction with steroid hormone producing cell antigens (5, 12-14, 132-135). Although SCAs are more specific than AOA, the accuracy is low because of lack of standardization of methods and use of antigens from various steroid hormone producing tissues (testes, ovaries, placenta or adrenal cortex) (136).

Use of specific immunoprecipitation methods such as Radio-Ligand Binding Assay (RIA) and Enzyme-linked immunosorbent assay (ELISA) have identified precise ovarian target antigens against several steroidogenic enzymes: 21-OH, SCC, 17-OH, and 3βHSD. Especially autoantibodies towards 21-OH have repeatedly demonstrated a high sensitivity in diagnosing autoimmune POI (3, 13, 120, 133, 134, 137-140). Approximately 0-13% of women with POI are positive for 21-OH autoantibodies, a

frequency significantly higher than the expected in the general population (<0.6%)(141, 142).

Associated autoimmune disease

POI is more common in women with autoimmune adrenal insufficiency (Addison's disease) and autoimmune polyendocrine syndrome (APS-1) (15, 112). Between 6-20% of women with autoimmune adrenal failure (Addison's disease) have POI, while approximately 2-3% of women with POI develop adrenal autoimmunity (3, 14, 88, 112, 143, 144). This association might be a consequence of a common adrenogonadal primordium and autoantibodies cross-reacting against antigens of steroid producing cells in both the adrenals and ovaries. The correlation with POI is strongest in the context of autoimmune polyendocrine syndrome type-1 (APS-1), an autosomal recessive disease caused by mutation in the AIRE gene involved in regulatory T cell selection (145). APS-1 predominantly manifests as adrenal insufficiency, mucocutaneous candidiasis, hypoparathyroidism and 50–60% of women with AIRE mutations will develop POI (145, 146).

POI often occurs in association with other autoimmune diseases, especially thyroid hormone disorders are common, with a prevalence of approximately 20% vs 5% in the general population. Other organ specific disease such as rheumatoid arthritis, coeliac disease, myasthenia, pernicious anemia, vitiligo, multiple sclerosis, and systemic autoimmune disease occur more often in POI women (112, 147-149). It is uncertain whether this association is due to an overlapping inflammatory autoimmune process involving common antigens or if it is caused by a general immune dysregulation triggered by changes in the hormonal milieu induced by POI. There is a sexual dimorphism related to the risk of developing autoimmune disease, with a preponderance among females. In addition onset and progression of autoimmune disease are related to the female transition periods with adverse effects on the immune system registered during puberty, in relation to pregnancy and after menopause (150, 151). The complex interaction between hormones and the immune system is partly impacted by estrogen withdrawal. After menopause changes in lymphocyte composition take place and there is a continuous rather than cyclical secretion of

cytokines with increased production of IL1, IL6, TNF-α, and decreased secretion of anti-inflammatory cytokines INF-gamma (5, 152-154). In POI women abnormalities of the cellular immunity with decreased numbers of effector regulatory T-cells and increased $CD4^+$ $CD69^+$ activated T cells in peripheral blood have been shown (112, 155).

In conclusion; an autoimmune etiology of POI should be evaluated in the presence of associated autoimmune disorders, lymphocytic oophoritis, or the existence of associated autoantibodies (112). The uncertainty with regards to prevalence of autoimmune POI (0–30%), probably reflects heterogenic study populations as well as use of variable autoantibody assays (112, 149).

Infectious oophorittis

Infectious oophoritis due to tuberculosis, myxovirus (mumps) cytomegalovirus, and Human immunodeficiency virus (HIV) have been reported (156-159). Though less common in our part of the world, this may occur more frequently in women from high-endemic areas. Screening is not recommended as part of a routine examination (15, 88).

There have been proposed safety concerns regarding human papilloma virus (HPV) vaccination and POI, based on case reports (160). Suggested mechanisms for the association involve the vaccine provoking immunological processes and/or toxic effects on ovarian cells (161). A large Danish cohort study of 996 300 girls and women did, however, not find any association between HPV vaccination and primary ovarian insufficiency (162).

Following the Covid-19 pandemic, there have been reports of menstrual disturbances and discussions regarding infertility after corona virus infection as well as vaccination (163, 164). Several hypothetical mechanisms have been proposed, including involvement of the Renin-angiotensin-aldosterone system (RAS) which has proven to be an important pathogenic mechanism for the corona virus (165). RAS is abundant in the female reproductive system including the steroidogenesis in ovarian follicles, suggesting vulnerability to corona virus infection (166). There have been case reports

of POI following coronavirus infections (167, 168). However the observation time is limited and there is a need for more studies before causality can be established.

Environmental factors

Numerous epidemiological studies support the precipitating role of environmental factors influencing timing of endocrine transition periods and female fertility (80).

Iatrogenic POI

An iatrogenic etiology is related to medical treatment or surgical oophorectomy. It is estimated that every third woman under the age of 30 who has received cancer treatment with chemotherapy or radiation in Norway will develop iatrogenic POI (169, 170). The risk varies considerably depending on age at treatment, ovarian toxicity of the medication and radiation dose to the pelvis (171). Alkylating agents, used in treatment of cancer and some rheumatological conditions, are the drugs most frequently associated with reduced ovarian function. These drugs do not rely on cell proliferation for effect and can therefore damage dormant primordial follicles (172). Oocytes are very sensitive to radiation and, though direct pelvic radiation gives the highest risk of POI, substantial ovarian damage can be caused also by scatter radiation (173).

Smoking

Although the association between cigarette smoking and earlier menopause is well established, studies on the association with POI are inconsistent (174-176). Animal experiments have shown that exposure to cigarette smoke can affect the follicle reserve (177). In humans, maternal smoking was found to impact the fetal ovary mediated through ER and activating aryl hydrocarbon receptor (AHR) among others, reducing germ cell proliferation via downstream promotion of apoptosis (178, 179). However, further studies in humans are required to determine the exact mechanisms behind the depletion of the ovarian follicle pool that occurs after exposure to cigarette smoke and whether this constituents a cause of early menopause and POI.

Endocrine disrupting chemicals

In 1962 Rachel Carson meticulously described the detrimental effects that the pesticide dichlorodiphenyltrichloroethane (DDT) had on animal and human biology (180). Increased production of industrial chemicals over the past century has resulted in exposure to mixtures of chemicals, some of which have been identified as endocrine-disrupting chemicals (EDCs). EDCs are defined by the Endocrine Society as "*an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action*" (181) . These are typically chemicals found in everyday products such as plastic bottles, cosmetics, toys, clothes, hygiene articles, flame retardants, and pesticides. They also include pharmaceutical drugs such as contraceptives, diethylstilbesterol (DES) originally prescribed in the 1970s to prevent miscarriage, and cancer treatments such as tamoxifen (182).

Concerns have been raised regarding the possible harmful effects of EDCs on human health including reproductive development and function i.e. genital malformations, increase in hormone sensitive cancers, reduced fertility, earlier puberty and menopause (183, 184). Among EDCs associated with ovarian function are pesticides, Phthalate's, Bisphenols (e.g. BPA), Phytoestrogens, Polycyclic aromatic hydrocarbons (PAH), including Polychlorinated biphenyls (PCBs), and newer chemicals replacing substances that are being banned or phased out such as polyfluoralkylsubstances (PFAS) and polybrominated diphenyl ethers (PBDEs). Disruption of the complex network of hormones of the signal pathways involved in regulation of ovarian function may accelerate age at onset of menopause and lead to POI (90).

Although demonstrating a definite causal relationship is challenging in a clinical setting, recently several governmental and international organizations have emphasized EDCs as factors of concern in human reproduction and advise awareness in future policy goals and research strategies (185-188).

2.3.4 Clinical aspects of ovarian failure

Clinically, women with POI present with menstrual disorders and infertility (15). In primary amenorrhea, menarche has not occurred and incomplete or lacking pubertal development may be present. In secondary amenorrhea, the woman has previously had

menstruations, but has stopped menstruating. Several of the symptoms and complications that occur in POI can be attributed to estrogen withdrawal (189). (Figure 6).

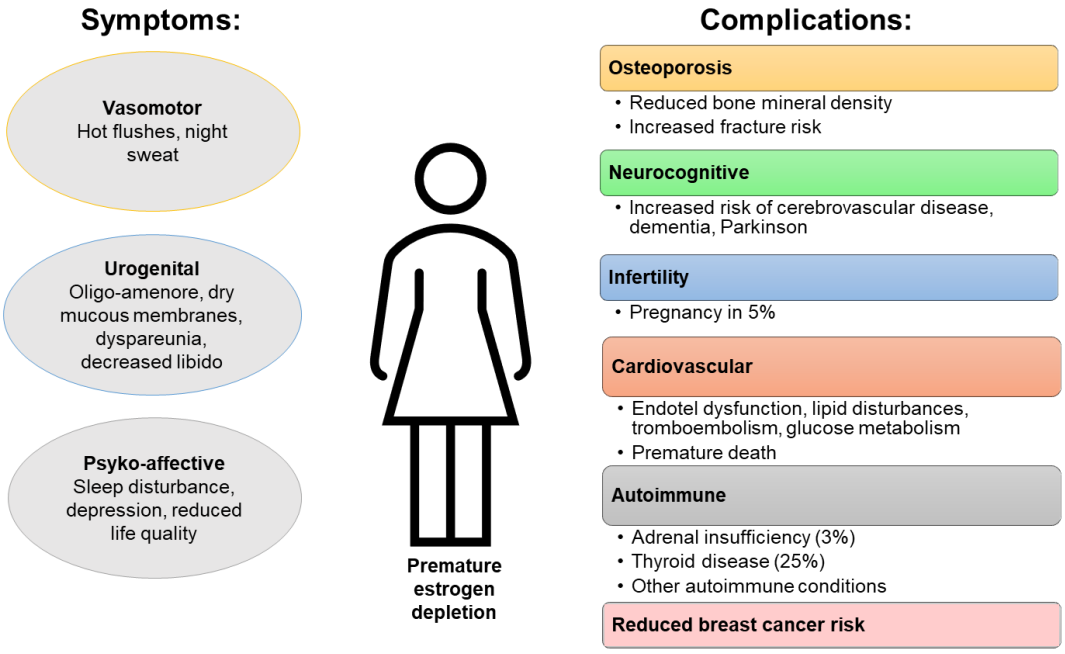

Figure 6 : Clinical consequences of POI. Symptoms and long term complications of POI.

Symptoms and complications

Vasomotor symptoms

Vasomotor symptoms are common among POI women and characterized by transient hot flushes and/or night sweats (190). Changing estrogen levels impact temperature homeostasis in the body. A proposed mechanism for vasomotor symptoms is that estrogen withdrawal causes a burst of activity in the GnRH releasing neurons, simultaneously modulating the neighboring thermoregulatory center in the hypothalamus. A narrowing of the thermoneutral zone takes place, resulting in minor elevations in core temperature triggering inappropriate compensatory episodes of

peripheral vasodilatation, increased cutaneous blood flow and heat loss. Perspiration occurs following the rapid heat loss before restoration of normal temperature (191, 192).

Vasomotor symptoms are also associated with other symptoms, the most common being sleep problems, fatigue, aching in muscles and joints (193).

Genitourinary symptoms

Decrease in estrogens and other sex steroids are involved in genitourinary symptoms such as dry mucous membranes, irritation, itching, dyspareunia and urinary incontinence (194). ERs are abundant in the vulva, vagina and bladder (195). Hypoestrogenism can cause vaginal pH to increase above 5, altered microbial profiles and loss of mucosal vascularization causing urogenital atrophy (196). ERs in the bladder and urethra are involved in maintaining sensory thresholds during bladder filling. Low levels of estrogen as well as progesterone are associated with a decrease in leak-point pressures in the bladder, resulting in urgency and incontinence problems (195, 197).

Most studies on prevalence and treatment of genitourinary symptoms have included women undergoing regular menopause while few studies have specifically been concerned with POI. It is therefore possible that aging and other contributing factors are involved in the pathogenesis of genitourinary symptoms in hypoestrogenic women (189).

Osteoporosis

Throughout life, bone mass evolves according to the shifting sex hormone balance. During the growth phase, bone formation (osteoblast activity) exceeds bone resorption (osteoclast activity). The precise mechanisms governing bone homeostasis remain unknown but likely involve both genomic and nongenomic pathways as well as a multitude of factors, including the receptor activator of nuclear factor kappa-B ligand (RANKL) signaling system, vitamin D and calcium balance (198). Estrogens are among the key regulators inhibiting osteoclasts and stimulating osteoblasts. In late puberty, increasing estrogen levels are crucial for epiphyseal closure in both females

and males. Peak bone mass is reached in the early 30s, after this age a gradual decrease in estrogen secretion results in bone resorption exceeding bone formation. During the sharp decline of estrogen levels around menopause, osteoclast activity dominates, resulting in net bone resorption (199).

Women with POI have an increased risk of osteoporosis compared to women with regular age at menopause (odds ratio 2.54; 95% CI 1.63–3.96) (200, 201). The underlying mechanisms for reduced BMD in this group are likely both insufficient peak bone mass accumulation in addition to increased bone resorption due to estrogen deficiency (95, 199).

Bone density measurement with "Dual-energy X-ray absorptiometry" (DXA) is recommended at the time of diagnosis. If bone density is low, DXA measurement should be repeated after 2-5 years (88). There are limited data available regarding fracture risk in women with POI, but treatment with continuous estrogen replacement is effective in preventing osteoporosis (15, 202).

Cardiovascular disease

Hypoestrogenism correlates with risk factors for endothelial dysfunction and atherosclerosis such as insulin resistance, unfavorable lipid profiles, hypertension, central obesity and nitric oxide disturbance (15, 203). The risk of cardiovascular disease (coronary heart disease or stroke) has been shown to be increased in women with POI (hazard ratio [HR] 1.55, 95% CI 1.38–1.73; $p<0.0001$). Each year of decrease in age at menopause is associated with a 3% increased risk of cardiovascular disease (72). Recently a large study of 1 201 175 postmenopausal women documented that a history of premature menopause was associated with a higher risk of incident heart failure (HR, 1.33; 95% CI, 1.26–1.40) and atrial fibrillation (1.09; 95% CI, 1.02–1.16), compared to women with regular menopausal age, after adjusting for the traditional cardiovascular risk factors (204).

A Norwegian longitudinal cohort study of 19 731 women found an inverse relationship between menopausal age and mortality, and concluded that early menopause is associated with premature death (205). Later meta-analyses have supported the

association between POI and increased risk of morality (206, 207). Hormone replacement therapy has shown beneficial effects on cardiovascular risk factors in women with POI (15).

Neurological and psychological complications

Several studies have shown an increased risk of Parkinsonism, dementia, depression and anxiety symptoms after surgical POI (208, 209). This association is stronger in younger woman, and is attributed to the loss of estrogen's and possibly also progesterones neuroprotective effect (59, 210). The psychological tension following a POI diagnosis can be great in light of possible infertility and potential health problems. In addition, some women experience a changed self-identity of aging early (211). Some studies also show reduced sexual function and satisfaction among women with POI. Sexuality is, however, complex and can be affected by both psychological factors and quality of life as well as genitourinary symptoms. (92, 212). The need for psychological support must receive attention in the care of in these women.

Fertility

Fertility is greatly reduced in POI; however ovarian function is fluctuating in the early stages and pregnancy after natural conception does occur in 5-10% (213). Most of these pregnancies take place within one year after diagnosis. Evidence of ovarian activity assessed by an ultrasound count of antral follicles and measurable s-AMH and inhibin-B levels are positive predictive factor, especially in cases of iatrogenic POI (214, 215).

Treatment of POI

Early diagnosis and initiation of adequate therapy is important in order to relieve symptoms, reduce the health risks and improve fertility.

Menopausal hormonal replacement therapy

Many of the health consequences of POI are directly related to premature estrogen deficiency. Hormone replacement therapy (HRT), in newer literature also called Menopausal hormone therapy (MHT), alleviates symptoms and complications of POI (216). If no contraindications are present, HRT with continuous oral or transdermal estrogen treatment is recommended . Serum estradiol levels in the reference range 250-

350 pmol / L (0.25-0.35 nmol/L), usually provide symptom relief and prevent osteoporosis (217). In young women with primary amenorrhea, gradual increase in dosage of transdermal 17β-estradiol may be used for puberty induction (70). Transdermal estradiol preparations present less risk of thromboembolism and hepatic effect than oral preparations (15). Combination birth control pills contain supraphysiological doses of ethinyl estradiol and there is uncertainty regarding the protective effect on bone density. If birth control pills are used, they should be taken continuously/for longer cycles to avoid intermittent estrogen deprivation. In women with intact uterus, progesterone should be given to protect against endometrial hyperplasia. This can be prescribed orally or transdermal in continuous or cyclic regimens. Intrauterine (IUD) with progesterone is preferred for contraceptive needs, and if the woman wants to avoid bleeding (15, 88).

The risk of breast cancer is low and the controversy surrounding menopausal hormone replacement therapy does not apply to women with POI (15). In these women, we replace hormones that are lacking and should strive for the most physiological treatment possible until regular menopausal age. Despite clear advice, there are studies demonstrating under-treatment of the condition (218). Regular follow-up is important to optimize replacement therapy in order to provide symptom relief and prevent complications.

Androgen hormone treatment

In women, the androgens are produced by the ovaries and the adrenal cortex. Thus, women with POI are not only estrogen deficient but also androgen deficient (219).

Despite evidence supporting a role for androgens in folliculogenesis, data from clinical studies on women with POI remains unconvincing and today there is no general recommendation for androgen therapy in POI women (15, 220). Some subgroups might, however, benefit from androgen replacement therapy.

Women with surgically induced POI experience an abrupt loss of ovarian steroid hormones. Improvement in sexual function including desire has been demonstrated after testosterone therapy in this group and women with surgical menopause and

hypoactive sexual desire disorder should be considered for androgen replacement therapy in addition to HRT (66, 221, 222). Women with Addison's disease and POI lack both adrenal and ovarian androgen production, consequently they have considerable lower levels of androgens than other postmenopausal women (223). Clinical trials of androgen replacement therapy in women with Addison's disease have so far shown inconsistent results regarding effect on subjective health status and sexuality, but none of the trials have specifically evaluated women with Addison's disease and POI (2, 224-226).

Fertility treatments

There are case reports and small studies on POI women that have evaluated the fertility-preserving effect of HRT and various ovarian-stimulating regimens, without convincing results (15, 190). Traditional assisted reproductive techniques involving controlled ovarian stimulation with exogenous gonadotropins are ineffective because women with POI generally lack follicles that can respond to gonadotropins.

Use of donor oocytes have proven effective, results showing a cumulative pregnancy rate after four cycles of 70-90% in POI women (213). Prophylactic cryopreservation of oocytes, embryos or ovarian tissue may be relevant options in women at high risk of developing POI. Although there are still challenges regarding revascularization of transplanted ovarian tissue, this strategy has been successful in several cases (227, 228).

3. Aims of the study

The overall aim of this thesis was to investigate epidemiology and to determine, clinical characteristics and biochemical markers of POI in order to identify the underlying etiology in more women.

The secondary aims were to:

- Assess the prevalence of autoimmune POI in a large cohort of women with Addison's disease and to describe their clinical, immunological, and genetic characteristics (Paper I)

- Assess the prevalence of premature menopause and autoimmune POI in a large cohort of women by hormonal and immunological evaluation (Paper II)

- Assess clinical and biochemical characteristics in women with newly diagnosed POI and to evaluate the underlying etiology by screening for immunological, genetic, and environmental markers (Paper III)

4. Materials and methods

Brief overviews of the materials and methods most central to this thesis are described in this section. Complete descriptions are given in the respective papers (I-III).

4.1 Participants and design

Paper I

In this observational population-based cohort study individuals with POI were identified among 461 women with Addison's disease from the National Registry of Organ-Specific Autoimmune Diseases (ROAS). The registry was established in 1996 with particular interest in Addison's disease and polyendocrine conditions. ROAS contains clinical data regarding autoimmune manifestations and concurrent disease, autoantibody profiles, and genome-wide single nucleotide polymorphism (SNP) results. The Norwegian Prescription Database was used to assess prescription of menopausal hormone replacement therapy (HRT).

Paper II

This was a retrospective cross-sectional study. The study population consisted of 6870 women from the second follow-up of the European Community Respiratory Health Survey carried out 2010-2012 (ECRHS III) and The Respiratory Health in Northern Europe, Spain and Australia generation study (RHINESSA) carried out 2013-2016 (www.ecrhs.org and www.rhinessa.net).

Paper III

In this prospective cross-sectional study 100 women with newly diagnosed POI of unknown cause referred for evaluation to the Endocrine or Gynecology outpatient clinics at Haukeland University Hospital, Bergen, Norway, between January 2019 to December 2021, were consecutively included and systemically evaluated. All participants were seen by the same examiner.

4.2 Laboratory

4.2.1 Assays of hormones

FSH and LH, prolactin, adrenocorticotropin (ACTH), thyroid stimulating hormone (TSH), free thyroxine (fT4), vitamin D, sex hormone binding globulin (SHBG) and Anti-Muller hormone (AMH) were analyzed using chemiluminescent immunoassays (Haukeland University Hospital, Bergen, Norway. Paper I and II) and (Demeditec Diagnostics, Kiel, Germany. Paper II). The steroid hormones (17β-estradiol, progesterone, testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEA-S) and cortisol were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (University of Bergen, Norway, 2017) (223)(35).

4.2.2 Assays of autoantibodies

Radio-Ligand Binding Assays (RIA) (All papers)

All autoantibody assays were performed in the laboratory at the Faculty of Medicine (University of Bergen, Norway). Serum samples were analyzed using recombinant proteins marked with a radioactive tracer (229). In summary, full-length cDNA clones encoded genes of interest were subcloned into expression vectors, transcribed and translated *in vitro*, thereby enabling the incorporation of ^{35}S- labelled methionine. Radiolabeled antigens were incubated with serum samples and immune complexes were precipitated with protein-A Sepharose in 96-well filter plates. Radioactive decay was measured in a liquid scintillation counter (230). Samples were tested as duplicates or triplicates. Positive cut-offs were calculated using positive and negative controls. Normal limits were given as three standard deviations above the mean of 100 healthy controls to avoid over-diagnosis. Samples were tested in duplicates and index values calculated as ((counts per minute (cpm) sample-cpm negative control)/(cpm positive control-cpm negative control)) x 1000. Index thresholds were >57, >200, >102 and >65 for 21-OH, SCC, 17-OH and NALP-5, respectively. Positivity for ovarian associated autoantibodies was perceived as likely autoimmune POI.

Enzyme-Linked Iimmunosorbent Assays (ELISA) (Paper I and III)

Autoantibodies against thyroid peroxidase (TPO) were analyzed at the Hormone laboratory at Haukeland University Hospital, by electrochemiluminescence immunoassay (Roche Cobas), accredited by NS-EN ISO 15189:2012.

4.2.3 Genetic tests

GWAS (Paper I)

Whole-genome genotype data were retrieved for 432 women with Addison's disease from ROAS, of whom 44 had POI (231). One hundred and four candidate genes were selected based on the Genomics England list of POI genes and risk loci for Addison's disease (106, 231). For each candidate gene, the longest gene transcript region (defined by the University of California Santa Cruz Gene Browser) was selected, and all SNPs within these regions were extracted from quality-controlled imputed genotype data (for details on input data and imputation (231). The variant sets were pruned for triallelic markers and markers in linkage disequilibrium , and a set-based association test was performed in Plink (232). This allows the assessment of SNPs in a set (here, all SNPs in any given gene) in aggregate, increasing the power relative to testing them individually.

Next generation sequencing (Paper III)

DNA was extracted from whole blood EDTA and sonicated to fragments for sequencing library preparation. Libraries for whole exome sequencing (WES) were prepared using SEQCap EZ HyperCap (v1.2) or KAPA HyperCap (v3.0) library preparation kits, with SeqCap EZ MedExome- or KAPA HyperExome target enrichment kits, respectively (all kits and reagents from Roche). Libraries were sequenced on Illumina NextSeq500 or Illumina NovaSeq 6000 instruments using paired-end 150 bp or 100 bp reads, respectively. Data processing, alignment to GRCh37 and variant-calling were performed essentially as described (233), except that GATK v.3.8.1 were used according to GATK's Best Practices guidelines (234, 235). Variant annotation and interpretation were performed using Alissa Interpret (Agilent Technologies) and Alamut Visual 2.15 (Sophia Genetics). Gene panel-based filtration of variants were performed using the expert curated Primary Ovarian Insufficiency

(Version 1.60) panel from Genomics England PanelApp, and an in-house designed panel for general hypogonadism (partly based on the Hypogonadotropic Hypogonadism panel from Genomics England PanelApp).

Karyotyping (Paper III)

G-banded chromosome analysis was performed using phytohemagglutinin-stimulated 3-day blood lymphocyte cultures according to standard protocols(236). At least 10 metaphases were screened for structural and numerical abnormalities. In four cases of suspected mosaicism for structural abnormalities on the X chromosome, chromosomal G-banding was also performed using cultured skin fibroblasts.

Copy number variant analysis (Paper III)

Submicroscopic genomic copy number variations (CNVs) such as deletions and duplications, and long stretches of homozygosity (LOH) were investigated by the CytoscanHD array (Thermo Fisher Scientific) following the supplier's protocol and analysis software.

FMR1 premutation testing (Paper III)

CGG repeat numbers in the 5' untranslated region of the *FMR1* gene were determined by the AmplideX® *FMR1* PCR kit from Asuragen using both fluorescent gene-specific and CGG repeat-specific primers. Amplified PCR products were mixed with HiDi™ Formamide and Rox1000 Size Ladder (Asuragen) and run on an ABI3130XL Genetic Analyzer (Applied Biosystems). Fragment analysis was performed using GeneMapper v5.0 software (Applied Biosystems). Alleles containing between 55 and 200 CGG repeats were defined as premutations.

4.3 Statistics

Continuous data were presented as medians and range (interquartile range (IQR)/minimum–maximum), or mean and standard deviation (SD) or 95% confidence interval (CI). Categorical statistics were presented as absolute numbers and percentages. For between-group comparisons we used the independent sample t-test or the Mann–Whitney independent sample U test as appropriate depending on data

distribution. A chi-squared test for independence (with Yates continuity correction) was used for assessing the association between categorical variables (All papers). Correlations were investigated using Pearson product moment correlation or the Spearman rank correlation (Papers I and III). Multivariable regression models adjusted for confounders were used to assess relationships between variables (paper II). Cohort co-dependency (family clustering) was examined by generalized estimate equations (GEE) and separate cohort analyses. A two-way between-group ANOVA was used to explore the difference between groups (paper II). Bonferroni-adjusted significance was included for between-group differences to avoid type I errors (false positives) (paper I). The statistical analyses were performed using IBM SPSS Statistics 22/23.

4.4 Ethics

All studies included in this thesis were conducted in accordance with the Declaration of Helsinki and approved by the regional Committee for Medical and Health Research Ethics: 2018/1537/REK Vest (paper I), 2012/1077/REK Vest (paper II), 2018/1206/REK Midt (paper III). All participants signed informed consent forms for registration approved by ROAS (2013/1504/REK Vest) (paper I and III) or from the appropriate ethics committees of each study center (paper II).

4. Results and summary of papers

4.1 Paper I: 'Primary ovarian insufficiency in women with Addison's disease'

In this study we assessed the prevalence of POI in a large national population-based cohort of women with Addison's disease (n=461) and described clinical, immunological, and genetic characteristics.

The prevalence of POI among women with Addison's disease was 10.2%, and one-third developed POI before 30 years of age. There was a strong positive correlation between age at diagnosis of Addison's disease and age at menopause. We found that POI preceded or coincided with the diagnosis of Addison's disease in more than half of the women. Concomitant autoimmune diseases were abundant among all women in the study, with 72% exhibiting at least one autoimmune comorbidity, hypothyroidism being the most common condition. The frequency of associated autoimmune disease did not differ between women with and without POI.

All women with Addison's disease had been prescribed glucocorticoid therapy for systemic use and the majority (95.0%) mineralocorticoid replacement therapy. Among the women with Addison's and POI, two-thirds were treated with HRT.

The women with POI had more autoantibodies than women without POI (≥2 autoantibodies in 78% vs 25%). Autoantibodies against SCC had the highest accuracy with a negative predictive value for POI of 96%. Autoantibodies against 17-OH were the third most common autoantibodies among the women with POI, but the specificity and sensitivity was low (71% and 49%). None of the women with Addison's disease tested positive for NALP-5 autoantibodies.

We also tested associations to 104 POI- or Addison disease-associated genes. Three loci (FOXL2, PNO1, and DDX4) reached nominal significance with a minor allele frequency >5% and are gene variants of interest in future studies of Addison and POI.

To conclude, we demonstrated a high prevalence of POI among women with Addison's disease. Diagnosing autoimmune POI remains challenging and relies on clinical, biochemical and immunological testing. Although POI diagnosis precedes/coincides with Addison's disease in more than half of the women, later POI debut was also common, emphasizing the need for conscientious follow up. As autoantibodies against SCC seem to be the most specific marker for autoimmune POI in women with Addison's disease, we recommend testing all women with Addison's disease <40 years who experience menstrual disturbances or fertility concerns for autoantibodies against SCC.

4.2 Paper II: 'Premature menopause and autoimmune primary ovarian insufficiency in two international multi-center cohorts'

In this study we reported the prevalence as well as clinical, endocrine and immunological markers of premature menopause (<40 years) and autoimmune POI in a general population of 6870 European women.

The prevalence of premature menopause was 2.8%. Approximately half of these women (47%) had iatrogenic reasons for premature menopause (ovarian surgery, cancer treatment), while non-ovarian reasons (hypothalamic amenorrhea, eating disorders and pituitary failure) were identified in 14%. The etiology was unknown in 39%.

In total the prevalence of idiopathic POI was 1.1%. We found immunological markers suggesting an autoimmune etiology in 4.5% of women with POI, using specific autoantibody assays. Positive 21-OH autoantibodies were found in three women with POI, one of whom also had positive SCC autoantibodies. Autoantibodies against 17-OH were abundant in both groups. None had positive NALP 5 autoantibodies.

We identified several reproductive and lifestyle-related factors associated with the timing of menopause. Women with premature menopause had a higher frequency of nulliparity, obesity, osteoporosis, use of HRT, and never smokers, compared to

menopause ≥40 years of age. Nulliparity is the variable most strongly associated with POI, but no determinant that could distinguish autoimmune POI from other causes of premature menopause.

4.3 Paper III: 'Improving diagnostic precision in primary ovarian insufficiency using comprehensive genetic and autoantibody testing'

In this study we evaluated the phenotypes and distribution of underlying etiologies in 100 women with newly diagnosed POI of unknown cause.

Standard recommended diagnostic investigations including screening for chromosomal anomalies and FMR1 premutations were performed. In addition we added a large POI specific NGS gene panel and specific immunoprecipitating assay of steroid cell autoantibodies.

The median age at menopause was 33 [29-39] years, and 15% of the women had primary amenorrhea. A family history of POI among first degree relatives was reported in 20% and approximately half of the women had previously been pregnant (51%). Reduced bone mineral density was common (23%). The frequency of concomitant autoimmune disease was also high with 23% exhibiting at one or more conditions including hypothyroidism, diabetes type 1 and multiple sclerosis (MS).

By including genetic and autoimmune markers in screening, we were able to increase the etiological diagnosis from 11% to 45%. Chromosomal aberrations were found in 5%, FMR1 premutations in 3% and POI associated genetic variants in 34%. Autoimmune POI was identified in 3%, by positive 21-OH and SCC autoantibodies.

Of particular interest, we found two siblings that were both homozygous carriers for a loss-of-function variant in the *ZSWIM7* gene. Furthermore, in three unrelated women we detected rare variants in the *SOX8* gene. Both these genes have been associated with female and male infertility

There were no specific phenotypical markers that predicted the underlying cause of POI, actualizing the discussion of which tests should be a part of diagnostic screening in clinical practice.

5. Discussion

This thesis explores clinical, immunological, and genetic markers of the pathogenetic mechanisms of POI.

5.1 Challenges in finding the underlying cause of POI

Since F. Albright first described the clinical characteristics of POI there have been considerable advances in our understanding of the condition. While it is clear that POI is inducible by internal and external factors the specific pathogenic mechanisms behind POI remain largely unidentified. An increasing understanding of the synergistic interplay between genetic susceptibility, environmental triggers, and innate immune regulatory mechanisms is starting to emerge, advocating a new model of understanding of POI (Figure 7).

The disease triangle concept was formalized as an empirical tool in disease epidemiology in the 1960s by George McNew "*to study the interrelationship of various factors*" (237). The parameters of the disease triangle are described as "*the inherent susceptibility of the host* (genetics)*, the inoculum potential of the pathogen* (environmental factors, lifestyle, infections, toxins etc)*, and the impact of the host environment* (immune system, autoantibodies) *on pathogenesis"* (238). This resonates with current advances in the understanding of the complexity of POI etiology. Genetic susceptibility modulated by recognized environmental factors can trigger an ubiquitous immune response resulting in damage of ovarian function, all factors contributing to disease etiology.

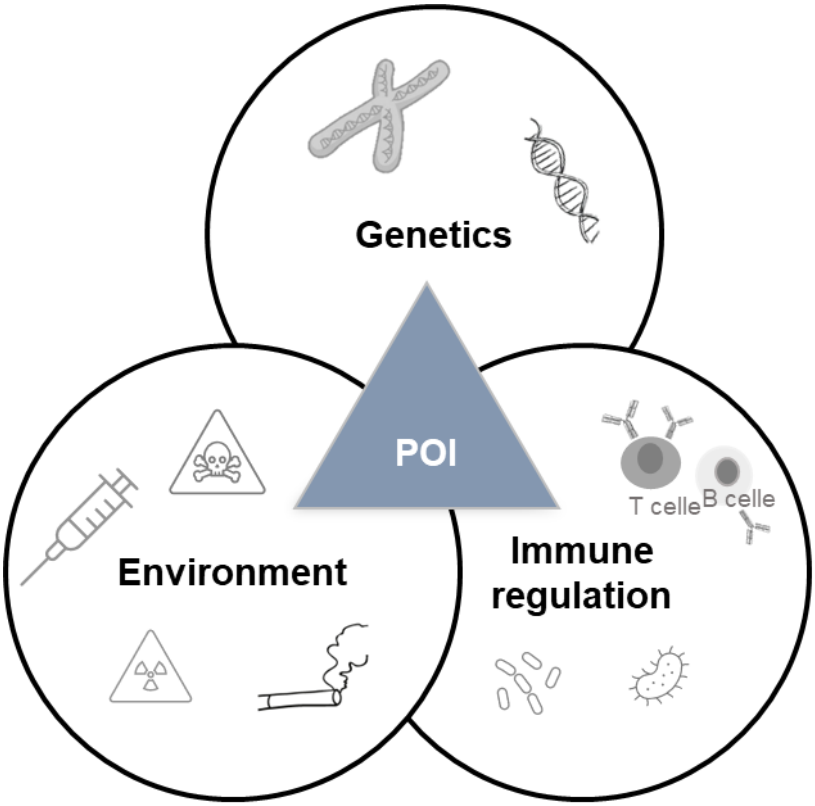

Figure 7. Disease triangle of POI. Interplay of genetic, environmental and immune factors in precipitation of POI.

5.1.1 Diagnosing genetic POI

The introduction of NGS technology has dramatically altered the availability and effectiveness of genetic testing. At present, extended testing of women with POI using NGS gene panels are not included in routine assessment (15, 88, 93). NGS has however proven to be a powerful tool unraveling genetic variants in numerous genes that are constantly expanding, as illustrated by both our findings and previous studies (16). We found that screening for genetic POI variants was valuable both in generating hypothesis (Paper I) and in diagnostic evaluation (Paper III).

Tailored NGS POI gene panels may be useful in diagnostic testing of women affected, as well as in predictive genetic screening of family members and women at risk of POI (239). Obviously, a dynamic revision of which genes to include in disease specific NGS gene panels is important. Custom panels must be modified over time, allowing for the addition of novel genes found to be involved in POI or the removal of genes that upon reevaluations are found not be associated with POI.

Advancement of precision and personalized medicine have, however, raised several ethical dilemmas regarding use of NGS. First of all there is a considerable degree of uncertainty regarding pathogenic accuracy as well as variable penetrance of autosomal genetic disease. Not everyone with a POI associated genetic variant develops the disease. This must be considered both in diagnostic and predictive testing, especially regarding screening of healthy relatives. On the other hand, information about the risk of POI can enable these women to make adjustments in their lives in order to deal with potential fertility issues. Awareness of the implications and limitations of genetic testing as well as clinical counselling is essential. Clinicians should have a clear understanding of the patients' phenotype, as well as the medical- and family histories, in order to ensure appropriate interpretation of variants.

The question of *Who should be genetically tested* remains controversial and cost-effectiveness analyses are required to aid sustainable diagnostics in clinical guidelines.

5.1.2 Diagnosing autoimmune POI

The recommended diagnostic autoimmune workup for POI is today restricted only to screening for autoimmune disease and the measurements of steroid cell autoantibodies (SCA/21-OH) as well as thyroid autoantibodies (15, 88, 93).

We found ovarian-associated steroidogenic cell autoantibodies in 3-4.5% of women with idiopathic POI in two different populations, pointing to a prevalence of autoimmune POI in the lower range of what has previously been reported (Paper II and Paper III). This could be due to use of specific immunoprecipitating assays in the present studies, as opposed to most previous studies that have applied sensitive but less specific indirect immunofluorescence methods for autoantibody detection.

Establishing a definite autoimmune causal mechanism for POI remains challenging and relies on clinical, immunological, and histological features. First of all, autoimmune POI is rarely a dichotomous event. Several years of fluctuating ovarian function may precede complete ovarian failure (85). The diagnosis of autoimmune POI based on criteria of positive ovarian associated autoantibodies is therefore not optimal as it represents the end-stage exhaustion of ovarian reserves. Autoantibody detection can be hampered by the fact that the diagnostic benchmarks of POI with amenorrhea and increased FSH levels, appear late in the disease course, thereby paralleling the loss of the antigenic target (128). Identifying autoimmune oophoritis at an early stage is important as the theca cells of the late-stage follicles seem to be the primary autoimmune target while the granulosa cells and the earlier stage follicles are initially spared, thus representing a potential therapeutic *window of opportunity* for immunotherapeutic intervention (112).

Although use of immunoprecipitating assays allow for detection of specific autoantibodies, such assays are limited to *a priori* knowledge of the autoantigens. In addition, universally standardized autoantibody assays are missing. As previously discussed, multiple ovarian autoantigens have been proposed as possible targets for autoimmune POI. In the latter years explorative approaches for identifying novel autoantigens have been developed. One such method is use of antigen arrays of proteins or peptides, offering the possibility to screen for serum reactivity against hundreds of autoantigens (240). This technology has potential to discover novel biomarkers of autoimmune POI and even for development of antigen-specific therapies (241).

Further, the complex interplay between immunological and genetic mechanisms are being unraveled. There is a strong hereditary component in autoimmune disease and several genes with a role in immune function and POI have been identified through GWAS (84). Many genes involved in immune regulation are located on the X chromosome and genetic risk loci predisposing for autoimmune disease have been identified in POI women with X chromosome anomalies, including Turner syndrome (242-245). Although most risk variants have subtle effects on disease susceptibility, they provide support for possible autoimmune mechanisms, including pathways for

antigen presentation, cytokine and TNF signaling and transcriptional regulation (246, 247).

5.1.3 Diagnosing environmental factors

Iatrogenic POI caused by gonadotoxic medical treatment or surgery is usually easy to recognize. Identifying a link between lifestyle environmental factors and POI can however be challenging. Although it is well-established that environmental contaminants can have endocrine-disrupting effects on the ovaries, distinguishing the specific environmental triggers in a clinical setting is complicated (248, 249).

First of all, EDCs challenge the traditional toxicological dogmas of monotonic dose-response effects, as U- or inverted U-shape **non-monotonic dose response curves** are often observed. This means biological effects can be found at low exposure levels, similar to the response of low concentration endogenous hormones with high hormone receptor affinity (182). In addition there can be a **long time-lag** between exposure and effect. Early-life exposure to EDCs during **sensitive windows of susceptibility** such as *in utero,* childhood or puberty may disrupt development and increase risk of developing diseases in adulthood (250, 251). Today the chemicals measured in experiments and studies represent only a small fraction of the **"cocktail**" of chemicals we are exposed to in real life. Hence, there is a need to develop efficient methods and risk assessments for mixtures of chemicals in addition to the current strategy of single chemical exposures (252).

Animal studies of EDCs' effect on ovarian function have provided additional insights into POI etiology and pathogenesis (90). Despite limited possibilities of confirming direct causal relationships between EDC exposure and POI in individual cases, caution is warranted on a population level. There is enough evidence of association between EDCs and reproductive function that public preventive measures to minimize exposure should be taken.

5.2 Why is it important to find underlying cause of POI?

Current challenges regarding diagnosis of POI include identification of efficient markers of genetic, immunological and environmental factors. Understanding the underlying mechanisms of POI are essential not only in order to allow precise etiological diagnosis but also in order to tailor new treatment options for POI. Medical technology is advancing and the commercial interests are strong. New opportunities for precision medicine have opened up areas of pharmacogenomics, i.e. regarding cancer treatments. Increased understanding of the molecular mechanisms involved in the development of POI can enable innovative engineered treatment options for this condition also.

Gene therapy

Novel stem cell technologies are challenging the conventional concept that the ovary cannot produce new oocytes after birth (253-255). Increased understanding of the major signaling pathways and the various autocrine and paracrine intra-ovarian regulators governing follicle recruitment has opened the way for targeted interventions to prolong the reproductive lifespan of women with POI. Treatment strategies include in vitro activation of ovarian residual follicles (IVA), autologous stem cell ovarian transplantation and intra-ovarian infusion of platelet-rich plasma (256, 257).

IVA techniques involve the recruitment of dormant primordial follicles into folliculogenesis for eventual ovulation. Crucial intracellular signal transduction pathways of follicle activation are concentrated in PTEN-PI3K-Akt-FOXO3 and Hippo pathways (104). Techniques for modulating these pathways *in vitro* include pharmaceutical treatment protocols and laparoscopic fragmentation of ovarian cortical tissue (258). IVA approaches have been used in human patients with a small number of live births reported (30).

Autologous stem cell ovarian transplantation has the potential to increase reproductive capacity in patients who are poor responders. Stem cells are pluripotent cells with the ability to self-renew and differentiate into specific tissues according to signals from the surrounding environment (259). Autologous stem cells can be retrieved from bone

marrow, adipose tissue, peripheral blood cells, amniotic fluid, placenta, among others. Stem cell therapy has shown promising results in restoring reproductive tissue in animal POI models (260, 261). Although initial findings from therapeutic studies on women with POI appear promising, there remain technical issues and ethical concerns. Further research and clinical trials need to be conducted to standardize the details of the transplantation process (262).

Platelet-rich plasma may be considered as a putative alternative strategy for treating POI. Platelet-rich plasma is a preparation of autologous human plasma including a multitude of growth factors and cytokines produced by platelets with the potential to stimulate existing ovarian stem cells through paracrine mechanisms. The concentrate is injected directly into the ovarian cortex by laparoscopy (263). Studies have demonstrated improved markers of ovarian reserve, menstrual cycle restoration, improved oocyte yields as well as pregnancies in POI women. Still, these results should be interpreted with caution given small and variable study designs (264).

In the future identification of POI gene variants could also potentially open for genetic editing using CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats and CRISPR associated nuclease 9)-related technologies to correct underlying gene defects (265).

Immune modulating therapy

Observational data suggest that immunosuppressive therapies may be beneficial in restoring ovarian function, especially in autoimmune POI. In autoimmune POI primordial follicles are spared, while growing preantral and antral follicles are destroyed. Granulosa cells appear viable for a period of up to several years after diagnosis as evidenced by normal levels of AMH and inhibin, although they are eventually depleted (120). Immunosuppressive therapy could potentially enable ovulatory cycles to recur, and prolong the *window of opportunity* to conceive in women with autoimmune POI.

A few case reports and small clinical studies using glucocorticoids have shown promising results on ovarian markers and fertility (266-269). Immune-modulating

therapy with monoclonal antibodies against CD20, a marker of B-cells, have been tried in a few other autoimmune endocrine diseases such as Graves's disease, diabetes type 1 and Addison's disease (270-272). A trial with Rituximab for diabetes type 1 was found to transiently reverse the decay of β-cell function (273). A pilot study using Rituximab in recent-onset Addison's disease showed various outcomes for the few included patients (272). Currently Rituximab is being tested to restore ovarian function in POI (EudraCT Number: 2017-004532-10).

The treatment options discussed above are likely just the tip of the iceberg, towards tailoring future personalized therapy options. Continuing to unravel pathogenetic mechanisms is therefor of clinically relevance to women with POI.

5.3 Methodological considerations

From a methodological point of view, several considerations apply to the studies in this thesis and have already been mentioned in the above as well as in the respective papers.

Some limitations apply to all the studies in this thesis, including the use of candidate markers of genetic variants and autoantibodies. This approach allows us only to identify already known markers of interest, limiting the possibility to discover new and potentially more specific immunological and genetic markers of POI.

The study designs also did not allow for longitudinal follow-up and repeated testing of diagnostic markers. POI is a continuum of ovarian dysfunction and may proceed through several biochemical stages.

Finally, the Covid-19 pandemic resulted in a delayed recruitment of patients to study III. The originally planned full scale etiological assessment of the women in this study included analysis of EDCs and immune markers. These analyses are still ongoing and could unfortunately not be included in this thesis.

6. Conclusion

In accordance with the specific aims of this thesis the following conclusions can be drawn:

- The prevalence of POI among Addison women is high (10.2%), compared to the prevalence in the general population (1.1%).
- POI diagnosis most often precedes, but can also debut after Addison's disease (1/3 of cases), emphasizing the need for conscientious clinical follow-up.
- Women with Addison's disease and POI have a higher frequency of positive autoantibodies than Addison women without POI. Autoantibodies against SCC are the most specific marker for autoimmune POI in women with Addison's disease, and we recommend testing younger women with menstrual disturbances or fertility concerns for SCC autoantibodies.
- We identified three genetic variants of interest in Addison women with POI.
- By extensive work-up it is possible to increase the identification of a possible underlying cause in women with idiopathic POI from 11%-45%
- Underlying genetic defects explain POI in almost half the cases: X chromosome anomalies found in 5%. FMR1 premutations identified in 3%, while POI associated genetic variants account for 34%. .
- Autoimmunity explains 3-4.5% of spontaneous POI cases judged by steroidogenic autoantibodies. Autoantibodies against 21-OH are the most useful markers for autoimmune POI in women with idiopathic POI.
- Several reproductive and lifestyle-related factors are associated with timing of menopause, but nulliparity is the variable most strongly associated with POI.
- No association was found between the POI phenotype and genotype.

7. Future perspectives

This thesis investigates the epidemiology, clinical characteristics and biochemical markers of POI, aiming to identify the underlying etiology in more POI women.

Studying genetic markers has shown us the huge potential novel genetic techniques have to improve diagnostic precision in POI. There are however still challenges in genetic screening, not least regarding evaluation of relevance of genetic variants explaining and predicting POI. Growing genetic sample collections and **collaborative interpretive approaches** will hopefully enable more precise classification of pathogenic genetic variants in the coming years. Also, as the trend of upward shift in childbearing age in many populations continues, future **genic risk scores** predicting the risk of POI could be valuable for women planning pregnancies.

One of the central questions in autoimmune research is: What governs immune system selection of autoantigens? We are planning a follow-up the women from *study III* to explore the repertoire of immune mediators in blood. Our research group has recently established new methods to identify biochemical inflammatory markers, including protein platforms with **proximity extension assays, mass cytometry and single cell RNA sequencing of immune cells.** We hope to phenotypically map the immunological fingerprint in POI women compared to fertile healthy women of the same age. Also we want to explore subgroups with autoimmune POI by **advanced antigenic profiling of ovarian biopsy tissue**, hopefully contributing to further uncover pathogenic mechanisms of autoimmune oophoritis. The ultimate goal being to find immunological markers that can identify these women before they develop ovarian failure.

Consistent with findings that EDCs affect reproductive aging, we have extended investigations of the study cohort in *study III* to include a panel of **EDCs** examining the association between serum per- and polyfluoroalkyl substances (PFAS). Age adjusted groups of never-pregnant women as well as blood donors are used as control groups. Blood tests are currently being analyzed at the Environmental Pollutant Laboratory, Department of Laboratory Medicine, University Hospital of North Norway

(Tromsø, Norway). We thus hope to demonstrate whether PFAS may be potential risk factors for POI.

One of the consequences of more precise diagnostics of POI etiology is the possibility to offer precision medicine. In collaboration with Karolinska Institute in Sweeden we are conducting a study assessing whether **immunosuppressive treatment** can halt the autoimmune destruction of the ovaries in women with autoimmune POI. We have currently included 10 women in this open label proof-of concept study doing controlled ovarian hyperstimulation before and four months after two infusions of 1-gram Rituximab (Mabthera®). Preliminary results are promising with restoration of ovarian function in approximately half of the women evaluated by reestablishment of spontaneous menstrual cycles and/or egg retrieval. We are looking forward to further results.

The obvious extension of our clinical POI study is to continue inclusion of more patients and follow-up the patients already included in our national registry ROAS. Ultimately a collaborative effort towards an international **POI database** could facilitate high quality data for future research.

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Clinical Research Article

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Primary Ovarian Insufficiency in Women With Addison's Disease

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Abbreviations: 17OH, 17-alpha hydroxylase; 21OH, 21 hydroxylase; AAD, autoimmune Addison's disease; ATC, Anatomical Therapeutic Chemical; FSH, follicle-stimulating hormone; GAD, glutamic acid decarboxylase; HRT, hormone replacement therapy; MAF, minor allele frequency; NALP-5, NACHT leucine-rich-repeat protein 5; NorPD, Norwegian Prescription Database; OER, observed to expected ratio; ROAS, National Registry of Organ-Specific Autoimmune Diseases; SCC, side chain cleavage enzyme; SNP, single nucleotide polymorphism; POI, primary ovarian insufficiency; TPO, thyroid peroxidase.

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Abstract

Context: Primary ovarian insufficiency (POI) is defined by menopause before 40 years of age. POI prevalence is higher among women with autoimmune Addison's disease (AAD) than in the general population, but their clinical characteristics are insufficiently studied.
Objective: To assess the prevalence of POI in a large cohort of women with AAD and describe clinical, immunological, and genetic characteristics.
Methods: An observational population-based cohort study of the Norwegian National Addison Registry. The Norwegian Prescription Database was used to assess prescription of menopausal hormone replacement therapy (HRT). A total of 461 women with AAD were studied. The primary outcome measure was prevalence of POI. Secondary outcomes were clinical characteristics, autoantibodies, and genome-wide single nucleotide polymorphism variation.
Results: The prevalence of POI was 10.2% (47/461) and one-third developed POI before 30 years of age. POI preceded or coincided with AAD diagnosis in more than half of the women. The prevalence of concomitant autoimmune diseases was 72%, and AAD women with POI had more autoantibodies than AAD women without (≥2 autoantibodies in 78% vs 25%). Autoantibodies against side-chain cleavage enzyme (SCC) had the

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highest accuracy with a negative predictive value for POI of 96%. HRT use was high compared to the age adjusted normal population (11.3 % vs 0.7%).
Conclusion: One in 10 women with AAD have POI. Autoantibodies against SCC are the most specific marker for autoimmune POI. We recommend testing women with AAD <40 years with menstrual disturbances or fertility concerns for autoantibodies against SCC.

Key Words: primary ovarian insufficiency, primary adrenal insufficiency, 21-hydroxylase, side-chain cleavage enzyme, hormone replacement therapy

Primary ovarian insufficiency (POI) is characterized by decreased follicle function and deficient steroid hormone production and is diagnosed by amenorrhea for >4 months and follicle-stimulating hormone (FSH) in the menopausal range before 40 years of age (1). It is a condition of multiple etiologies including genetic and immunological factors, although the majority of cases remain idiopathic (2). Autoimmunity is estimated to be responsible for approximately 5% to 30% of POI cases, reflecting heterogeneity of patient cohorts studied (3).

The prevalence of POI has been reported to be higher among women with autoimmune Addison's disease (AAD) (6-20%) than in the general population (1-2%) (1, 4-6). POI in these women is understood to be a consequence of an autoimmune oophoritis with immune infiltrate selectively involving the theca cells (7). The initial sparing of granulosa cells of primordial follicles is reflected by detectable levels of anti-Müllerian hormone and inhibin, as well as fluctuating ovarian function during the first years after debut (8, 9). Hormonal replacement treatment is recommended to prevent complications of estrogen deficiency (1).

Ovarian biopsy is not advised in routine diagnostic workup. Instead, autoantibodies are used as surrogate markers of ovarian autoimmunity (1). Earlier methods based on indirect immunofluorescence could detect reactivities towards tissue components, but not the specific autoantigen. These methods, which are tissue and investigator dependent and therefore difficult to standardize, have largely been replaced by more sensitive immunoassays testing the presence of specific autoantibodies (10, 11). Good correlations between histologically verified autoimmune oophoritis and autoantibodies against the steroidogenic enzymes 21 hydroxylase (21OH), side chain cleavage enzyme (SCC), and 17-alpha hydroxylase (17OH), as well as NACHT leucine-rich-repeat protein 5 (NALP-5), have been published (12-15). As steroidogenic enzymes are expressed in both the adrenal cortex and ovaries, the specificity of these autoantibodies in women with autoimmune adrenal cortex deficiency is uncertain.

Spontaneous POI has been linked to multiple genetic loci involved in oogenesis, folliculogenesis, DNA damage repair, homologous recombination, and meiosis (16, 17). To date, there are limited data on any potential genetic basis of autoimmune POI, but a recent genome-wide association study on AAD can perhaps provide hints to the pathogenesis (18).

To overcome previous limitation of smaller and potentially biased cohorts, we here provide data on epidemiology, clinical features, autoantibodies, and genetic background of autoimmune POI from a national registry on Addison's disease. We find that 1 in 10 women with AAD develop POI, a result that has widespread clinical implications.

Patients and Methods

Patients, Registries, and Data Collection

Individuals with POI were identified among AAD patients in the National Registry of Organ-Specific Autoimmune Diseases (ROAS), encompassing the Norwegian Addison Registry. The population is almost exclusively Caucasian/Scandinavian. Of the 540 women registered with Addison's disease, 57 were excluded because of nonautoimmune causes or incomplete data. We also excluded 22 women with known autoimmune polyendocrine syndrome type-1, leaving 461 women with AAD who all had autoantibodies against 21OH. The registry contains information on age at AAD diagnosis, age at menopause and other autoimmune manifestations autoantibody profiles, and genome-wide single nucleotide polymorphism (SNP) data (18, 19). POI was diagnosed based on standard clinical and biochemical criteria (1) oligo/amenorrhea, and (2) an elevated FSH level in menopausal reference range (1). The information was confirmed with self-reported biobank data and telephone interviews regarding time of menopause.

Autoantibody Assays

ROAS biobank serum samples are routinely analyzed for autoantibodies against 21OH, SCC, 17OH, glutamic acid decarboxylase (GAD), and NALP-5 using radiobinding ligand assays as described previously (20). Autoantibodies against thyroid peroxidase (TPO) were analyzed by electrochemiluminescence immunoassay (Roche Cobas).

Genetic Analyses

Recently generated whole-genome genotype data were retrieved for 432 women with AAD from ROAS, of whom 44 had POI (18). Due to the low number of patients with POI, we selected 104 candidate genes based on the Genomics England list of POI genes and risk loci for AAD (18, 21) (see supplementary table for details (22)). For each candidate gene, the longest gene transcript region (defined by the University of California Santa Cruz Gene Browser) was selected, and all SNPs within these regions were extracted from quality-controlled imputed genotype data (for details on input data and imputation, see (18)). The variant sets were pruned for triallelic markers and markers in linkage disequilibrium , and a set-based association test was performed in Plink (23). This allows the assessment of SNPs in a set (here, all SNPs in any given gene) in aggregate, increasing the power relative to testing them individually.

Medication Records From the Norwegian Prescription Database

The use of hormone replacement therapy (HRT) among women with AAD younger than 40 years was obtained using The Norwegian Prescription Database (NorPD), which contains information of quantity, dosage, expenditure, and reimbursement of all prescription drugs that have been dispensed from pharmacies to individual patients (24). NorPD does not contain information on diagnosis or indication for treatment and the data was anonymous linked to ROAS using personal identity numbers assigned to all individuals living in Norway. Age- and sex specific reference populations are provided. All drugs are classified according to the Anatomical Therapeutic Chemical system (ATC number). We used data on glucocorticoid therapy for systemic use (H02AB), mineralocorticoid replacement therapy (H02AA), hormonal contraceptives for systemic use (G03A), estrogens (CG03C), and progesterone and estrogens in combination (G03F), prescribed ≥1 time between 2004 and 2018. The expected number of users were found by calculating the proportion of women aged 20-39 years from the general population (n = 161 969) who had been prescribed drugs in the mentioned ATC groups, and multiplying by total number of women with AAD in the same age group (n = 194). Observed to expected ratio (OER) of users was found by dividing observed number of women with AAD prescribed a drug in the ATC groups with the expected number of users. Thus, an OER above 1 indicates an increased use, and an OER below 1 indicates a reduced use of the respective drugs in women with AAD.

Statistical Analyses

Categorical statistics were presented as absolute numbers and percentages while continuous data were presented as medians and range (minimum–maximum), or mean ± SD or 95% CI, as appropriate. For between-group comparisons, we used the independent sample t-test or the Mann–Whitney independent sample U test as appropriate depending on data distribution. A chi-squared test for independence (with Yates continuity correction) was used for assessing the association between categorical variables. Correlations were investigated using Pearson product moment correlation or the Spearman rank correlation.

Ethics

All study participants gave informed and written consent. The study was approved by The Regional Committee for Medical and Health Research Ethics (permit no. 2018/1573/REK Vest). The study was conducted in agreement with the local and international guidelines and regulations, including the Declaration of Helsinki (2013 version) and the principles of good clinical practice (CPMP/ICH/135/95).

Role of Funding Source

Regional health authorities of Western Norway and the University of Bergen covered the salaries and cost connected to the registry. Stiftelsen Kristian Gerhard Jebsen and the Norwegian Research Council covered immunological and genetic analyses.

Results

Prevalence and Clinical Characteristics

Altogether 47 of 461 women (10.2%) with AAD in The Norwegian Addison registry had POI (Fig. 1) with a mean age at menopause of 33.2 years (95% CI 31.5-35.0 years). The corresponding age for women with AAD without POI was 48.9 years (95% CI 48.1-49.8) compared with the general reference population 52.7 years (95% CI 52.6-52.8 years) (25). Women with POI were nonsignificantly younger at AAD diagnosis (34.5 vs 37.0 years, $P = .129$), but had a longer AAD disease duration than those without POI (26.8 vs 20.1 years, $P = .003$). There was a strong positive correlation between age at diagnosis of AAD and age at menopause ($P < .001$), with lower age at AAD diagnosis associated with lower age at menopause. We found that POI was diagnosed before or at the same time as AAD in 27 women (57%), and after AAD in 15 women (32%) (Fig. 2). In 5 women the chronological timing of events was uncertain.

The prevalence of associated autoimmune diseases was high among all women with AAD, with 72% exhibiting at least 1 autoimmune comorbidity. Hypothyroidism was most frequent affecting more than half of the women in both groups. The frequency of associated autoimmune disease did not differ between women with and without POI (Table 1).

Autoantibodies

There were significantly higher frequencies of autoantibodies against both SCC and 17OH in AAD women with POI than in AAD women without (P = 0.01 and $P \leq$ 0.001, respectively) (Table 2). Except for 21OH autoantibodies, those against SCC were the most common in AAD women with POI (specificity 84% [341/406]; sensitivity 72% [33/46]), giving a negative predictive value of 96% and a positive predictive value of 34%. Longitudinal data on SCC autoantibodies were limited with multiple measurements available only for 17 AAD women with POI. We did however find stable SCC autoantibody titers in 15 of these women, indicating a long-lasting presence of the autoantibodies similar to what has been reported for 21OH autoantibodies in Addison's disease (26). Autoantibodies against 17OH were the third most common autoantibodies among AAD women with POI, but the specificity (71%, 179/251) and sensitivity (49%, 22/45) was lower. Likewise, the negative (92%) and positive (16%) predictive values were lower than autoantibodies against SCC. Combining the results from SCC and 17OH autoantibody testing did not increase the accuracy compared with testing autoantibodies against SCC alone. None of the women with AAD tested positive for NALP-5 autoantibodies. The AAD women with POI where positive for more autoantibodies towards the 3 steroidogenic enzymes than AAD women without POI, exhibiting ≥2 positive autoantibodies in 78% vs 25% of cases and ≥3 positive autoantibodies in 37% vs 5% (P < .01).

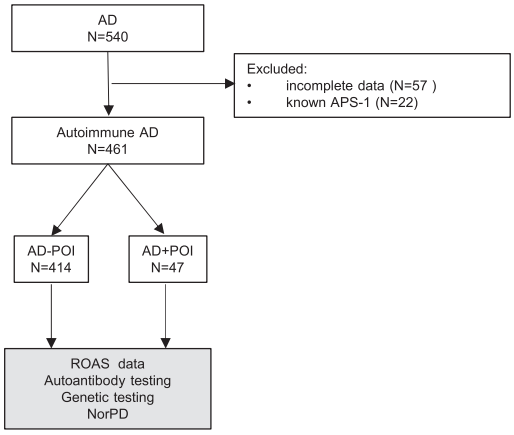

Figure 1. Study design. AAD, Addison's disease; POI, primary ovarian insufficiency; ROAS, National Registry of Organ-Specific Autoimmune Diseases.

Genetic Associations

We tested associations to 104 POI- or AAD-associated genes. When applying the Bonferroni-adjusted significance level of 0.00048, no between-group differences were detected for any of the 104 genes tested for. Eight of the 104 loci reached nominal significance, including 3 (FOXL2, PNO1, and DDX4) with a minor allele frequency >5% (Table 3 and (22)). Since genotyping and imputation of rare alleles (defined as <5%) are prone to inaccuracy for technical reasons, the genotypes reported for the remaining

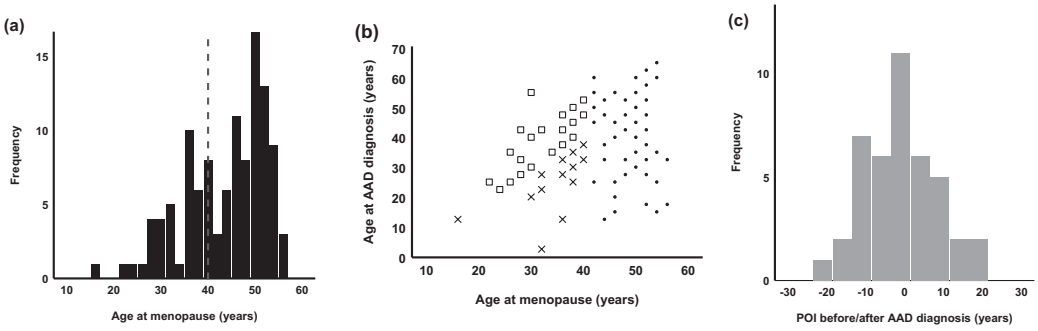

Figure 2. Age at menopause. (A) Age at menopause in women with AAD. (B) Age at menopause compared to age at AAD diagnosis. (X) POI after/same time as AAD, (□) POI before AAD, (·) menopause after 40 years. (C) Timing of POI diagnosis in relation to AAD diagnosis. POI, primary ovarian insufficiency; AAD, Addison's disease.

Table 1. Associated autoimmune conditions in women with Addison's disease (AAD) with and without Primary Ovarian Insufficiency (POI)

	AAD with POI	AAD without POI
	n = 47	n = 414
	Frequency (%)	Frequency (%)
Hypothyroidism	24 (51.1)	217 (52.8)
Hyperthyroidism	5 (10.6)	35 (8.5)
Diabetes type 1	5 (10.6)	50 (12.2)
Hypoparathyroidism	0 (0.0)	1 (0.2)
Alopecia	0 (0.0)	19 (4.6)
Vitiligo	7 (14.9)	46 (11.2)
Vitamin B12 deficiency	6 (12.8)	36 (8.8)
Celiac disease	2 (4.3)	27 (6.6)

Table 2. Autoantibodies in Addison's disease (AAD) women with and without primary ovarian insufficiency (POI)

	AAD with POI	AAD without POI
	n = 47	n = 414
	Positive/Tested (%)	Positive/Tested (%)
21 OH	47/47 (100)	414/414 (100)
SCC	33/46 (71.7)[a]	65/406 (16.0)[a]
17 OH	22/45 (48.9)[b]	72/251 (28.7)[b]
NALP-5	0/34 (0.0)	0/28 (0.0)
TPO	14/33 (42.4)	99/214 (46.3)
GAD	11/35 (31.4)	67/247 (27.1)

[a]$P < .001$, [b]$P = .01$ (chi-squared test).
Abbreviations: 17OH, 17-alpha hydroxylase; 21OH, 21 hydroxylase; GAD, glutamic acid decarboxylase; NALP-5, NACHT leucine-rich-repeat protein 5; SCC, side chain cleavage enzyme; TPO, thyroid peroxidase.

5 rare alleles were not reliable in our POI cohort and were therefore not reported.

Use of Hormone Replacement Therapy

All women with AAD had been prescribed glucocorticoid therapy for systemic use (H02AB) and 459 (95.0 %) mineralocorticoid replacement therapy (H02AA). The use of menopausal HRT (G03C or G03F) was higher in women with AAD <40 years (n = 22) than in the age-adjusted normal population (11.3 % vs 0.7%, $P < .00001$; OER 16.0). The use of hormonal contraceptives for systemic use (G03A) was also higher among women with AAD <40 years of age (n = 112) than in the general population of women 20-39 years (57.7% vs 35.2%, $P < .00001$; OER 1.6). Among the women with POI, two-thirds were treated with HRT.

Discussion

In this first comprehensive national survey of POI in AAD we found that 1 in 10 women with AAD developed menopause before 40 years of age, one-third of these before the 30 years of age. This has important implications for the women involved and should be communicated clearly so that females with AAD can take informed decisions with regard to family planning and HRT. Autoantibodies against SCC are useful biomarkers to identify POI in women with AAD, with a high negative predictive value.

Although the prevalence of POI in our cohort of women with AAD is higher than in the general population, it is in the lower range of what has been reported in previous studies on women with AAD (4-6). These differences can be explained by methodological factors, inclusion criteria or diagnostic precision. Here, we believe to have minimized the potential selection bias by recruiting women with AAD from a national registry, to our knowledge the largest cohort of women with AAD studied so far. We have validated the registered diagnosis to overcome reporting bias by verification of self-reported menopausal age, demonstrating good coherence on timing of menopause.

In clinical practice, endocrinologists need to be aware that women with AAD have a higher risk of POI and earlier menopause than the reference population which has a reported menopausal age of 52.7 years (95% CI 52.6-52.8 years) (25). Contrary to previous studies that showed POI to precede AAD, we here demonstrate that POI can emerge many years or even decades after AAD diagnosis, emphasizing the need for conscientious clinical follow-up (4). Importantly, we also confirm that POI precedes or coincides with AAD in most of the patients, highlighting the importance of screening for AAD with 21OH autoantibodies in women with newly diagnosed POI of unknown cause.

We confirmed a high frequency of concomitant autoimmunity against multiple endocrine organs among women with AAD (5, 19). Similar underlying autoimmune mechanisms have been proposed in both autoimmune adrenalitis and oophoritis (27). As there is a shared embryonic primordium of both organs, and similarities in steroidogenesis, the immunological mechanisms involved in destruction may be analogous (28). The fact that AAD women with POI in this study had a higher frequency of autoantibodies towards steroidogenic enzymes points towards a more aggressive immunological attack.

Autoantibodies towards the steroidogenic enzyme 21OH have repeatedly proven to have the highest sensitivity in diagnosing autoimmune POI in women without AAD, even though this enzyme is exclusively expressed in the adrenal cortex (4, 9, 12, 13). However, most women

Table 3. Nominal significant SNPs associated with primary ovarian insufficiency

Gene	Variants in signal	Function	Minor allele	Cases (MAF)	Controls (MAF)	European (MAF)	*P* value
FOXL2	**rs11924939**	**5′ UTR**	**T**	**0.341**	**0.178**	**0.211**	**.003069**
NR5A1	rs72761481	Intron	T	0.057	0.009	0.030	.00748
CYP17A1	rs45609333	Intron	A	0.045	0.006	0.030	.007841
PNO1	**rs10153623**	**Intron**	**T**	**0.136**	**0.314**	**0.229**	**.01311**
LHX8	rs115689198	Intron	T	0.045	0.006	0.000	.01557
LPP (mir28)	rs76686113	Introns	G	0.079	0.012	0.032	.02136
	rs115389480		A	0.079	0.014	0.046	
	rs62289608		T	0.079	0.016	0.048	
	rs143350743		A	0.045	0.006	0.012	
CTLA4	rs73993040	Intron	T	0.045	0.009	0.000	.04447
DDX4	rs111944699	Introns	A	0.136	0.041	0.046	.04612
	rs4865982		**C**	**0.205**	**0.124**	**0.098**	

Variants with population MAFs greater than 5% in bold. European MAF from European gnomAD, based on nearly 19 000 samples.
Abbreviations: MAF, minor allele frequency; SNP, single nucleotide polymorphism;UTR, untranslated region.
Genes in bold have a reported minor allele frequency > 5% (0.005).

with AAD will already have disease-associated 21OH autoantibodies and they can therefore not be used to diagnose POI. Our study confirms SCC as the most sensitive autoantibody for autoimmune POI in AAD (4, 13, 27). Although the negative predictive value of SCC is high, the positive predictive value is low. Thus, a negative test result predicts that POI is unlikely to develop, while a positive score predicts that it might happen, although many will not develop POI, limiting the use of SCC as a sole screening test. We therefore recommend testing young women with AAD with symptoms of menstrual disturbances or concerns about fertility for autoantibodies against SCC.

NALP-5 autoantibodies have previously been shown to be present in 7% of women with AAD and 12% of women with autoimmune POI (15). We could not replicate these findings in our study. Although prevalent in the cohort compared to the normal population, autoantibodies against TPO and GAD did not differ between AAD women with and without POI and are therefore not useful as markers of ovary-specific autoimmunity.

Elucidating the complex interplay between immunological and genetic mechanisms of autoimmune POI is essential, not only for understanding the connection between AAD and POI, but also in providing genetic counseling and fertility guidance. The lack of any statistically positive genetic associations in the current study may be due to several factors: (1) There may be no relevant genetic differences between AAD women with and without POI; (2) this element of the study is underpowered to detect any but the strongest of effects and any existing effects may be more subtle; or (3) the selected candidate gene set may not include variants that more strongly predispose patients to develop autoimmune POI. Nevertheless, the 3 variants of nominal significance in FOXL2, PNO1, and DDX4 may be of relevance. First, variants in the *FOXL2* gene are known to cause blepharophimosis–ptosis–epicanthus inversus syndrome, but are also associated with spontaneous POI in 1% to 2.9% cases, possibly by impairment of transcriptional repression activity on target genes involved in granulosa cell steroidogenesis and proliferation (17). Second, PNO1 (Partner of NOB1 Homolog) is involved in rRNA processing and has recently been linked with POI in women with autoimmune polyendocrine syndrome type-1 (29). DDX4 is involved in embryogenesis and germ cell development through alteration of RNA secondary structure (30). Thus, these candidate genes are of particular interest when investigating genetic susceptibility for POI in women with AAD and should be included in future larger studies.

In addition to menopausal symptoms of estrogen deficiency and infertility, POI increases the risk of osteoporosis, neurodegenerative disease, cardiovascular disease, and premature death (1). HRT with estrogen and progesterone alleviates these consequences to some extent, and their use is recommended until regular menopausal age (1). Merging data from ROAS with a high-quality prescription register has given us insight into the unique prescription pattern among women with AAD <40 years, demonstrating a significant higher prescription rate for HRT than the same age group in the general population. This indirectly confirms a higher prevalence of POI in women with AAD.

Women with AAD from this cohort have not reported reduced sexual activity or satisfaction compared with the general population, but their fertility is lower (6). In contrast to previous findings in a Swedish study, we found a higher prescription rate of hormonal contraceptives among women with AAD than in the general population, possibly

because of the younger age in our cohort study (31). It is important to be aware that the use of hormonal contraceptives potentially can camouflage menstrual disturbances resulting in delayed diagnosis of POI. The global trend of increasing maternal age at first time pregnancy emphasizes the importance of evaluation the risk of POI and infertility in young women with AAD (32).

Some limitations apply to our study, including the use of candidate markers of autoantibodies and genetic variants. This approach allows us only to identify already known variants of interest, limiting the possibility to discover new and potentially more specific immunological and genetic markers of POI in women with AAD. Another potential weakness is the lack of chronological autoantibody index levels. POI is a continuum of ovarian dysfunction and may proceed through several biochemical stages. Autoantibodies are also known to predate clinical symptoms in some cases and may not be detectable after some years (19). Also, the anonymous structure of the data from NorPD did not allow us to identify who did receive HRT or oral hormonal contraceptives.

To conclude, our national study of women with AAD has demonstrated a high prevalence of POI. Although POI diagnosis preceded AAD in more than half of the women, later POI debut was also common. Diagnosing autoimmune POI remains challenging and relies on clinical, biochemical and immunological testing. Autoantibodies against SCC seem to be the most specific marker for autoimmune POI in women with AAD and we recommend testing all women with AAD <40 years with menstrual disturbances or fertility concerns for autoantibodies against SCC. We also identified 3 possible gene variants of interest in AAD women with POI.

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Additional Information

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Data availability: All data generated or analyzed during this study are not publicly available but are available from the corresponding author on reasonable request. Supplementary data are available in the data repositories listed in References.

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Supplementary table

List	Gene	SNPs in gene (N)	Significant SNPs in gene (N)	Independently significant SNPs in set (N)	P-val	SNPs
POI	FOXL2	4	1	1	0,003069	rs11924939
POI	NR5A1	15	1	1	0,00748	rs72761481
POI	CYP17A1	4	1	1	0,007841	rs45609333
POI	PNO1	15	1	1	0,01311	rs10153623
POI	LHX8	13	1	1	0,01557	rs115689198
AD	LPP	466	27	5	0,02136	rs76686113|rs115389480|rs62289608|rs143350743|rs16863564
AD	CTLA4	6	1	1	0,04447	rs73993040
POI	DDX4	45	4	2	0,04612	rs111944699|rs4865982
POI	ATM	41	3	3	0,0917	rs4986839|rs189872799|rs4987923
POI	BNC1	14	3	3	0,1005	rs28523446|rs7173166|rs9630494
POI	CYP19A1	72	5	5	0,1005	rs147417040|rs4775934|rs146610032|rs4479171|rs3751592
AD	SIGLEC5	19	1	1	0,1005	rs10414149
POI	PSMC3IP	4	1	1	0,1167	rs117222387
POI	BRCA2	46	1	1	0,1333	rs9567582
AD	UBASH3A	42	2	2	0,1538	rs2277800|rs111809474
POI	KHDRBS1	15	1	1	0,156	rs12408664
POI	LMNA	21	2	2	0,1587	rs61813331|rs11578696
POI	FSHR	122	13	5	0,19	rs61296414|rs6545096|rs10194443|rs10176392|rs1882556
POI	POLG	11	1	1	0,2011	rs41556814
POI	LHCGR	82	2	2	0,2535	rs72820994|rs6755901
POI	NLRP5	113	7	5	0,2787	rs185969474|rs1612449|rs2903527|rs2867233|rs521700
POI	FANCM	24	1	1	0,2895	rs79571440
POI	FANCA	69	6	3	0,32	rs12445478|rs7206398|rs113548871
POI	EIF4ENIF1	27	2	2	0,3265	rs71331292|rs112058175
POI	RBM28	22	4	4	0,3721	rs117368079|rs73458923|rs359652|rs11972429
POI	CCDC169-SOHLH2	83	4	2	0,4118	rs7321429|rs9531603
POI	GNAS	49	3	3	0,4118	rs6026557|rs117968941|rs73915943
POI	LRPPRC	58	2	2	0,4118	rs35508693|rs35768060
POI	NBN	23	2	2	0,4118	rs14448|rs10464867
POI	LARS2	71	2	2	0,4516	rs148410569|rs17639261
AD	AIRE	17	1	1	0,4643	rs9974092
POI	HFM1	62	7	5	0,4667	rs281985|rs11165778|rs487069|rs12125586|rs79486667
POI	ESR2	53	3	2	0,4815	rs180742718|rs61984408
POI	NUP107	25	1	1	0,5455	rs74332187
POI	ADAMTS19	118	4	4	0,5789	rs111833396|rs587410|rs114843902|rs173490

AD	BACH2	195	10	5	0,5789	rs56238244|rs9362710|rs117134952|rs9451313|rs60319541
POI	DACH2	232	10	5	0,5882	rs5923541|rs55657926|rs189538997|rs183785285|rs148703061
POI	TP63	276	18	5	0,5882	rs74754828|rs74885811|rs75436877|rs73197825|rs149975958
POI	BLM	50	1	1	0,625	rs4932365
POI	BBS9	271	8	5	0,6429	rs17170243|rs1860601|rs112604081|rs191814955|rs10486532
POI	BMPR2	70	2	2	0,6429	rs112529457|rs146869774
POI	FANCC	42	1	1	0,6429	rs10821450
POI	MCM9	63	1	1	0,6429	rs144606482
POI	GUCY2C	27	5	5	0,6923	rs2114841|rs4764112|rs79442279|rs12304530|rs7307553
POI	MCM8	24	1	1	0,7273	rs77839128
POI	MSH4	64	5	4	0,7273	rs5745437|rs5745548|rs12024786|rs188207305
POI	BMPR1B	221	7	5	0,7778	rs12500446|rs11723423|rs28548879|rs4699361|rs76643668
POI	HSD17B4	42	1	1	0,7778	rs74883918
POI	ATG7	111	5	5	0,8571	rs75438291|rs116166360|rs114209470|rs17536147|rs2442781
POI	DIAPH2	257	14	5	0,8571	rs5921850|rs5967304|rs2168783|rs186458021|rs149278191
POI	ERCC6	42	3	2	0,8571	rs4253111|rs4253016
POI	WRN	58	4	4	0,8571	rs11574415|rs11574304|rs12544538|rs2553279
POI	AARS2	9	0	0	1	NA
POI	AMH	7	0	0	1	NA
POI	ATG9A	6	0	0	1	NA
POI	BMP15	2	0	0	1	NA
POI	CLPP	6	0	0	1	NA
POI	CYP11A1	11	0	0	1	NA
POI	DMC1	17	0	0	1	NA
POI	DTD1	105	1	1	1	rs6081257
POI	EIF2B4	6	0	0	1	NA
POI	ERAL1	1	0	0	1	NA
POI	ESR1	246	7	5	1	rs6935074|rs7761133|rs3020312|rs113429088|rs6939683
POI	FANCG	5	0	0	1	NA
POI	FIGLA	0	0	0	1	NA
POI	FMR1	0	0	0	1	NA
POI	FOXO4	4	0	0	1	NA
POI	GALT	2	0	0	1	NA
POI	GDF9	10	0	0	1	NA
POI	GJA4	5	0	0	1	NA
POI	HARS2	6	0	0	1	NA
POI	HID1	10	0	0	1	NA
POI	HSD3B2	11	0	0	1	NA
POI	INHA	10	0	0	1	NA
POI	KHDC3L	3	0	0	1	NA
POI	MORC2	14	0	0	1	NA
POI	MRPS22	7	0	0	1	NA
POI	MSH5	0	0	0	1	NA

POI	NANOS3	2	0	0	1	NA
POI	NOBOX	6	0	0	1	NA
POI	NOG	0	0	0	1	NA
POI	NOTCH2	11	0	0	1	NA
POI	PARP1	32	0	0	1	NA
POI	POF1B	17	0	0	1	NA
POI	POLR2C	6	0	0	1	NA
POI	POLR3H	8	0	0	1	NA
POI	POMC	9	0	0	1	NA
POI	POU5F1	1	0	0	1	NA
AD	PTPN22	20	0	0	1	NA
POI	RASIP1	7	0	0	1	NA
POI	RECQL4	4	0	0	1	NA
POI	SALL4	21	0	0	1	NA
POI	SGO2	27	0	0	1	NA
AD	SH2B3	9	0	0	1	NA
POI	SOHLH2	30	0	0	1	NA
POI	SOX8	9	0	0	1	NA
POI	SPIDR	50	0	0	1	NA
POI	SRSF8	3	0	0	1	NA
POI	STAR	2	0	0	1	NA
POI	SYCE1	16	0	0	1	NA
POI	TGFBR3	177	6	5	1	rs17131543|rs4658267|rs17886020|rs72714494|rs11165300
POI	TWNK	3	0	0	1	NA
POI	WDR62	29	0	0	1	NA
POI	WT1	21	0	0	1	NA

RESEARCH

Premature menopause and autoimmune primary ovarian insufficiency in two international multi-center cohorts

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Abstract

Objective: To investigate markers of premature menopause (<40 years) and specifically the prevalence of autoimmune primary ovarian insufficiency (POI) in European women.
Design: Postmenopausal women were categorized according to age at menopause and self-reported reason for menopause in a cross-sectional analysis of 6870 women.
Methods: Variables associated with the timing of menopause and hormone measurements of 17β-estradiol and follicle-stimulating hormone were explored using multivariable logistic regression analysis. Specific immunoprecipitating assays of steroidogenic autoantibodies against 21-hydroxylase (21-OH), side-chain cleavage

Key Words
- premature ovarian insufficiency
- premature ovarian failure
- premature menopause
- primary ovarian insufficiency
- autoimmune

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enzyme (anti-SCC) and 17alpha-hydroxylase (17 OH), as well as NACHT leucine-rich-repeat protein 5 were used to identify women with likely autoimmune POI.
Results: Premature menopause was identified in 2.8% of women, and these women had higher frequencies of nulliparity (37.4% vs 19.7%), obesity (28.7% vs 21.4%), osteoporosis (17.1% vs 11.6%), hormone replacement therapy (59.1% vs 36.9%) and never smokers (60.1% vs 50.9%) ($P < 0.05$), compared to women with menopause ≥40 years. Iatrogenic causes were found in 91 (47%) and non-ovarian causes in 27 (14%) women, while 77 (39%) women were classified as POI of unknown cause, resulting in a 1.1% prevalence of idiopathic POI. After adjustments nulliparity was the only variable significantly associated with POI (odds ratio 2.46; 95% CI 1.63–3.42). Based on the presence of autoantibodies against 21 OH and SCC, 4.5% of POI cases were of likely autoimmune origin.
Conclusion: Idiopathic POI affects 1.1% of all women and almost half of the women with premature menopause. Autoimmunity explains 4.5% of these cases judged by positive steroidogenic autoantibodies.

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Introduction

Menopause is defined as the permanent cessation of menstrual periods and usually occurs around 50 years of age (1). Timing of menopause is influenced by genetics in addition to environmental and lifestyle factors including smoking habits, nutritional status and general health (2, 3, 4, 5, 6, 7, 8).

Menopause before age 40 years is considered premature and may be caused by a defect in any part of the hypothalamic–pituitary–ovarian axis, with subsequent hypoestrogenism. If the deficiency is located in the ovary, it is referred to as primary ovarian insufficiency (POI), characterized by low estradiol levels promoting high follicle-stimulating hormone (FSH) levels, due to lack of negative feedback (9). Early estrogen deficiency has negative long-term health consequences, including infertility, osteoporosis, declined neurocognitive function, increased risk of cardiovascular disease and total mortality (10, 11, 12, 13, 14, 15, 16). Hormone replacement therapy (HRT) is recommended for these women (17, 18).

The prevalence of POI in the general population is estimated to 1–3% (19, 20, 21, 22, 23). Important causes of POI include iatrogenic treatment (surgical, chemotherapy or radiation), genetic, infectious or autoimmune etiology (17, 18). However, in the majority of cases the cause remains unknown (9). According to European guidelines diagnostic autoimmune workup for POI should include measurements of ovarian associated autoantibodies (17). Autoantibodies against the steroidogenic cell enzymes have shown consistent association with autoimmune POI, including autoantibodies against 21-hydroxylase (21-OH), side-chain cleavage enzyme (anti-SCC) and 17alpha-hydroxylase (17 OH), as well as NACHT leucine-rich-repeat protein 5 (NALP5), which are highly expressed in the ovaries (24, 25, 26).

Previously reported prevalence of autoimmune POI varies greatly (0–30%), the broad estimate probably reflecting heterogenic study populations as well as use of variable autoantibody assays (27, 28, 29, 30). Clinically women with autoimmune POI have a more fluctuating ovarian function during the first years after onset and follicular activity seems to be intact initially as judged by higher anti-Müllerian hormone levels compared to other forms of POI. Thus, early diagnosis of autoimmune POI could improve fertility possibilities (31, 32). Identifying these women using specific immunoprecipitating assays of steroidogenic cell autoantibodies is possible but has not to our knowledge been done in large population-based cohorts. Here, we describe potential markers of premature menopause and estimate the prevalence of autoimmune POI in two large multi-center international cohorts providing menopausal age and information on health-, lifestyle factors and reproductive hormone levels.

Methods

Study population

In this retrospective cross-sectional study, the study population consisted of the second follow-up of the European Community Respiratory Health Survey carried out in 2010–2012 (ECRHS III) and The Respiratory Health in Northern Europe, Spain and Australia generation study carried out in 2013–2016 (www.ecrhs.org and www.rhinessa.net). The latter comprises the maternal

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or paternal offspring of the initial participants of the former and potential co-dependence was considered in the subsequent analyses. Both studies collected data in the same standardized way, using an interview-led questionnaire on anthropometrics, reproductive health- and lifestyle factors. In addition, both cohorts provided serum samples for analyses of reproductive hormones and autoantibodies.

Data weres obtained from 6870 women from 15 study centers in eight European countries (Spain, France, Germany, Sweden, Denmark, Iceland, Estonia and Norway). The women were born between 1945 and 1998 and the mean age (S.D.) at inclusion was 40 (13.7) years (median 39 (28–52) years). A flow chart describing the study population is presented in Fig. 1.

Ethical approval was obtained from the appropriate ethics committees of each study center, and all participants provided their informed written consent.

Variables

Women were defined as postmenopausal if they had not had a menstrual period within the last 12 months prior to answering the questionnaire. Women who reported using HRT were included even if they reported having menstrual periods within the last 12 months. Women whose date of last menstrual period was missing (n = 263), who were using hormonal contraception (n = 1637) or who were either pregnant or breastfeeding (n = 374), were excluded.

The main dependent variables, prevalence of premature menopause (<40 years) and POI, were based on self-reported age and reason for menopause. Four categories of postmenopausal women were created based on the response:

(1) Idiopathic POI is acknowledged as premature menopause of unknown reason.
(2) Iatrogenic-induced menopause (bilateral oophorectomy and/or hysterectomy, after cancer treatment).
(3) Menopause due to non-ovarian reasons (eating disorder/underweight, pituitary failure, disorder of uterus).
(4) Menopause ≥40 years.

Our independent variables consisted of potential predictors of menopausal timing such as age at menarche, parity, current and former smoking (for ≥1 year), ever use of oral contraceptives (OC's), in addition to information on other illnesses and potential consequences of premature menopause including eating disorders, cancer and osteoporosis and use of HRT. Variable details are presented in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

Reported height and weight were used to calculate BMI (kg/m^2) and participants were classified as underweight (<18.5 kg/m^2), normal (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2) and obese (≥30 kg/m^2) (33). Because of the low number of underweight women (n = 15), these were not analyzed separately.

Hormone samples

Hormone measurements were available for 1134 postmenopausal women, of whom 994 did not use HRT. FSH and luteinizing hormone (LH), were analyzed using

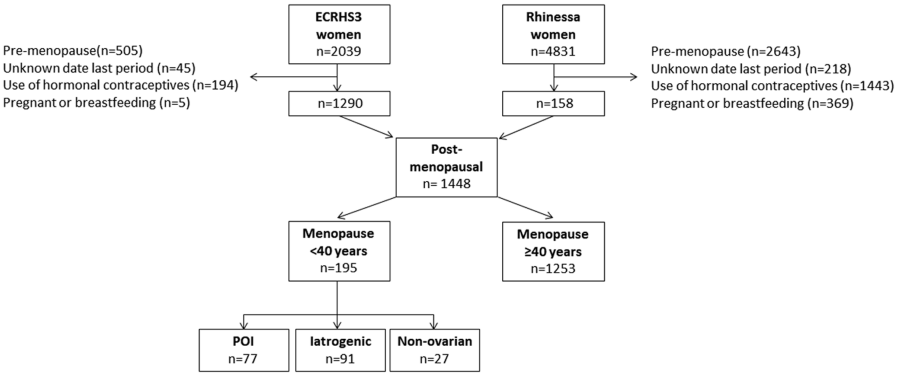

Figure 1
Study design. European Community Respiratory Health Survey (ECRHS3S). The Respiratory Health in Northern Europe, Spain and Australia (Rhinessa). Idiopathic premature ovarian insufficiency (POI).

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ELISAs (Demeditec Diagnostics, Kiel, Germany), and the steroid hormones (17β-estradiol, estrone, progesterone, testosterone and DHEA-S) were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Core Facility for Metabolomics (University of Bergen, Norway, 2017) (34). Concentrations below the lower limit of quantification (LLQ) for 17β-estradiol (3.6 pmol/L), estrone (2.1 pmol/L), testosterone (106 pmol/L), DHEA-S (0.21 μmol/L), FSH (5.0 IU/L), LH (10.0 IU/L) and sex hormone-binding globulin (SHBG) (4.0 nmol/L) were included as LLQ/2 (32).

Autoantibodies

Serum samples from 66 women with POI and a control group of 64 age-matched women with iatrogenic premature menopause (bilateral ovariectomized) were analyzed for the following autoantibodies: 21 OH, SCC and 17 OH. NALP-5. All autoantibody assays were performed in the laboratory at the Faculty of Medicine (University of Bergen, Norway) using radio-binding ligand assays (35). Positive cut-offs were calculated using positive and negative controls with index thresholds of >57, >200, >102 and >65 for 21 OH, SCC, 17 OH and NALP-5, respectively. Positivity for ovarian associated autoantibodies was perceived as likely autoimmune POI.

Statistical analyses

To determine potential predictors and consequences of premature menopause and POI, clinical variables and hormonal data were compared to women with menopause ≥40 years. Continuous and normally distributed variables were presented as mean and S.D., and t-tests were used to evaluate between-group difference. Continuous variables with a skewed distribution were presented as median and interquartile range (IQR), and Mann–Whitney *U* test was used to evaluate between-group difference. Categorical variables were presented as frequencies (*n*) and/or percentages (%), and chi-square test (with Yates continuity correction) was used to evaluate between-group differences.

The variables associated with POI were first investigated with univariable logistic regression. Thereafter, relevant predictors were included in a multivariable logistic regression analysis. Statistical significance was set to a *P* value <0.05. Cohort co-dependency (family clusters) was examined by generalized estimate equations (GEE) and separate analyses of the ECRHS 3 cohort separately.

Differences in hormone levels were investigated using multivariable logistic and linear regression analyses controlling for the time since menopause and BMI. A two-way between-group ANOVA was used to explore the difference between groups. Due to skewed distributions, hormone values were log10-transformed for all comparative analysis, and then back-transformed for ease of interpretation.

Results

In this population of women aged 18–66 years, 21.1% (1448/6870) were postmenopausal. The mean age at menopause was 47 (7.7) years, median age 49 (IQR 44–52) years and normally distributed though slightly skewed toward a younger age (Fig. 2). There was no difference in the median age of menopause between European regions (*P* = 0.168).

Among women with menopause ≥40 years, 79% (988/1253) reported spontaneous reasons, 21% (260/1253) stated surgical reasons and <1% (5/1253)) non-ovarian reasons for menopause.

Premature menopause

The prevalence of premature menopause was 2.8% (195/6870. Among the women with premature menopause, 47% (91/195) had iatrogenic reasons, of whom 81 reported previous surgery and 10 cancer treatment. Non-ovarian reasons were identified in 14% (27/195), including 11 women with hypothalamic amenorrhea, 12 with eating disorders and 4 with pituitary failure. The etiology was unknown in 39% (77/195) of women with premature menopause. Taken together, the prevalence of idiopathic POI was 1.1% (77/6870).

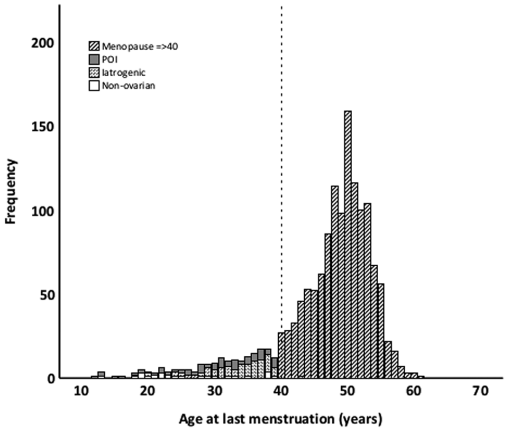

Figure 2
Timing and etiology of menopause.

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Table 1 Characteristics of reproductive and lifestyle factors by timing and reason for menopause. Continuous data are given as mean and s.d. and categorical data are given as percent (%).

	Menopause < 40 years (*n* =195)			**Menopause ≥40 years** (*n* =1253)	***P*-value Menopause <40 vs Menopause ≥40 years**
	POI (*n* =77)	Iatrogenic (*n* =91)	Non-ovarian (*n* =27)		
Age menopause, (years)	28.7 (7.3)	33.2 (4.9)	26.5 (7.7)	49.3 (4.1)	<0.001[a]
Age menarche (years)	12.9 (1.7)[c]	12.7 (1.6)	12.9 (1.9)	12.9 (1.5)	NS
Nulliparity	37.2	33.0	53.8	19.7	<0.001[b]
BMI, (kg/m^2)	26.75 (4.80)	27.69 (5.37)	24.80 (7.41)	26.36 (5.15)	NS
BMI categories:					
<18.5	0	0	27.3	1.2	NS
18.5–24.9	35.7	31.8	37.5	44.7	0.046[b]
25.0–29.9	35.7	38.6	10.2	32.8	NS
≥30.0	28.6	29.5	25.0	21.4	0.022[b]
Smoking status:					
Current	15.8	12.0	12.5	16.8	NS
Former	27.4	26.8	24.0	32.3	NS
Never	56.7	61.2	63.5	50.9	0.018[b]
Treated for cancer	7.6	9.9	7.7	8.4	NS
Osteoporosis	16.0	12.2	37.5	11.6	0.034[b]
Oral contraceptive use, ever	77.2	81.3	73.1	75.0	NS
HRT use, ever	60.8	58.2	57.7	36.9	<0.001[b]

[a]T-test; [b]Chi-square tests; [c]Six women reported primary amenorrhea.
NS, non-significant.

We found some differences in reproductive and lifestyle characteristics related to timing for menopause (Table 1). Women with premature menopause had a higher frequency of nulliparity (37.4% vs 19.7%, $P < 0.001$), obesity (28.7% vs 21.4%, $P = 0.002$), osteoporosis (17.1% vs 11.6%, $P = 0.034$), use of HRT (59.1% vs 36.9%, $P < 0.001$), never smokers (60.1% vs 50.9%, $P = 0.018$), and fewer women deviating from normal BMI (34.0% vs 44.7%, $P = 0.046$), compared to menopause ≥40 years of age.

We identified 20 family clusters (mother and daughter included in the study population), however, results of the applied GEE did not indicate altered results. Analysis restricted to ECRHS3 women showed very similar results as our main analysis, despite a higher frequency of cancer treatment among women with premature menopause (22.4 vs 8.6%, $P = 0.005$), and more former smokers (56.6 vs 54.2, $P = 0.935$) as well as lower HRT use (21.5 vs 17.7%, $P = 0.493$) in both groups.

Hormone levels did not differ between women with premature menopause and menopause ≥40 years adjusting for years since menopause and BMI (Table 2). The 17β-estradiol levels decreased with years since the last menstruation in all women (regression coefficient $\beta = -0.213$, $P < 0.001$), while no significant association was found for FSH. Women with higher BMI had higher levels of 17β-estradiol (regression coefficient $\beta = 0.127$, $P < 0.001$), and correspondingly decreased FSH levels (regression coefficient $\beta = -1.148$, $P < 0.001$) (Fig. 3).

Table 2 Hormone levels in premature menopause compared to menopause ≥ 40 years[b]. Hormone levels reported as median and interquartile range (IQR).

	Menopause <40 years (*n* =66)	**Menopause ≥40 years** (*n* =928)	***P*-value[a]**
FSH (IU/L)	102.5 (77.9–155.3)	125.9 (88.8–171.7)	0.222
LH (IU/L)	24.3 (13.5–31.2)	27.4 (19.7–36.6)	0.992
Estradiol (pmol/L)	13.3 (7.9–24.5)	11.6 (6.2–22.4)	0.395
Estrone (pmol/L)	64.9 (48.4–105.0)	69.1 (47.9–101.7)	0.982
Progesterone (nmol/L)	<0.21	<0.21	0.521
Testosterone (nmol/L)	0.57 (0.35–0.68)	0.53 (0.37–0.73)	0.338
DHEAS (umol/L)	1.86 (1.06–3.41)	1.16 (1.01–2.47)	0.790
SHBG (nmol/L)	65.4 (37.6–108.8)	65.4 (40.5–99.5)	0.911

[a]Mann–Whitney *U* test; [b]Women currently using OC or HRT and pregnant women were excluded.

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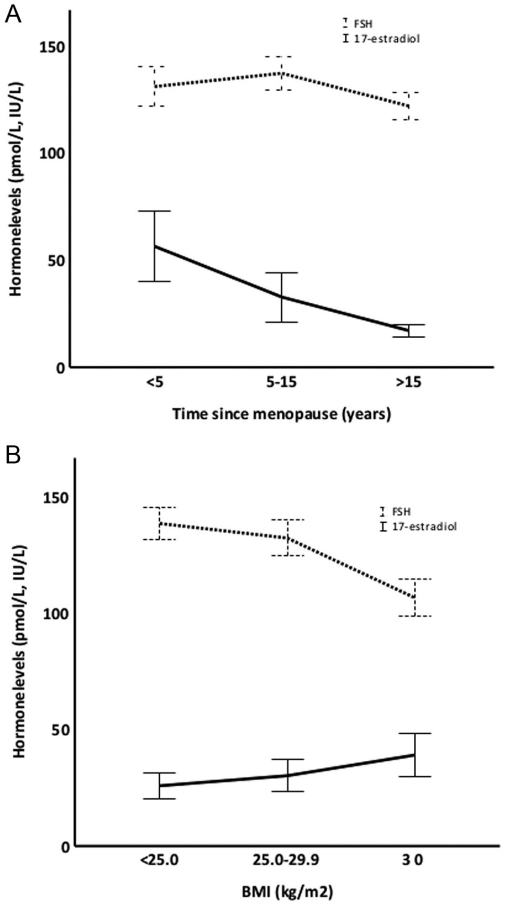

Figure 3
17β-estradiol and follicle-stimulating hormone (FSH) by (A) time since menopause and (B) BMI in all post-menopausal women (n = 1134). Hormone levels reported in mean and 95% CI.

Primary ovarian insufficiency

Nulliparity, obesity and smoking history were all independently associated with POI compared to women with menopause ≥40 years. However, after adjusting for BMI, smoking and age as well as study affiliation in a multivariable analysis, only nulliparity was statistically significantly associated with POI (adOR 2.46; 95% CI 1.63–3.42). Normal weight and never smoking showed a tendency toward being protective of POI (Fig. 4).

There were also some differences in reproductive and lifestyle characteristics related to the reason for premature

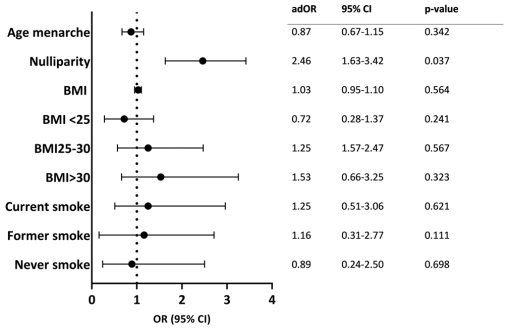

Figure 4
Multivariable logistic regression of reproductive and lifestyle factors associated with idiopathic primary ovarian insufficiency (POI) compared to menopause ≥40 years. Odds ratio adjusted for BMI, smoking, age and study affiliation (adOR) and 95% CI. Excluding women with surgically induced menopause ≥40 years (n = 261) or women with menopause at 40–44 years (n = 361) did not alter the results of the multivariable logistic regression analysis.

menopause (Table 1). We found that women with POI and women with iatrogenic reasons for premature menopause had similar characteristics, while women with non-ovarian reasons reported menopause at a slightly younger age, had a lower weight and a higher frequency of nulliparity and osteoporosis (P < 0.05).

Women with POI and iatrogenic reasons for premature menopause had comparable hormonal patterns with low levels of 17β-estradiol (median 15.6 (9.2–28.0) and 14.7 (9.0–27.2)) and corresponding high levels of FSH (median 111.6 (90.0–122.0) and 102.0 (87.3–119.1)), while women with

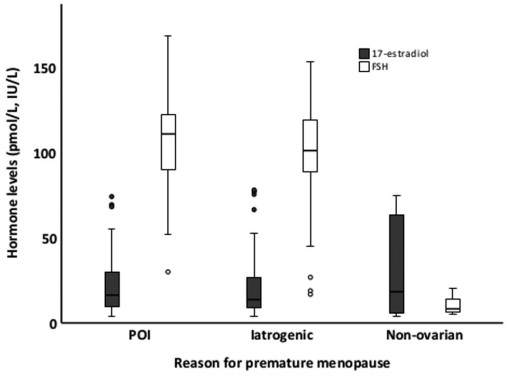

Figure 5
Hormonal patterns of 17β-estradiol and follicle-stimulating hormone (FSH) in three groups of women with different reasons for premature menopause. Idiopathic primary ovarian insufficiency (POI), iatrogenic and non-ovarian premature menopause.

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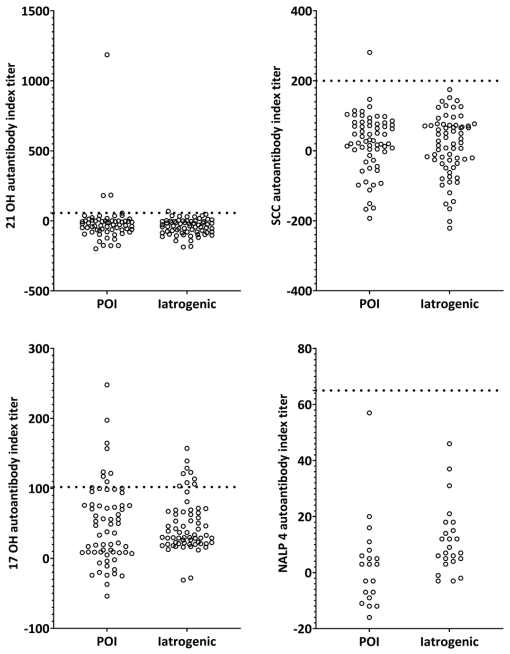

Figure 6
Autoantibody index levels in 66 women with idiopathic primary ovarian insufficiency (POI) and a control group of 64 age-matched women with iatrogenic premature menopause (bilateral ovariectomized). Dotted line shows the cut-off threshold for positive test in this radio-binding ligand assay.

non-ovarian reason for premature menopause had similar 17β-estradiol levels (median 18.3 (6.2–63.2)) but significantly lower FSH levels (median 11.1 (9.7–16.9)) ($P = 0.016$) (Fig. 5).

Autoimmune POI

In total, 4.5% (3/66) of POI cases were considered to have autoimmune POI based on presence of autoantibodies (Fig. 6). Positive 21 OH autoantibodies were found in three women with POI. One of these women also had positive SCC autoantibodies. Autoantibodies against 17 OH were abundant in both groups. None had positive NALP 5 autoantibodies. Case 1 had POI from age 28, positive autoantibodies against 21 OH (titer 181.0 IU/mL) and 17 OH (titer 157.25 IU/mL), known hypothyroidism and coeliac disease. Case 2 had POI from age 36, positive autoantibodies against 21 OH autoantibodies (titer 1186.0 IU/mL) and SCC (281 IU/mL), known hypothyroidism, hypertension, and scoliosis. Case 3 had POI from age 35 and positive 21 OH autoantibodies (titer 184.0 IU/mL). There was no information about intercurrent disease in general or adrenal insufficiency in particular regarding case 3. Hormonally these women did not differ from other women with POI.

Discussion

We found immunological markers suggesting an autoimmune etiology in 4.5% of women with POI, using specific autoantibody assays. We identified several reproductive and lifestyle-related factors associated with the timing of menopause, but no determinant that could distinguish autoimmune POI from other causes of premature menopause.

Finding the true prevalence of premature menopause and POI is challenging because of heterogenic etiology, terminology and diagnostic criteria as well as variation in study designs and statistical methods (19). Use of OC's can also disguise symptoms and delay diagnosis. We found a prevalence of premature menopause and POI that was in coherence with previous studies (19, 20, 21, 22, 23, 36). One in four of all postmenopausal women in our study reported surgically induced menopause and was even more common among women with premature menopause (47%). Although the frequency of surgically induced menopause varies across populations, in accordance with Dratva *et al.*, the determinants of age at surgically induced menopause, did not differ from other causes of menopause in our study (5).

The association between nulliparity and premature menopause is not unexpected (3, 37, 38). It was also the only variable directly associated with POI in our study. Infertility is an inevitable result of menopause but reproductive decline in the years preceding menopause is well known (39). Since the majority of women with premature menopause in this study had their final menstrual period between age 27 and 37 years, there was potentially time to conceive prior to the diagnosis indicating that infertility can be both a predictor and a consequence of early menopause. However, other reasons for lower fertility rates in women with premature menopause and POI, such as concomitant disease or a secular trend of lower birth rates, cannot be excluded.

We found that women with normal weight were less likely to report premature menopause than women who were overweight or obese. A U-shaped relationship between BMI and the risk of earlier menopause has been shown in previous studies (7, 8, 40). Underweight associated with malnutrition, over-exercising and chronic illness can cause

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premature menopause due to hypothalamic-pituitary deficiency (8, 41, 42, 43). Because of a relatively low number of underweight women in this study we did not have enough power to address this issue. Weight increases with age in a majority of women, but evidence regarding the association between BMI and timing of menopause has been inconsistent and remains controversial (2, 7, 8, 21, 43). Longitudinal studies have reported that weight gain during midlife is associated with a sedentary lifestyle and aging itself, not menopausal status (44). An increase in BMI with age is however accompanied by an adverse change of body composition that manifests during the first years of menopause with estrogen depletion resulting in a decline of lean body mass and increase in adipose abdominal fat (45).

Smoking is the predictive factor most consistently associated with younger age at menopause (2, 3, 6, 7). We could not confirm a direct association with current or former smoking. This could be due to fewer smokers among the younger birth cohorts in this population (5).

Use of HRT was more common among women with premature menopause (59.1%), but in total less than half of the women in this study had ever used HRT. According to European guidelines, HRT is indicated both for the treatment of symptoms related to hypoestrogenism and to prevent complications, that is osteoporosis (17). Variations in national recommendations and culture are factors that may influence use. With regards to the timing of the data collection in this study, the lower usage of HRT in the ECRHS3 cohort could be attributed to the debate following publications at the beginning of this millennium initially reporting an increased risk of breast cancer and venous thromboembolism in HRT users, resulting in a marked decrease in prescription the following years (46, 47).

We found no evidence that age at menarche is a predictor of the timing of menopause. It seems intriguing that these two events in a woman's reproductive life could be related. However, no consistent association between ages at menarche and menopause has been observed in epidemiological studies (2, 48, 49, 50, 51). This is further supported by newer genetic data revealing limited overlap in genomic regions associated with the timing of the two events (52, 53).

Major alterations in reproductive hormones take place through the menopausal transition and years following menopause (54, 55). Our results are consistent with others in finding that postmenopausal women with high BMI and shortest time since menopause had the highest levels of 17β-estradiol (45, 56). This was most pronounced in women with premature menopause. Our results suggest that BMI as well as years since the last menstrual period and age should be considered when interpreting hormone levels of postmenopausal women, although it is essential to be aware of the limitations of group differences when applied to individual patients.

We also demonstrated how hormone tests can be useful in confirming POI as compared to non-ovarian reasons for premature menopause in non-clinical settings. Women with idiopathic POI and iatrogenic premature menopause had high levels of FSH and low levels of 17β-estradiol, while women with non-ovarian reasons had low levels of both FSH and 17β-estradiol, pointing toward a pituitary–hypothalamic deficiency. In previous studies, FSH and 17β-estradiol concentrations have shown good confirmation with the classification of causes of menopause based on questionnaires (44).

We found ovarian-associated steroidogenic cell autoantibodies in 4.5% of women with idiopathic POI, pointing to a prevalence of autoimmune POI in the lower range of what has previously been reported (27, 28, 29, 30). This could be due to the unselected study population, supporting the diagnosis of POI on self-reported data, not clinical evaluation. However, it could also be due to use of specific immunoprecipitating assays in the present study, whereas most previous studies have applied sensitive but less specific indirect immunofluorescence methods for autoantibody detection (17, 18, 27, 30, 57, 58).

Multiple ovarian autoantigens have been proposed as possible targets for autoimmune POI. However, autoantibodies toward the steroidogenic cell enzymes have specific diagnostic value for autoimmune disease in the ovary as well as the adrenal gland. Primary adrenal insufficiency affects approximately 2–3% of women with spontaneous POI, a 300-fold increase compared with the general population (26, 28, 30). We found that positive 21 OH autoantibodies were significantly more common in POI women, compared to the general population, confirming its role as a potential marker for autoimmune POI. In our study, the women with positive 21 OH autoantibodies did not have clinical or biochemical markers of AD. This does however not exclude the diagnosis as POI can develop both before and after the onset of AD. Women with positive steroidogenic cell autoantibodies should therefore be assessed with adrenal function tests (17, 59). In contrast to autoantibodies against 21 OH and SCC, we showed that autoantibodies against 17 OH and NALP-5 did not differ between the groups and thus seem to be more unspecific markers in this setting and unsuitable for screening.

Autoimmune POI represents a continuum from impaired ovarian function to complete ovarian failure

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and the disease is often diagnosed at an end stage (30). Autoantibody detection could be hampered by the timing of testing in relation to the disease stage. At the final stages of an autoimmune disease, lower titers of autoantibodies are expected due to antigen elimination, however, autoantibodies against 21 OH have proven remarkably stable over time (60).

A major strength of this study was access to data from populations across different European regions, suggesting that our results are not biased by varying cultures and environmental factors. Another major strength of our analysis is the high sensitivity of the LC-MS/MS assay used for hormone measurements. However, some limitations need to be acknowledged. As our study population consisted predominantly of Caucasian women, our findings might not be generalizable to all women. Previous studies have shown that there are ethnic differences in the timing of menopause as well as levels of reproductive hormones (36, 61).

The cross-sectional design allows assessment of association but not demonstration of causality. Our study population is heterogenic with regards to postmenopausal chronology complicating interpretation of the influence of lifestyle factors and several exposures may mask the connection, including interactions between genetic and environmental influences. However, there was no indication that present family clusters altered our results. In addition, universally standardized autoantibody assays are missing. Retrospectively reported age at menopause can potentially suffer from recall bias. Misclassification is however less likely to occur among women with premature menopause as studies suggest that unusual events (such as menopause before normal age) are easier to remember (62). Several studies have also demonstrated high accuracy and reliability of self-reported age of menopause (63, 64).

In conclusion, POI affects 1.1% of all women and almost half of the women with premature menopause. Autoimmunity explains 4.5% of these cases as judged by the presence of autoantibodies. Nulliparity is the variable most strongly associated with POI. Evaluation of hypothalamic–pituitary–gonadal axis hormonal levels are useful in distinguishing different causes of premature menopause in cross-sectional studies. Future studies on the subject should include longitudinal data on hormone and autoantibody levels.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EC-22-0024.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Elinor Chelsom Vogt did statistical analysis and manuscript drafting. Kai Triebner and Marianne Øksnes participated equally in the methodology, statistical analysis and reviewal of the manuscript. Francisco Gómez Real took the initiative for the use of this dataset and has actively discussed and reviewed the study design and manuscript. The remainder authors participated in the study design, recruitment of participants and data collection as well as constructive contributions in reviewal of the manuscript. K Triebner and M Øksnes contributed equally to this study.

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