Advanced Ovarian Ageing:
Studies on Fertility and Vascular Health

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Advanced Ovarian Ageing: Studies on Fertility and Vascular Health

Verminderde Ovariële Reserve:
Studies naar Fertiliteit en Vasculaire Gezondheid
(met een samenvatting in het Nederlands)

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Felicia Yarde

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Promotor:	Prof. dr. F.J.M. Broekmans
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Copromotor:	Dr. W. Spiering

"It always seems impossible until it's done"

– Nelson Mandela –

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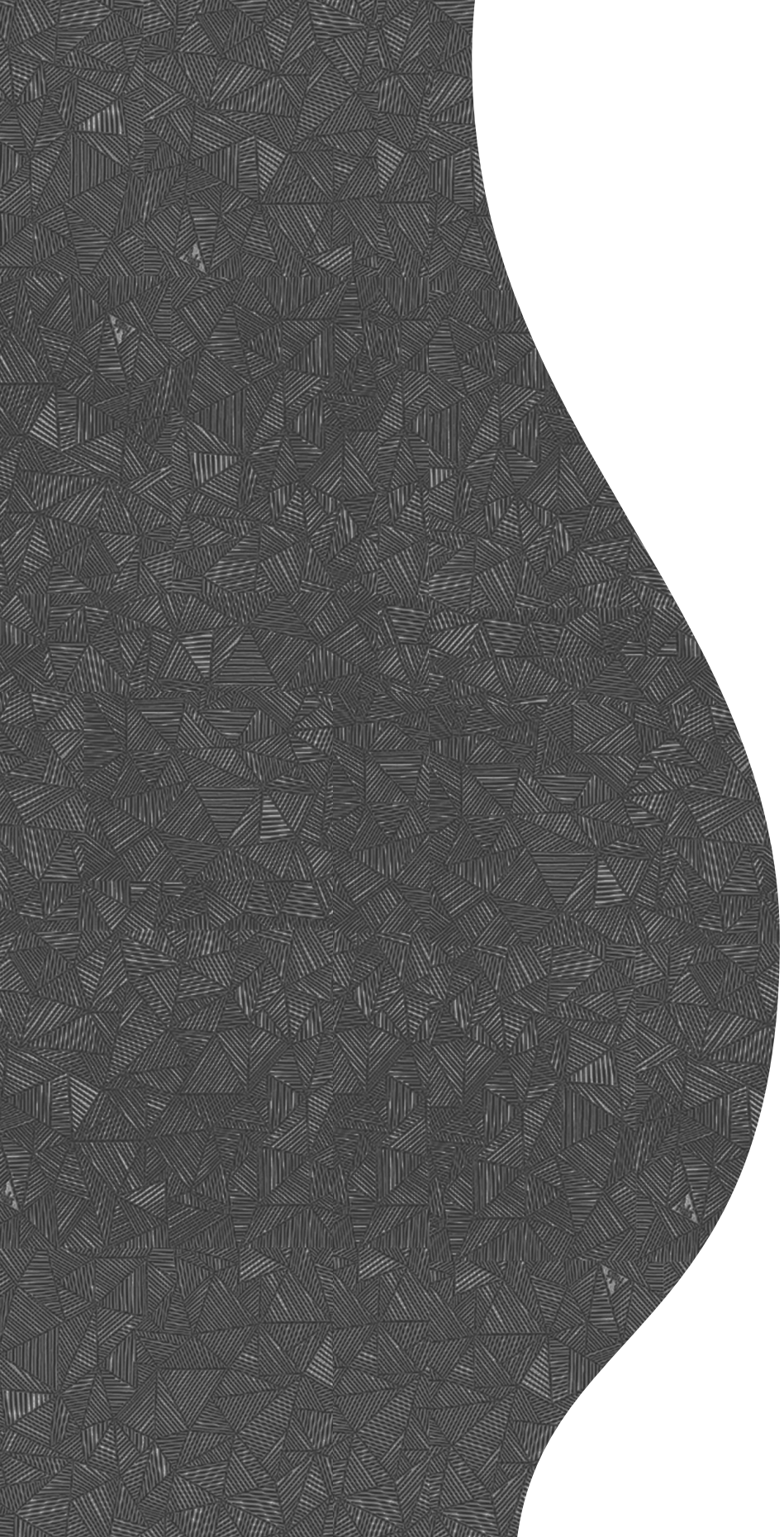

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General introduction

Based on a chapter published in "Intra-uterine insemination: an evidence based guideline for daily practice: Female age".

FEMALE REPRODUCTIVE AGEING

It has long been understood that female reproductive success is strongly associated with age. With increasing age, female fecundity (the ability to produce viable offspring) decreases. This insight is derived from studies in historical populations, where no consistent contraceptive methods were used (1) and on studies in women undergoing donor inseminations (2;3). These observations have shown the gradual decline in monthly fecundity rate after the age of 30 years (4;5). From 36 years onwards this decline occurs more rapidly, with the end of natural fertility occurring, on average, as early as 41 years. The onset of infertility takes place without any noticeable sign to the woman: menstrual cycles can still occur on a regular basis, although a tendency towards shorter cycles may be observed.

The human species can be considered as relatively infertile compared to many animal species, with a monthly fecundity rate of only 20% (6). This indicates that even optimally fertile couples may require many exposure months to conceive, with the age-related decline in fecundity further extending the necessary time frame for natural conception. Therefore, the proportion of infertile couples in their 20s is only 4%, compared to 10-20% in women over 35 years. These infertility rates can increase up to 50% for moderately fertile women over 35 years, who have unsuccessfully tried to conceive for many years (6-8).

Since the introduction of hormonal contraceptive methods in the 1960s, together with the growing economic wealth, women gained the opportunity to increase their education levels and participate in the labour force (9). As a result, maternal age at first birth increased considerably by postponing childbearing. Due to these trends, an increasing proportion of women will fail to conceive within a time frame of 12 months. This condition, referred to as infertility, causes a growing proportion of couples to seek assisted reproductive technology (ART). However, ART will only compensate for the decreased natural fertility to a limited extent, leaving many couples involuntarily childless (9;10).

OVARIAN AGEING

Changes in ovarian function, dominated by the gradual decline of both oocyte quantity and quality, are major contributors to the reproductive ageing process (11). The decrease in oocyte quality becomes apparent both in increasing rates of infertility observed at older age as well as increased aneuploidy rates, responsible for a higher risk of miscarriage and trisomic births with increasing age (12-14). An increase in the occurrence of meiotic disjunctions with increasing age is assumed to induce the decrease in oocyte quality. Studies on IVF pregnancies in women of advancing age have demonstrated that the majority of oocytes and embryos are chromosomally abnormal (15-18).

Underlying mechanisms may be inherited, and involve differences in germ cells at formation, or acquired, through the accumulation of oocyte damage or through changes in granulosa cell quality (11). Furthermore, the accumulation of oocyte damage with increasing age could lead to mitochondrial dysfunction which may cause chaotic mosaicism in human pre-implantation embryos (an expression associated with chromosomal abnormalities) (19).
The size of a woman's follicle and oocyte stock is already determined during early stages of foetal development, with the size of 6-7 million oocytes at the fourth month of foetal development (20). At birth, this primordial follicle pool consists of around 1-2 million oocytes (21). Due to a continuous process of apoptosis, follicle numbers are reduced to approximately 300,000-400,000 at menarche (22). Renewal of the primordial follicle pool from pluripotent stem cells has always been considered not to occur, although new insights have recently been proposed (23).
When follicle numbers fall below a critical threshold of a few thousand, the perimenopausal transition commences, which is characterised by cycle irregularity and altered cycle length. Finally, prolonged menstrual cycles proceed to cycle arrest, a milestone referred to as menopause, which marks the end of the reproductive lifespan and coincides with a near absence of primordial follicles in the ovaries (24-26). **Figure 1** demonstrates a model for the gradual loss of primordial follicles through the stages of reproductive life of the female.

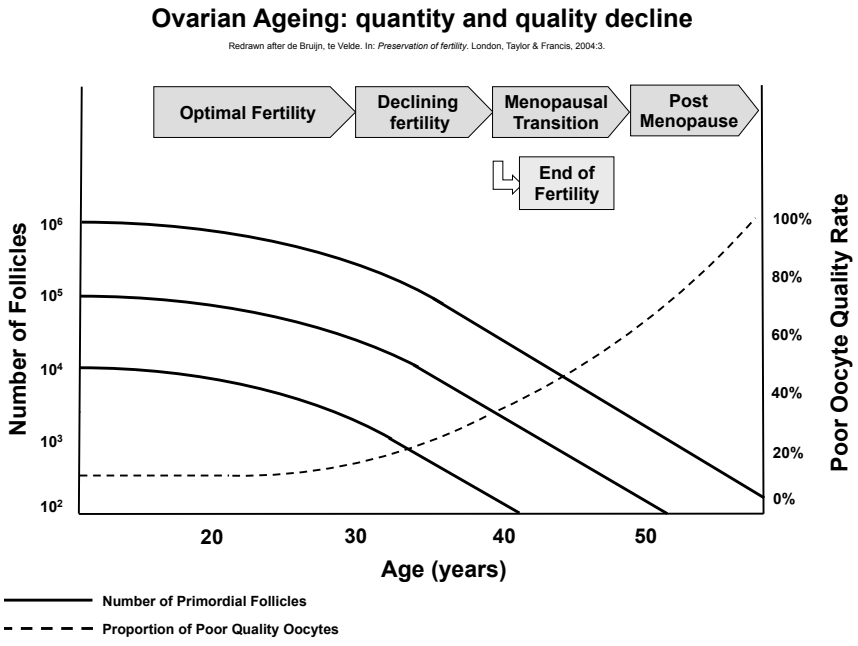
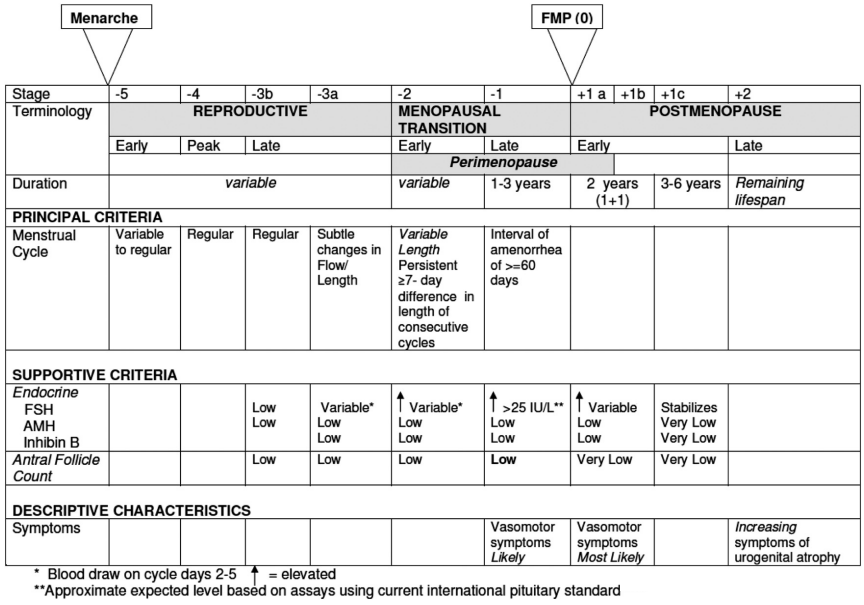


Figure 1. Gradual loss of quality and quantity of the primordial follicle pool.

The normal process of ovarian ageing varies considerably among women, with a peak fertility in their mid- to late twenties. The profound age-related decline in female fecundity, however, remains largely unnoticed until clinical signs of the perimenopausal transition are present. The staging system according to the stages of the reproductive ageing workshop (STRAW) is demonstrated in **Figure 2** (27). The reduction in the capacity to create an ongoing pregnancy leading to a live birth is accompanied by an increase in early follicular follicle-stimulating hormone (FSH) levels; this condition is known as imminent ovarian failure (IOF) or late reproductive ageing (**Figure 2**, stage -3a STRAW) (27). By definition this is the period before the onset of the menopausal transition and is characterised by a regular menstrual cycle, elevated FSH levels during the early follicular phase, in women with a high probability of being infertile (28) and cannot be easily recognised in an individual.
The onset of the menopausal transition, expressed by lengthened cycles due to deficiency of antral follicles capable of growing into dominance, is usually a woman's first overt notification of advanced ovarian ageing.
Next to the subtle changes in monthly menstrual cyclicity, changes in ovarian function may also become evident in the reduced ability to produce viable offspring.



Stage	-5	-4	-3b	-3a	-2	-1	+1 a	+1b	+1c	+2
Terminology	REPRODUCTIVE				MENOPAUSAL TRANSITION		POSTMENOPAUSE			
	Early	Peak	Late		Early	Late	Early			Late
					Perimenopause					
Duration	*variable*				*variable*	1-3 years	2 years (1+1)		3-6 years	*Remaining lifespan*
PRINCIPAL CRITERIA										
Menstrual Cycle	Variable to regular	Regular	Regular	Subtle changes in Flow/ Length	*Variable Length* Persistent ≥7- day difference in length of consecutive cycles	Interval of amenorrhea of >=60 days				
SUPPORTIVE CRITERIA										
Endocrine FSH AMH Inhibin B			Low Low	Variable* Low Low	↑ Variable* Low Low	↑ >25 IU/L** Low Low	↑ Variable Low Low		Stabilizes Very Low Very Low	
Antral Follicle Count			Low	Low	Low	Low	Very Low		Very Low	
DESCRIPTIVE CHARACTERISTICS										
Symptoms						Vasomotor symptoms *Likely*	Vasomotor symptoms *Most Likely*			*Increasing* symptoms of urogenital atrophy

* Blood draw on cycle days 2-5 ↑ = elevated
**Approximate expected level based on assays using current international pituitary standard

Figure 2. The Stages of Reproductive Ageing Workshop. Harlow SD et al. Executive summary of the Stages of Reproductive Aging Workshop + 10: addressing the unfinished agenda of staging reproductive aging. J Clin Endocrinol Metab 2012 Apr;97(4):1159-68, by permission of The Endocrine Society.

Therefore, a clinical condition like unexplained infertility may be a reflection of advanced ovarian ageing, expressed by a low response to ovarian hyperstimulation in IVF.

OVARIAN RESERVE TESTS

Direct measurement of the actual primordial follicle pool is impossible, as it would implicate the removal of one or both ovaries. However, several markers have been identified which can predict the quantitative aspect of current ovarian reserve status.

Historically, FSH was the first tool to be identified for the assessment of ovarian reserve and therefore often routinely measured in the early follicular phase in the diagnostic work-up of infertile couples. FSH acts as a quantitative marker of ovarian reserve and increases with advancing age due to the loss of negative feedback on pituitary FSH release by the reduction of Inhibin B and oestradiol (29). In the majority of women, elevated FSH levels are the result of inadequate gonadal feedback due to reduced ovarian reserve (30). However, other causes should be kept in mind, including physiological causes of prolonged quiescence of the HPO axis in lactational amenorrhea or post oral contraceptive use (31;32) or an increase in secretory drive, as seen in mothers with familial dizygotic twins (33). Finally, a FSH receptor variant can be the cause of elevated serum FSH levels, where higher FSH levels are required to compensate for a less active receptor to obtain normal function (34;35). The value of an elevated basal FSH remains unclear in clinical practice. Should these women be advised to start infertility treatment immediately or is expectative management justified?

The ovarian response to controlled ovarian hyperstimulation (COH) in IVF is another way to express the quantity of the ovarian reserve. A poor response in IVF, defined as the retrieval of a small number of oocytes, is considered a sign of diminished ovarian reserve. As a result, only a few oocytes are retrieved, a reduced number of embryos is available for transfer and consequently a poor pregnancy rate. These poor responders are thought to reach all four reproductive milestones earlier and are therefore considered to have an advanced biologic age for their chronological age. In line with this, they tend to reach menopause earlier than women with a normal response (36). Furthermore, a poor responder is believed to have reduced pregnancy rates as a result of this advanced biologic ageing (37;38). However, reasonable prospects of pregnancy have been published (39;40). This suggests that besides chronological age other factors are involved with the ovarian ageing process.

The use of Anti-Müllerian hormone (AMH) is a novel method to reflect female ovarian reserve and is gaining importance in reproductive medicine. Originally, AMH is known for its role during embryonic development where it plays an significant

role in male sexual differentiation (41). In the presence of AMH, regression of the Müllerian ducts is induced. Whereas in the absence of AMH in the early stages of female foetal development, the Müllerian ducts develop into the uterus, fallopian tubes and the upper part of the vagina (42). Next to its role during embryogenesis, this dimeric glycoprotein plays an important endocrine role in the recruitment of primordial follicles. Produced by the granulosa cells of antral follicles, AMH is highly related to the antral follicle count (AFC) and the size of the primordial follicle pool (43). Due to the gradual loss of primordial follicles in the ovaries, serum AMH shows a consistent decline with increasing female age (44-47).
Some small studies have suggested that in young women with elevated basal FSH levels, the combined information of age and AMH could identify a subset of couples with still reasonable pregnancy prospects (48-50).
Both an elevated basal FSH level and a poor response to IVF are expressions of limited follicle quantity of the ovarian reserve status. However, it seems that not all of these women are similar in terms of quality loss. There is an urgent need to identify those women with a reasonable prognosis as well as the ones with pregnancy prospects close to zero. Balanced decision making on the management of these women is desirable and may include individualising stimulation protocols as well as justifying or denying additional cycles. Besides the fact that ART will only compensate for the decreased natural fertility to a limited extent, it is also a financial burden to society and has a substantial emotional impact on couples facing fertility problems, and should therefore only be offered if justified (51;52). The possible inconsistency between the quantity and quality of the primordial follicle pool as observed in women with advanced ovarian ageing evokes the question whether AMH or patient characteristics can identify those women with a still acceptable prognosis.

VARIATION IN THE REPRODUCTIVE AGEING PROCESS

The variation in age at normal menopause is considerable and covers a range from 40 to 60 years of age, with a mean age of 51 years (53). A small subset of women will experience their final menstrual period before the age of 40, which is referred to as primary ovarian insufficiency (POI) (54).
A fixed time interval is believed to be present among the stages of reproductive ageing (**Figure 3**) (11;55). In line with this, age at menopausal transition ranges from 35 to 54 years, with an average of 46 years (56). In addition, from natural population studies an apparent variation in the age at last child birth has been observed, with a distribution shape highly similar to the one for menopause; an average age of 41 years, ranging from 23 and 51 years (1;7). The presumed fixed time interval between menopause and natural sterility may prove of great clinical importance to provide

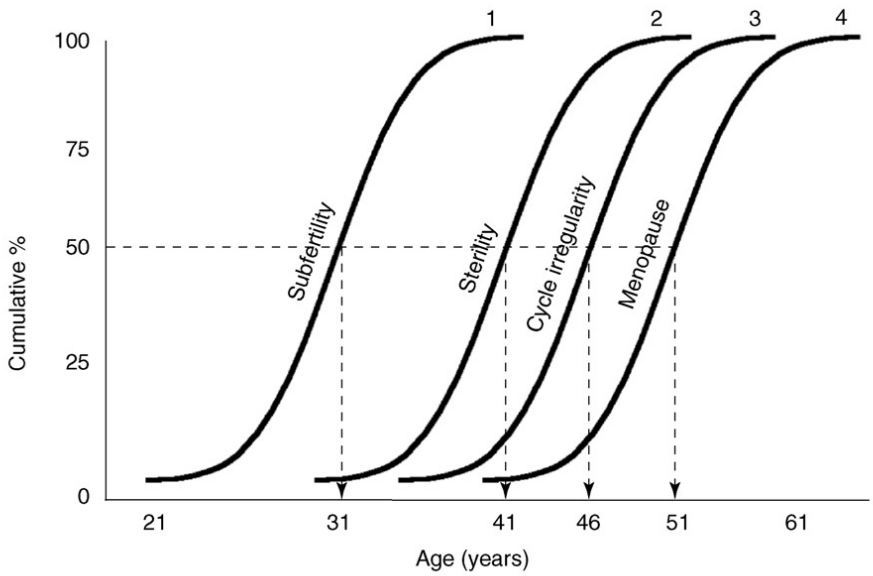


Figure 3. The distributions of age at the onset of subfertility, natural sterility, menopausal transition and menopause. Reproduced from Broekmans et al. Female reproductive ageing: current knowledge and future trends., 2007 Mar;18(2):58-65 (55), by permission of Elsevier.

information concerning a woman's individual fertility lifespan. An early menopause suggests an early loss of natural fertility resulting in infertility at young age, and vice versa (36;55). Chronological ageing and biological ageing, therefore, do not always coincide.

Timing of menopause has great implications for women's health and fertility. Early menopause is associated with an increased risk of cardiovascular disease (57-61), osteoporosis (62) and colorectal cancer (63). While on the other hand, late menopause is associated with an increased risk of ovarian, endometrial and breast cancer (64-66).

DETERMINANTS OF AGE AT MENOPAUSE

The variation in age at normal menopause is considered to be highly attributable to genetic factors. Although the effect sizes may be small, several genes have been found to be associated with age at natural menopause. Notably, these genes are implicated in DNA repair (MSH6, MCM8, EXO1, HELQ, UIMC1, FAM175A, FANCI, TLK1, POLG, PRIM1) and immune function (IL11, NLRP11, BAT2) (67;68), whereas evidence for an association between genes directly associated with ovarian function and menopausal age has been inconsistent (69).

Next, several environmental and life-style factors can contribute to the rate of the reproductive ageing process. Smoking and nulliparity have been associated with an early age at menopause (70;71). Still, the inter-individual variation in menopausal age cannot be fully explained by these factors altogether.
The association between early menopause and vascular health as a possible causative factor has only recently received attention.

VASCULAR HEALTH - CAUSE OR CONSEQUENCE?

The association between age at menopause and an increased risk of cardiovascular disease has been described previously. Van der Schouw et al. found that for each year of delay in menopausal age, the cardiovascular mortality risk decreased by 2% (61). In addition, Lisabeth et al described that women with menopause before the age of 40 have a twofold increase in the risk of having a stroke compared to women with a later menopause (59).
The inverse relation between cardiovascular health and menopausal age has been attributed to the deprivation of endogenous oestrogens that occurs after menopause. This is supported by observational studies such as the Nurses' Health Study, which have suggested a beneficial effect of exogenous oestrogen suppletion (72). However, the results from clinical trials have failed to confirm that oestrogen replacement therapy prevents the development of cardiovascular disease. The randomised controlled Women's Health Initiative study even reported a significantly increased morbidity and mortality from myocardial infarction and stroke in the oestrogen supplementation group (73). Therefore the deleterious effect of endogenous oestrogen decline after menopause as a causative factor for cardiovascular disease remains under debate and provides room for an alternative hypothesis: compromised vascular health as a cause instead of a consequence of menopause.
Kok et al. were the first to investigate the alternative scenario that atherosclerosis promotes early menopause. In a prospective cohort study they demonstrated that a premenopausal unfavourable cardiovascular risk profile was associated with early menopause (74). In addition, atherosclerotic plaque sizes were shown to be negatively correlated with ovarian reserve status in premenopausal female primates (75). This reverse association may be plausible considering that irreversible vascular damage leads to compromised vascularisation of the ovarian tissue with increased loss or limited survival of primordial follicles as a result.
Atherosclerosis is a progressive, systemic disease, which has its origin early in life when fatty streaks and fibrous plaques are formed in the intima of arteries (76;77). The continued lipid deposition thereafter increases these vessel lesions, which can eventually result in the clinical manifestation of atherosclerotic disease by diminished vascular diameter and elasticity, but also by sudden events like arterial

thrombosis and embolism. Therefore, although clinically manifest vascular disease in women generally does not manifest before menopause, the process most likely originates during the premenopausal years.

Genetic studies have provided further evidence that vascular health is associated with age at menopause. Genome wide association studies (GWAS) have demonstrated that loci associated with age at menopause are located in domains of genes related to pathways for DNA repair and cell death (67;68). In addition, associations have been found between a variant of the APO-E gene, which is associated with longevity and atherosclerosis, and age at natural menopause (78;79).

Finally, a foetal origin of advanced ovarian ageing may also be considered. An adverse prenatal environment is thought to affect health in later life by irreversibly affecting the physiology, metabolism and organ structure of the developing foetus (80). In this context, caloric restriction during pregnancy has been associated with an increased risk of cardiovascular disease in adult life (81). Female reproductive function involves a complex interplay of both hormonal and physical events. As such, it has been hypothesised that caloric restriction during pregnancy may also affect the prenatal development of the reproductive organs and thereby result in an earlier menopause (82). This is supported by the observations of an earlier menopause following exposure to famine in early childhood (83).

Identifying markers of vascular ageing that are associated with advanced ovarian ageing may help to predict a woman's reproductive lifespan. The detection of a causal link between vascular and ovarian ageing is notably difficult and cannot be identified with a cross-sectional study design. However, if a robust association would be demonstrated, this would justify the design and execution of interventional studies, such as animal models with induced vascular damage to study subsequent reproductive lifespan and performance. Elucidation of these associations will additionally create more understanding about the variation of the reproduction ageing process. With this information, individualised preventive strategies can be made with regard to early fertility loss and menopause related diseases in terms of oocyte vitrification or lifestyle changes.

AIMS AND OUTLINE OF THE THESIS

The studies presented in this thesis focus on pregnancy forecasting based on information regarding quantitative ovarian reserve status, as well as the role of vascular health and unfavourable conditions during intra-uterine life in female reproductive ageing patterns. We studied these issues in several clinical cohorts that serve as an extreme phenotype for compromised ovarian reserve, vascular health status or foetal environment.

Part One:
Identification of patient characteristics and ovarian reserve markers that forecast pregnancy or live birth in women with a diminished ovarian reserve status.

Part Two:
Investigation of the role of unfavourable cardiovascular health in the timing of ovarian ageing, using several phenotypes of compromised vascular health as a model.

OUTLINE

Part One:

Chapter 2 presents a systematic review of the existing literature regarding patient characteristics and ovarian reserve tests in poor responders and their pregnancy prospects.

Chapter 3 investigates the role of serum Anti-Müllerian hormone levels as a predictor of live birth and reproductive stage in subfertile women with elevated basal FSH levels.

Part Two:

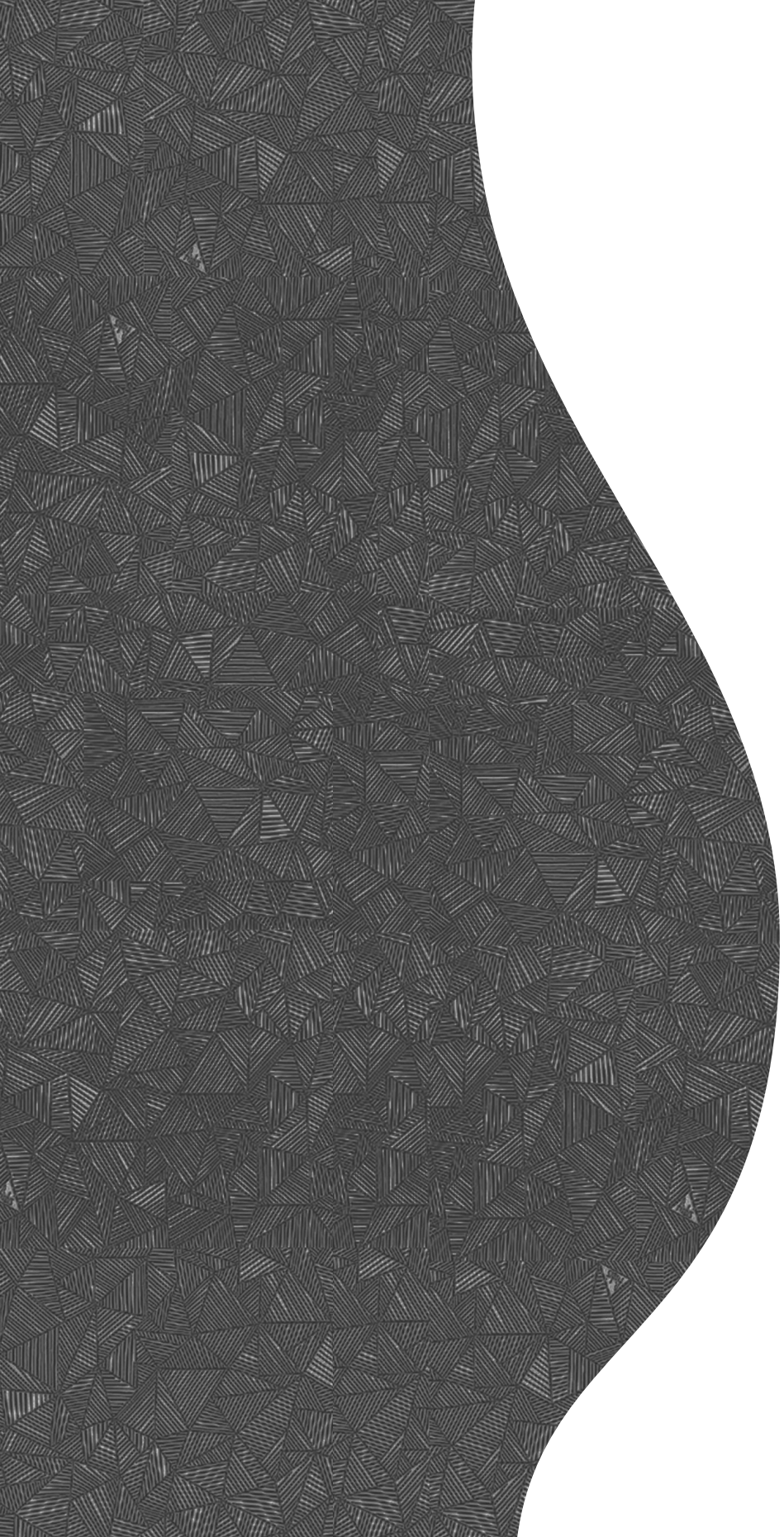
Chapter 4 discusses the results of a retrospective cohort study into the role of vascular factors as a potential determinant in the variation of the ovarian ageing process with preeclampsia as
a model.

Chapter 5 describes the results of a cross-sectional patient-control study on the association between vascular health and ovarian reserve status in women with type 1 diabetes.

Chapter 6 studies the role of prenatal famine exposure on birth weight, reproductive performance and age at menopause in a retrospective cohort study.

Chapter 7 discusses the results of a patient-control study which studied age at natural menopause in women with type 1 diabetes compared to a general Dutch population.

Chapter 8 summarises the results of the studies presented in this thesis and discusses the implications for clinical practice and future research.



2

The poor responder in IVF: is the prognosis always poor?

A systematic literature review.

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ABSTRACT

Background

In IVF treatment a considerable proportion of women is faced with a low number of oocytes retrieved. These poor responders have reduced pregnancy rates compared to normal responders. However, this may not be applicable to all poor responders. This review aims at identifying patient characteristics and ovarian reserve tests that will determine prognosis for pregnancy in poor responders.

Methods

A systematic search was conducted in PubMed, Embase, Cochrane and SCOPUS databases in April 2010. Studies regarding patient characteristics or ovarian reserve tests in poor responders and their pregnancy prospects were included. All included papers were summarized in descriptive tables.

Results

Nineteen studies were included. Pooled data of six studies comparing poor and normal responders demonstrated clearly lower pregnancy rates in poor responders (14.8% versus 34.5%) Ten studies indicated that older poor responders have a lower range of pregnancy rates compared to younger (1.5%- 12.7% versus 13.0%– 37.5%, respectively). Four studies showed that pregnancy prospects become reduced when fewer oocytes are retrieved (0%- 7% with 1 oocyte versus 11.5% -18.6% with 4 oocytes). Five studies concerning pregnancy rates in subsequent cycles suggested a more favourable outcome in unexpected poor responders, and if ≥ 2 oocytes were retrieved.

Conclusions

Poor responders are not a homogeneous group of women with regards to pregnancy prospects. Female age and number of oocytes retrieved in particular will modulate the chances for pregnancy in current and subsequent cycles. Applying these criteria will allow the identification of couples with a reasonable prognosis and balanced decision making on the management of poor responders.

INTRODUCTION

The birth of a normal healthy infant girl after replacement of a human embryo after IVF, reported by gynaecologist Steptoe and physiologist Edwards in the Lancet in 1978, resulted from the use of a single oocyte from a spontaneous ovarian cycle (84;85). Soon after this ground breaking event, controlled ovarian hyperstimulation (COH) was introduced, and the availability of a high number of oocytes boosted the pregnancy rates of IVF treatment (84;86). However, after 40 years of experience there are still women who respond poorly to COH, resulting in only few oocytes at retrieval, a reduced number of embryos available for transfer and a poor pregnancy rate (87). The prevalence of poor responders is reported to vary between 5.6% and 35.1% (37;39;88-91), depending on differences in the definition of poor response. In general, poor responders have a lower pregnancy rate compared to normal responders (37-39;88;92-96), although reports on poor responders with reasonable prospects of pregnancy have also been published (39;40). The physiology behind ovaries responding poorly to hyperstimulation is the presence of a reduced number of FSH-sensitive follicles, most frequently linked to the condition known as diminished ovarian reserve. In some cases, however, poor response may be associated with suboptimal exposure to gonadotropins, for example in obese women (97), or the presence of FSH receptor subtypes which render the follicles less sensitive to exogenous gonadotropins (98).

Although declining ovarian reserve with age is associated with a reduction in oocyte quality, exemplified by poorer chances of implantation and higher rates of early pregnancy loss, a solid link between the remaining quantity of antral follicles and the quality of the oocytes held within these follicles seems missing. Hence, it can be assumed that not all poor responders are similar in terms of loss of oocyte quality and the question arises of whether patient characteristics can be identified that mark poor responders who still have an acceptable prognosis, both in the current cycle as well as in subsequent cycles. Once identified, these couples could be counselled on whether it is worthwhile to start or continue with IVF.

The aim of this literature review was therefore to identify the prognostic value of patient characteristics and ovarian reserve tests (ORT) for pregnancy in poor responders to COH in the current or subsequent cycle.

METHODS

Literature search

A systematic search was conducted in PubMed, Embase, Cochrane and SCOPUS databases using synonyms for ‘in vitro fertilisation’, ‘intracytoplasmic sperm injection’ or ‘assisted reproduction treatment’ and ‘poor response’ or ‘number of oocytes’ and ‘pregnancy rate’. A period of all years through April 2010 was covered

by the search. The search was conducted independently by two researchers (J.O and S.B.) No limits were used in the advanced search. If necessary and applicable, authors were contacted for any missing data. The Statement of Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) was followed as far as applicable, although the authors realised that the nature of the studies was likely to make adherence to this protocol difficult, as our aim was not to make a comparison between predefined groups but to investigate associations between predictive factors and the outcome.

Study selection

Studies were selected if the target population consisted of patients with a poor response to COH for IVF or ICSI and inclusion followed if two basic conditions were met. First, probability of pregnancy in the present cycle and/or subsequent cycles had to be reported or analysed. Second, the role of patient characteristics, such as age and/or BMI and/or ORTs and/or treatment characteristics, in the probability of a pregnancy occurring had to be documented. As ORTs, we preferred to study FSH, antral follicle count (AFC) and/or Anti-Müllerian hormone (AMH) because these tests are superior to ovarian volume or any other test as a marker for ovarian reserve. However, ovarian volume as a predictive factor was still included in the search. All possible studies that could meet these two basic conditions were included in the search (**Figure 1**).

Currently, there is no international consensus regarding the definition of a poor response. Many definitions have been used since the 'poor responder' was first described in 1983 (99;100). Definitions vary from using the number of oocytes retrieved, the number of follicles at the end of COH, the peak oestradiol (E2) level after standard stimulation or a combination of these all. Every possible definition of poor response was included in this review so that a complete search, selection and analysis in this review could be performed. However, proxy definitions of poor response, such as prior elevated basal FSH or advanced female age, were not included in the present review as a considerable proportion of these so called predicted poor responders will not demonstrate a poor response in the actual first cycle. Furthermore, all possible definitions used for the main outcome (pregnancy) in poor responders to COH were included. Reviews and case-reports did not meet the inclusion criteria and were excluded from the original search.

Study selection occurred in two stages. First, titles and abstract were screened by two researchers (J.O., F.Y.) in order to provide a selection of full text papers likely to meet the predefined selection criteria (**Figure 1**). Second, final inclusion and exclusion occurred after examination of the full text by three independent researchers (J.O, F.Y and S.B). Any disagreement about inclusion was resolved by consensus or a fourth reviewer (F.B.).

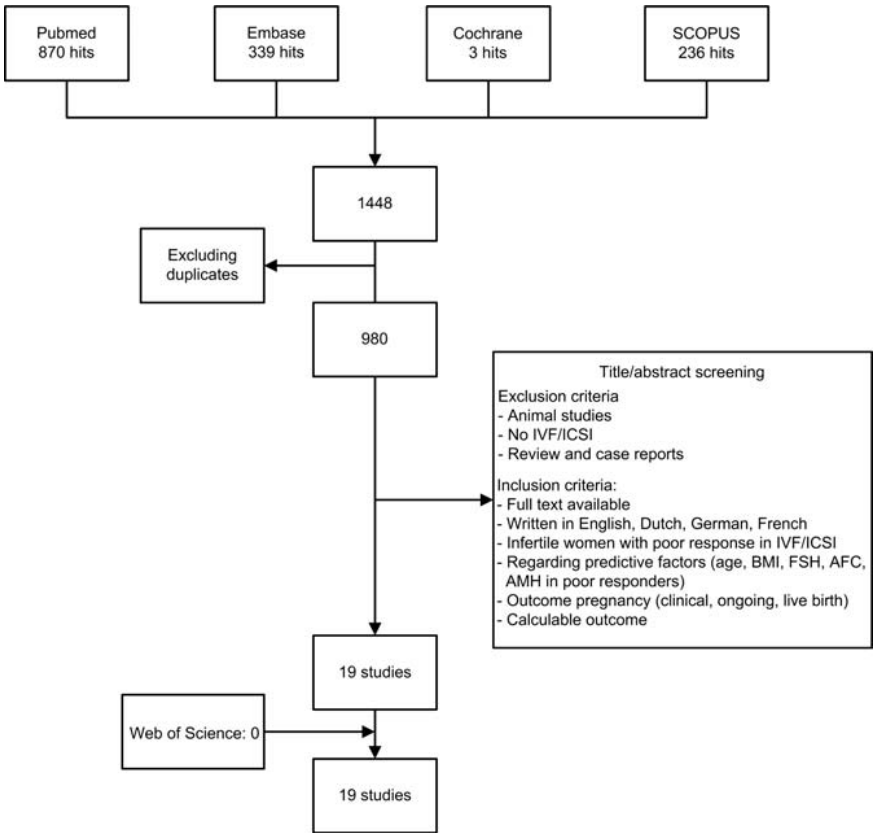


Figure 1. Search and selection strategy for systematic review of the literature to identify patient characteristics and ovarian reserve tests that will determine prognosis for pregnancy in poor responders in IVF. AFC: antral follicle count, AMH: anti-Mullerian hormone

Data extraction

Relevant data from all included studies were summarized in descriptive tables containing the study design, the number of included patients, the definitions applied and the patient characteristics or ORTs analysed (**Table 1**).

Meta-analysis

The extracted data from the selected studies were converted into 2x2 tables for the predictive factor studied, patient characteristics or ORTs, versus pregnancy (yes or no), using the cut-off value for the predictive factors as stated in the studies. Odds ratios (OR) and measures of statistical uncertainty were calculated from the 2x2 tables. In case of similar cut-off values, statistical pooling was considered feasible,

Table 1. Characteristics of the included studies

Author	Year	Design	*n*	Definition Poor Response	Definition Pregnancy	One or consecutive cycles	Predictive Factor studied	Compared to normal resp.
Baka et al.	2006	RC	96c	≤ 3 oocytes or E2 level <500pg/ml day of hCG injection or FSH<20IU/L	Clinical	One	Number of oocytes	No
Biljan et al.	2000	PC	828w	≤ 3 follicles US	Clinical	One	Age	Yes
Gaast, van der et al.	2006	RC	7422w	Not specified	Unclear	One	Number of oocytes	No
Galey-Fontaine et al.	2005	RC	163c	< 5 follicles >14mm + E2 < 1000pg/ml before HCG injection	Clinical	One	Age, FSH	No
Hanoch et al.	1998	RC	143c	E2 level <1000pg/ml day of HCG injection	Clinical	One	Age	No
Hellberg et al.	2004	RC	1699w	< 5 oocytes retrieved	Live birth	Consecutive	Number of oocytes	No
Hendriks et al.	2008	PC	222w	< 4 oocytes or cancellation	Ongoing	Consecutive	Ovarian reserve tests	Yes
Inge et al.	2005	RC	805c	≥1 oocyte and ≤ 5 oocytes	Live birth	One	Age	No
Klinkert et al.	2004	RC	225w	< 4 oocytes or < 3 follicles	Ongoing	Consecutive	Ovarian reserve tests	No
Orvieto et al.	2009	RC	397w	< 5 oocytes	Clinical	One	BMI	No
Rooij, van et al.	2003	PC	93w	< 4 oocytes	Ongoing	One	Age	No
Saldeen et al.	2007	RC	1706c	≤ 5 oocytes	Clinical	One	Age	Yes
Schimberni et al.	2009	RC	294w	previous cycle ≤ 1 follicle	Clinical	Consecutive		No
Sutter, de et al.	2003	RC	9644c	≥1 oocyte and < 5 oocytes	Ongoing	One	Age	Yes
Timeva et al.	2006	RC	1017c	≤ 5 oocytes	Clinical	One	Number of oocytes	Yes
Ulug et al.	2003	RC	209c	≤ 4 follicles >10mm US	Clinical	One	Age, number of oocytes	No
Veleva et al.	2005	RC	45w	≤ 3 oocytes	Clinical	Consecutive	Poor response consistency	No
Yih et al.	2005	RC	4862c	≤4 oocytes	Clinical	One	Age	No
Zhen et al.	2008	RC	944c	< 4 oocytes	Clinical	One	Age	Yes

n= number of women(w)/ cycles(c). Study design: RC = retrospective cohort, PC = prospective cohort. US = ultrasound. E2 = estradiol

and inverse variance weighted pooling of the log (OR) was performed. When the differences between cut-off values were considered too large, no statistical pooling was performed.

RESULTS

Systematic review

The initial database search resulted in 1448 hits. Duplicates were removed using Refworks, which resulted in 980 remaining articles. After reading the title and abstract a total of 19 full-text articles were detected. These articles reported on the prognosis of poor responders related to patient characteristics, compared to normal responders or concerning the pregnancy rate in the current cycle and the subsequent cycles (for overview of search strategy and results, see **Figure 1**). These complete papers were read, and results were extracted and summarized. Six studies revealed the pregnancy rate for poor responders compared to normal responders, ten studies showed the influence of female age on the pregnancy rate for poor responders and only one article reported the prognosis of poor responders and the influence of BMI. One study reported on the value of basal FSH as prognosticator of pregnancy. Four studies investigated the pregnancy rate by comparing classes of oocyte number obtained and five studies investigated the pregnancy rate for the present and consecutive cycles in first cycle poor responders. Three studies offering multivariable prediction models for outcome pregnancy were identified and considered suitable for data extraction (39;40;101).

Cut-off values of age and FSH varied among the included studies as well as the definition of poor response. As a consequence of this, the results from these individual studies could not be compared and their data could not be aggregated. However, to point out a general tendency we pooled the data (without correcting for heterogeneity) concerning the comparison of poor responders and normal responders. For the analysis of predictive factors within the poor responder group, structured tabulations were performed in order to demonstrate the interpretations of the data.

We decided to refrain from contacting the authors for additional information as this would be more appropriate for an analysis of individual patient data and our aim was to give an overview of the current literature.

No publications were found regarding the prognosis for poor responders specified for smoking, AFC, AMH or ovarian volume. By cross-checking references from the articles utilising the Web of Science, no additional studies were located. In **Table 1** the studies selected for reading the full paper and subsequent data extraction are listed.

Table 2. Comparison of pregnancy rate in poor responders versus normal responders

Author	*n*	Definition poor response	Definition of Pregnancy	PR/cycle poor resp.	PR/cycle normal resp.	*p*-value	Age poor responders	Age normal responders	*p*-value
Biljan et al.	805w	≤ 3 follicles US	Clinical	14.3%	33.0%	Not stated	37.3* 41.4 **	34.5* 42.3**	0.003* NS**
Hendriks et al.	222w	< 4 oocytes + cancellation	Ongoing	7.6%	25.9%	0.001	39	35	0.01
Saldeen et al.	1706c	≤ 5 oocytes	Clinical	9.0%	32.6%	<0.0005	35.9	33.7	<0.0001
Sutter, de et al.	9644c	≥1 oocyte and < 5 oocytes	Clinical	17.5%	35.3%	<0.0001	Not stated	Not stated	
Timeva et al.	1017c	≤ 5 oocytes	Clinical	12.1%	29.5%	<0.05	Not stated	Not stated	
Zhen et al.	944c	< 4 oocytes	Clinical	14.8%	36.7%	<0.05	36.6	33.3	<0.05
Pooled estimate	**14.338**			**14.8%**	**34.5%**				

n = number of women(w)/cycles(c) included. PR= pregnancy rate. *group <40 years, **group ≥40 years. NS = not significant.

Prognosis for poor responders compared to normal responders

A literature overview of the pregnancy rate for poor responders compared with normal responders is shown in **Table 2**, demonstrating that poor responders have a pregnancy rate varying from 7.6% to 17.5% compared to normal responders varying from 25.9% to 36.7%. After pooling the data, without a correction for heterogeneity, a total of 14,338 patients could be included. From the pooled data analysis the estimate for the pregnancy rate for poor responders was 14.8%, as opposed to 34.5% for normal responders. The effect of age on the difference in pregnancy prospects could be questioned. Unfortunately, this could not be taken into full account in this review, as individual patient data were lacking for multivariable analysis. However, a trend towards an older age for poor responders becomes apparent from the listed studies.

Female age

Ten studies were located regarding the prognosis of poor responders in subgroups based on female age (**Table 3**). All 10 studies (37;87-89;93;94;96;101-103) showed a decrease in pregnancy rate for the older poor responder and in five of these studies these differences were statistically significant (37;87;88;93;96;102). For example, de Sutter and Dhont (2003) compared the pregnancy rate between women of 36 years and younger with women older than 36 years and demonstrated a significant difference of $p<0.0001$ (pregnancy rate of 23.0% versus 12.0%, respectively). Overall, the effect of female age on the prognosis in poor responders shows that older poor responders have lower pregnancy rates (ranging between 1.5% and 12.7%) compared to younger poor responders (ranging between 13.0% and 35%). Owing to the heterogeneity in age class distribution applied in the different studies, pooling of data for an overall meta-analysis could not be justified.

BMI

Only one study was found concerning pregnancy rates for poor responders and the influence of BMI. Orvieto et al. (2009) described the pregnancy rate in subgroups for BMI below or above 30 kg/m^2. A significant decrease in pregnancy rate was found for the poor responders with a BMI>30 kg/m^2 versus a BMI <30 kg/m^2 (4.5% versus 23%, respectively). The age distribution of the patients in the two subgroups was similar (32.4 SD ± 5.5 years versus 32.7 SD ± 4.5 years, for BMI>30 and BMI <30, respectively).

Basal FSH

Galey-Fontaine et al. (2005) compared the pregnancy rates for poor responders according to the basal FSH level. In the analysis of 163 poor responders with either normal or elevated basal FSH levels (cut off 12.0 IU/L), a significant decrease in pregnancy rates for women with an elevated basal FSH versus those with normal FSH (4.0% versus 14.8%, respectively) was demonstrated. After correction for

Table 3. Female age and pregnancy rate per cycle started

Author	*n*	Definition poor response	Female age	*p*-value	Note
			28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 >46		
Zhen et al.	472c	<4 oocytes	18.5% | 2.8%	<0.001	
Rooij, van et al.	47w	< 4 oocytes	13.0% | 4.0%	NS	
Biljan et al.	42w	≤ 3 oocytes US	27.8% | 4.2%	>0.05	
Saldeen et al.	290c	≤ 5 oocytes	14.0% | 3.0%	NS	PR per ovarium pick up
Inge et al.	173c	≥1 oocyte and ≤ 5 oocytes	27.1% | 12.7%	NS	DR per cycle
Sutter, de et al.	1280c	≥1 oocyte and < 5 oocytes	23.0% | 12.0%	<0.0001	
Galey-Fontaine et al.	163c	<5 foll. + oestradiol <1000pg/ml before HCG	14.6% | 4.9%	<0.04	PR per retrieval
Ulug et al.	209c	≤4 follicles >10mm US	19.5% | 7.2% | 1.5%	<0.04	PR per embryo transfer
Hanoch et al.	143w	E2 level <1000pg/ml day of HCG injection	19.3% | 6.0% | 6.5%	0.004	
Yih et al.	525w	≤4 oocytes	35% | 21% | 17% | 11%	NS	PR per retrieval

n = number of women(w)/ cycles(c) included, PR = pregnancy rate, DR = delivery rate, US = ultrasonographically, SD = standard deviation, NS = not stated

female age category (< versus ≥ 36 years), the effect of elevated basal FSH on the prospects in this poor responder group was still significant.

Number of oocytes retrieved

Four papers summarized the outcome of IVF in poor responders in subgroups based on the actual number of oocytes retrieved (**Table 4**). Three groups investigated the predictive value of the number of oocytes retrieved for poor responders with one, two and three oocytes retrieved (87;92;95): women with one oocyte retrieved showed a very low pregnancy rate in all three studies (0%, 0% versus 2.3%, respectively), while in cases with two oocytes retrieved higher numbers of pregnancies (15%, 10.8% versus 4.3%, respectively) were observed. The same was true for cases with three oocytes, showing a further increase in pregnancy prospects, with the exception of the study by Timeva et al. (2006) and Baka et al. (2006). Timeva et al (2006) and Ulug et al (2003) also reported on four oocytes retrieved, which resulted in even better results (11.5% versus 15.9%, respectively) and finally Timeva et al. (2006) also included women with five oocytes retrieved, with a pregnancy rate of 22%.

Table 4. Number of oocytes retrieved and pregnancy rate per first cycle started

Author	*n*	1	2	3	4	5	*p*-value	Note
Baka et al.	96c	0.0%	15.2%	12.5%			0.41	*p*-value on 3 oocytes vs 1
Gaast, van der et al.	7422w	7.0%	11.5%	15.6%	18.6%	21.7%	–	Data extracted from figure
Timeva et al.	1017c	0.0%	10.8%	8.7%	11.5%	22%	<0.05	
Ulug et al.	209c	2.3%	4.3%	11.5%	15.9%		<0.05	PR per embryo transfer

n = number of women(w)/ cycles(c) included, PR= pregnancy rate, NS = not stated

Several studies (38;95;103) investigated the predictive value of the number of oocytes retrieved, including all women undergoing IVF/ICSI, with regard to pregnancy rate. The authors assumed that there is an optimal range of oocytes for achieving pregnancy. According to the results of van der Gaast et al. (2006) thirteen oocytes at retrieval resulted in the highest pregnancy rate of 28% . Furthermore, they showed a clear correlation in pregnancy rate for poor responders depending on the number of oocytes retrieved, however data could not be accurately obtained from the graph shown.

Performance in subsequent cycles

Five studies have described the pregnancy rate for poor responders in the current and subsequent cycles (**Table 5**). Hendriks et al. (2008) and Klinkert et al. (2004) analysed the pregnancy rate for expected poor responder compared to the unexpected poor responder in three consecutive cycles.

In both Hendriks et al. (2008) and Klinkert et al. (2004) a decrease in pregnancy rate was observed for expected poor responders in the subsequent cycles from 7% to 9% in the second cycle to 0% in the third subsequent cycle, with a cumulative pregnancy rate in the second and third cycle of 11.5% to 19%. This contrasted to unexpected poor responders, who showed an increasing pregnancy rate from 11% to 22% in the second cycle to 21% to 25% in the third cycle, with a cumulative rate of 25.9 to 47% in the third cycle. Moreover, Schimberni et al. (2009) investigated the pregnancy rate for poor responders, defined as a cancelled cycle in the previous cycle, who underwent a series of natural cycle IVF treatment (104). An increasing pregnancy rate was seen until the third cycle (9.5% to 12%), however after the third cycle the chance of becoming pregnant falls to 10.2% in the fourth until 7.2% in the fifth cycle, with an overall cumulative pregnancy rate of 16.7%.

Hellberg et al. (2004) studied the birth rate in 1699 women with two subsequent IVF cycles, 898 of whom had three successive IVF cycles. The number of oocytes retrieved in the first IVF cycle was used to predict the outcome in the second or third treatment cycle. When one to two oocytes were retrieved in the first cycle, the birth rate in the second cycle was 9.5%. The retrieval of three to four oocytes resulted in birth rates of 16.5% in the second cycle. Moreover, irrespective of the number of oocytes retrieved in the second cycle, a decline in birth rate (mean 7.3%) was demonstrated in the third cycle when originally one to three oocytes were retrieved in cycle one.

Finally, Veleva et al., (2005) reported on the predictive value of a poor response in the first cycle on pregnancy rates in two subsequent cycles. In 54% of the women, an initial low response was followed by a normal response in at least one cycle. However, with a pregnancy rate of only 10.1% per cycle, they concluded that a poor response was an indicator of a poor prognosis. In the studied population, consisting mostly of women less than 40 years of age, a consistent low ovarian response was observed in only 2.5%, with a live birth rate of 16.7% per cycle of two cases stimulated three times. As only data on three cycles completed have been analysed in retrospect, selection bias is likely to have influenced the observations.

Table 5. Poor responder and pregnancy rate in subsequent cycles.

Author	*n*	Cycles		PR per cycle (cumulative PR)			Note
				Expected poor responder (EPR)	Unexpected poor responder (UPR)	Definition Pregnancy	
Hendriks et al.	79w	3	Cycle 1	8%	7%	Ongoing	(un)expected poor responder: 'tri-variable-model' [12 x FSH (IU/L)] – [14 x AFC (n)] – [1 x inhibin B (pg/ml)]
			Cycle 2	7% (11.5%)	11% (14.8%)		
			Cycle 3	0% (11.5%)	21% (25.9%)		
Klinkert et al.	225w	3	Cycle 1	11%	9%	Ongoing	EPR: ≥41 FSH≥15 IU/L
			Cycle 2	9% (19%)	22% (29%)		UPR: <41 years old and FSH <15IU/L
			Cycle 3	0% (19%)	25% (47%)		
				Poor responder in Cycle 1			
Schimberni et al.	294w	≥5	Cycle 2	9.7% (12.9%)		Clinical	Natural cycle IVF after cycle 1 Poor responder if in previous cycle ≤ 1 follicle
			Cycle 3	12.0% (15.0%)			
			Cycle 4	10.2% (16.3%)			
			Cycle 5	7.1% (16.7%)			
Hellberg et al.	1699w	2	Cycle 2	1-2 oocytes cycle 1	9.5% (NS)	Live birth	Poor responder if < 5 oocytes retrieved
				3-4 oocytes cycle 1	16.5% (NS)		
Veleva et al.	45w	3	Cycle 2 and/or Cycle 3	LR 10.1% (*n*=43w)		Clinical	LR= low response
			Cycle 2 and/or Cycle 3	NR 16.7% (*n*=2w)			NR= normal response

n = number of women(w)/ cycles(c) included, PR= pregnancy rate, NS = not stated.

DISCUSSION

Main findings

This systematic literature review is the first to summarize the available evidence regarding the prognostic value of various patient characteristics and ORTs to predict the pregnancy rate in the current or subsequent cycle for poor responders after COH for IVF/ICSI in cycle one. The review confirms that poor responders have a diminished pregnancy rate compared to normal responders but also demonstrates that several factors modulate the prognosis in this patient group, with possible implications for clinical practice.

The aim of this review was to determine patient characteristics that differentiate poor responders with pregnancy prospects close to zero from those poor responders who still have a reasonable prognosis. In clinical practice, this latter category of poor responders should not be judged based solely on their poor response and no limitations in number of IVF treatment cycles should be applied. In contrast, balanced decision making on the management of poor responders that could be identified as having poor pregnancy prospects is desirable. The question is whether additional cycles are justified or continuation of treatment should be discouraged. Unfortunately, this review does not allow us to make this clear differentiation between favourable and unfavourable poor responders. The clinical value may lie in the possibility to counsel couples on the different prospects.

Not all women who respond poorly to COH have poor pregnancy prospects. The reduced prospects in poor responders for pregnancy may partly be attributed to the effect of female age, as in several studies reported in this review the poor responders are of higher age than control normal responders. The gradual decline in oocyte quality with advancing age in parallel to follicle number reduction, will explain the effect of poor response on reproductive capacity (105). However, a full in-depth analysis into this subject appeared not to be possible and comparison of data for individual patients may be the only way to fully assess the role of female age in the comparison of normal versus poor responders.

Among the factors predicting pregnancy outcome within the poor responder group, female age appeared to play a distinct role. The trend that older poor responders have a lower pregnancy rate compared to younger poor responders, is exemplified by the fact that in half of the studies a significant difference in pregnancy rate between age groups was noted. Not one publication contradicted this observation. Much like for the role of age in the difference in pregnancy rates between normal and poor responders, the age effect on oocyte quality may explain the effect of age within the poor response group. Several studies have shown that quantitative measures of ovarian reserve will relate to measures of quality but more so in older women. For example, poor response to stimulation was predictive of both pregnancy loss and the occurrence of a trisomic pregnancy after IVF (106;107). However, ORTs,

such as the AFC and AMH, failed to be predictive of pregnancy loss in an infertile population, in contrast to the steady relationship of female age with pregnancy loss (108). This indicates that the relationship between quantity of follicles and quality of oocytes is far from elucidated, with age being a driver behind decline in oocyte quality but partly independent of changes in quantity.

A second factor of relevance for prognosis in poor responders was the degree of poor response. As a result of the lower number of oocytes, there are fewer embryos to transfer and subsequently a lower prospect for pregnancy, in addition to the assumed overall negative effect of poor ovarian capacity on oocyte quality. An increased number of oocytes improves the prospects for pregnancy, varying from close to zero with one oocyte up to almost 15% when four oocytes had been retrieved. In the present review, the role of female age could not be analysed from the existing data, thereby leaving open the possibility that the effect of number of oocytes is mainly explained by age effects. In general, more oocytes will increase the chances for obtaining high quality embryos, an effect that has been shown to be independent of age. Such independence has also been demonstrated for the poor responder group separately (94). Larger datasets using individual patient data from published literature may allow for additional analyses of this subject. It should be noted, however, that there seems to be an optimal range for the number of oocytes retrieved: in addition to low numbers, the retrieval of high numbers of oocytes has consistently been associated with less favourable, although not poor, pregnancy prospects (38;109).

The present review has also demonstrated that patient characteristics other than age and ORTs have not been investigated properly as yet. Obese poor responders might have a lower pregnancy rate than non-obese poor responders, as the results in this review supported current views on the role of body weight in female reproduction (110;111). However, as the evidence for a role of body weight in poor responders is only based on one paper, it is not possible to reach a final conclusion (90). Likewise, for the only ORT studied (basal FSH level), evidence was found that it affects pregnancy rates in poor responders, based on only one study. The literature has previously shown a link between a raised basal FSH and a reduced reproductive capacity (112), which also fits the concept of the expected poor responder where, combined with an abnormal FSH test result prognosis is clearly decreased (40).

The systematic review on the prospects in subsequent cycles has yielded a limited number of studies. The question was raised what prognosis can be expected for women with a poor response in their first cycle, if they would continue treatment. Is it worthwhile proceeding for these women? From the limited studies available it emerges that some poor responders may still have reasonable prospects, depending greatly on their ovarian reserve status and age (39;40;91;104;113). Larger studies are necessary to support the concept of the expected poor responder, who may have a prognosis so poor that further treatment should be avoided.

Limitations

Results obtained in this review could have been influenced by some limitations in the search strategy. First of all there was no international consensus for the definition of a poor response. Different definitions and cut-off values were used, varying from ≤2 oocytes at retrieval to ≤5 oocytes at retrieval, ≤4 follicles >10mm to <6 follicles just before retrieval (87), a previous cancelled cycle (104), E2 level <1000pg/ml on the day of HCG injection (102), basal FSH level >10 IU/L, FSH stimulation with >450IU or a combination of any of these (39;92;93). Consequently, various patient groups were included in our search, affecting the homogeneity of the data and thereby the generalizability of the findings. As a result, articles were selected only when the authors used a low number of oocytes retrieved or when the mean number of oocytes was low, as this definition of poor responder is the one most commonly used. Milder ovarian stimulation protocols, with individualised dosing or the use of GnRH antagonist LH peak prevention, are currently promoted in order to decrease adverse effects (114;115): such an approach will result in a more modest number of oocytes at retrieval, with the inclusion of more cases that will formally yield a poor response but within fact optimal implantation rates (115). Obviously, in such responders the addition of the word ''poor' may be considered a misnomer. Also, various cut-off values were applied for age categories, which has prevented a more robust overall analysis of the effect of the characteristics on pregnancy prospects. Second, no studies were found regarding AMH and AFC in the prediction of outcome in poor responders. Therefore, only the predictive value of FSH was reported. Studies regarding AMH and AFC in outcome prediction in poor responders are highly desirable, as these tests have been shown to be superior over FSH in the prediction of response in an IVF population. If the AMH and AFC tests would help to identify so called expected poor responders, estimation of the prognosis may become more meaningful, as demonstrated previously (39). Outcome results on pregnancy were reported in many different ways, and presented as rate per cycle, per retrieval, per embryo transfer or per implantation, and as clinical pregnancy, ongoing pregnancy rate or live birth. Conversion could be performed with some of the results but ultimately pooling of data was greatly jeopardised. Still, comparisons within the studies were valid as the same units of outcome were applied.

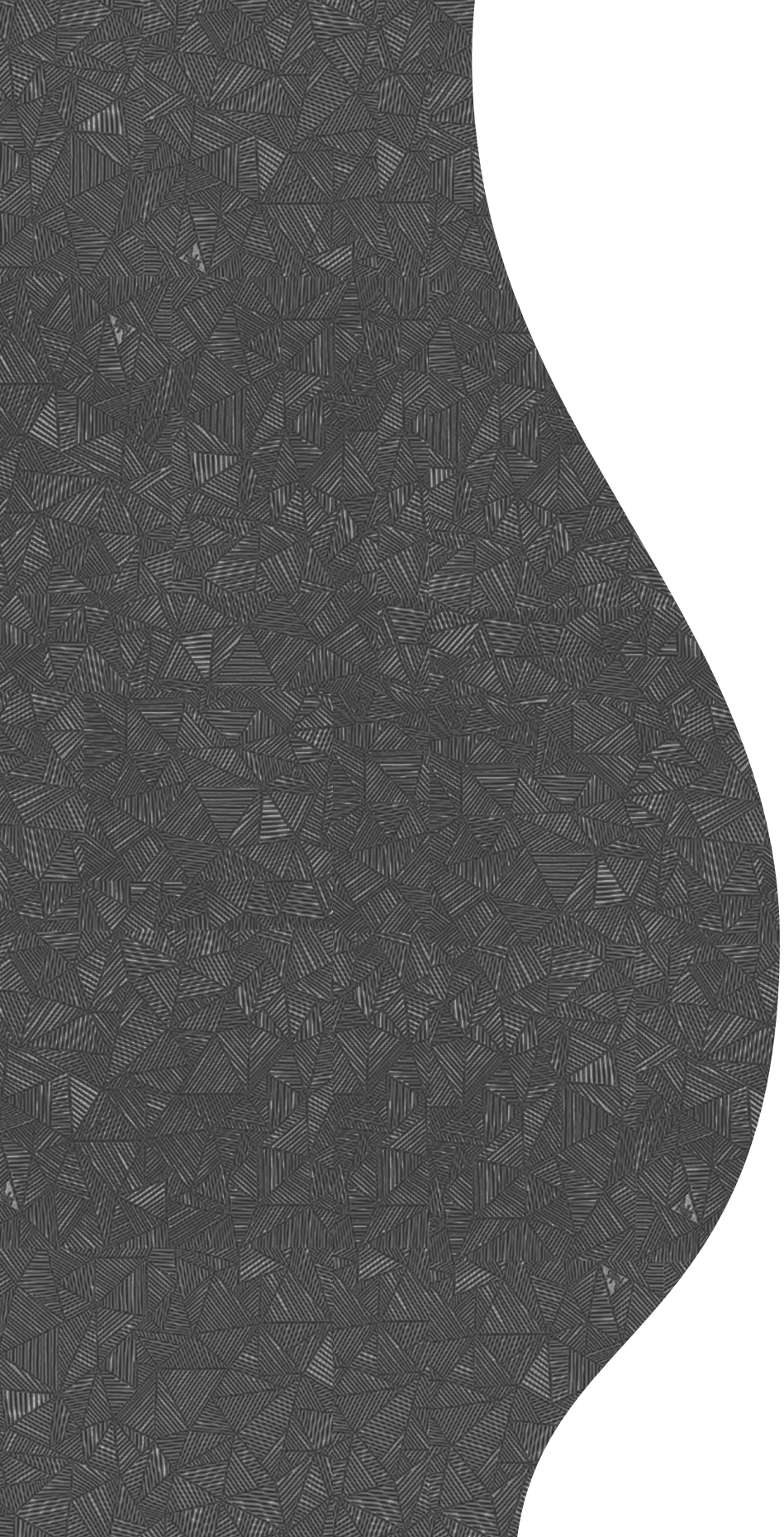
The results were further influenced by the inclusion or exclusion of cancelled cycles, or cycles with no eggs retrieved. Excluding these cases may lead to overestimation of the performance of poor responders, and currently there are no means of correcting for this phenomenon. Moreover, the cut-off value utilised in determining when to cancel a cycle differed greatly, varying from less than 3 mature follicles visualized ultrasonographically (39;88;91) to up to no more than five follicles present and an E2 level <1000pg/mL (103). As a result of these considerations, the conclusions in this review must be used with the assumption that cancelled cycles would have produced the same results as those cases that did undergo final maturation triggering and ovum retrieval in comparable circumstances of follicle growth.

Clinical implications

The clinical value of the present findings may lie in the counselling of couples that face a poor response to COH for whom, in general, the prospects are clearly different from cases with a normal response to hyperstimulation. However, this may be less obvious in younger women with 3 or 4 oocytes obtained and previously normal results in ORTs. Such poor response may be accidental and unrelated to a loss in oocyte quality. The question then will be which approach will be preferable. Ready cancellation of the cycle and repeating the stimulation with higher dosages of FSH may be driven by the hope for a normal response, with possibly better prospects. Proceeding to the follicle aspiration and dealing with the limited number of oocytes available that may still show sufficient quality in younger patients may be justifiable. In presumed poor ovarian reserve patients (abnormal ovarian reserve, high age) proceeding to retrieval may be the best way, as prognosis may not be altered anyway. Studies on the management of predicted or observed poor responders so far have not delivered the solid evidence for a preferred strategy. As such, proceeding to follicle aspiration may be justified in cases with a so called unexpected poor response.

If the poor response is observed after the oocyte retrieval, the same factors may direct our counselling on the question of whether additional cycles are justified. Female age and ovarian reserve status in concert deliver the tools for correct decision making, as a subgroup with still favourable prognosis can be identified. Nevertheless, robust studies on the management of predicted or actual poor responders are greatly needed, with a focus on the cost effectiveness of various strategies.

In summary, this literature review demonstrates that poor responders are not a homogeneous group and that the prognosis for these patients may vary greatly depending on patient characteristics, such as age or the actual number of oocytes obtained. These factors may help in decision making regarding continuation of treatment. Continued research using individual patient data from the published literature will enable more firm conclusions to be reached on the role of predictive factors in the poor responder in IVF.



3

AMH as predictor of reproductive outcome in subfertile women with elevated basal FSH levels: a follow up study

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ABSTRACT

Objective
To investigate the role of serum AMH as a predictor of live birth and reproductive stage in subfertile women with elevated basal FSH levels.

Design
A prospective observational cohort study conducted between February 2005 and June 2009.

Setting
Tertiary fertility center.
Patient(s): Subfertile women with 1) a regular menstrual cycle (mean cycle length 25-35 days), 2) basal FSH concentrations ≥12.3 IU/L and 3) younger than 40 years (n=96).

Intervention(s)
none.
Main Outcome Measure(s): Live birth and reproductive stage according to the Stages of Reproductive Ageing Workshop (STRAW).

Result(s)
A cumulative live birth rate of 63.5% was observed during a median follow-up of 3.3 years (n=85). AMH level was significantly associated with live birth. There was evidence of a non-linear prediction pattern, with an increase in chances of live birth up till an AMH level of 1 microgram/L. Other ovarian reserve tests and chronological age appeared of limited value in predicting live birth. Moreover, AMH was significantly associated with the timing of reproductive stages (n=68), i.e. the occurrence of menopausal transition or menopause during follow-up.

Conclusion(s)
The present findings suggest applicability of AMH determination as a marker for actual fertility in subfertile women with elevated basal FSH levels.

INTRODUCTION

Female reproductive ageing is a process dominated by the gradual decline of both oocyte quantity and quality (11). With increasing chronological age, female fecundity decreases (105). The progressive follicle decline is accompanied by notable changes in menstrual cycle regularity with menopause as the final step in the ovarian ageing process (24-26). Before cycle irregularity marks the onset of the perimenopausal transition, an increase in early follicular FSH level occurs, a clinical condition referred to as late reproductive ageing (stage -3a) according to the Stages of Reproductive Ageing Workshop (STRAW) classification (27). By definition this is the period before the onset of the menopausal transition, characterized by the presence of a regular menstrual cycle and elevated basal FSH levels, in women with a high probability of being infertile (28). Historically, FSH was the first tool to be identified for assessing ovarian reserve and, as a result, it is often routinely measured in the early follicular phase in the diagnostic work-up of infertile couples. An elevation in FSH is generally thought to imply lower chances of pregnancy (30). However, alternate explanations for elevated basal FSH exist, including physiological causes where prolonged quiescence of the hypothalamic-pituitary-ovarian axis, such as during lactational amenorrhea (32) or post oral contraceptive use (31) elicits an overshoot secretion of FSH at resumption of the menstrual cycle. Moreover, in mothers with familial dizygotic twins, elevated FSH levels are associated with an increase in the secretory drive of FSH instead of inadequate gonadal feedback (33). Another possible reason for slightly elevated FSH levels is the FSH receptor variant, where higher FSH levels are required to compensate for a less active receptor to obtain normal function, but these adjusted FSH levels are usually around the upper limit of the normal range (34;35;116). Therefore, in the vast majority of women with regular cycles, elevated early follicular FSH will either be based on reduced ovarian reserve or increased secretory drive.

An ongoing debate exists about the value of an elevated basal FSH in clinical practice. Is expectative management with regard to pregnancy prospects justified or should these women be advised to start infertility treatment immediately? Also, the long term outcome in hypergonadotropic women with regard to fertility remains unknown.

Anti-Müllerian hormone (AMH) is a novel method to reflect a woman's ovarian reserve. Recent studies suggest this dimeric glycoprotein to be superior and more reliable in comparison to FSH in predicting ovarian reserve (117-122). Synthesis and release from the later antral follicle stages will allow the build-up of serum levels, in a cycle independent fashion (47;119;123-125). Due to the gradual loss of primordial follicles from the ovaries, which in turn affects the number of antral follicles at any given time, serum AMH shows a consistent decline with increasing female age (44-47). Some small studies have suggested that in young hypergonadotropic women

the combined information of age and AMH could identify a subset of couples with still reasonable pregnancy prospects (48;49).
In this context the question arises whether measuring AMH could identify a subgroup of women with manifest advanced ovarian ageing and as such could be useful in the clinical management of subfertile hypergonadotropic women. The aim of this study therefore is to investigate the role of AMH as predictor of live birth and reproductive stage according to STRAW in subfertile, regularly cycling women with elevated basal FSH levels.

MATERIALS AND METHODS

Study design

The proposed study, focussing on the role of AMH in predicting live birth and reproductive stage according to STRAW in subfertile women with elevated basal FSH levels, was designed as an observational follow-up cohort study.

Participants

Women under treatment at the Reproductive Medicine Unit of the University Medical Centre (UMC) Utrecht with infertility and elevated FSH levels (≥ 12.3 IU/L) at initial ovarian reserve screening were subjected to the so-called COLA (Cycle disorders, OLigo- and Amenorrhoea, WHO III) screening. All consecutively screened women were registered in the COLA database. We selected those women with a regular menstrual cycle, i.e. an average cycle length between 25 and 35 days, who were younger than 40 years of age, and had serum FSH concentrations exceeding 12.3 IU/L in the early follicular phase (cycle day 2-5) who were screened between February 2005 and June 2009. Exclusion criteria were poor ovarian response in a previous IVF cycle (< 5 oocytes at retrieval) or cycle cancellation (<3 developing follicles of at least 12 mm in size), endocrine disease and use of sex steroid medication (**Figure 1**).

In the COLA screening procedure (t=0) data on the medical history, obstetric and gynaecologic history, as well as smoking status and use of medication were recorded. Physical examination was performed, including body height and weight. Transvaginal ultrasound was performed to assess the antral follicle count (AFC) using the 7.5-MHz transvaginal probe on an Aloka SSD-4000 (Hitachi-Aloka Medical, Japan). AFC was calculated by adding up the follicles with a diameter of 2 to 5 mm from both ovaries as these sizes of antral follicles show the strongest correlation with ovarian reserve status (126). For the counting procedure a standard systematic approach was used by the operators (127).

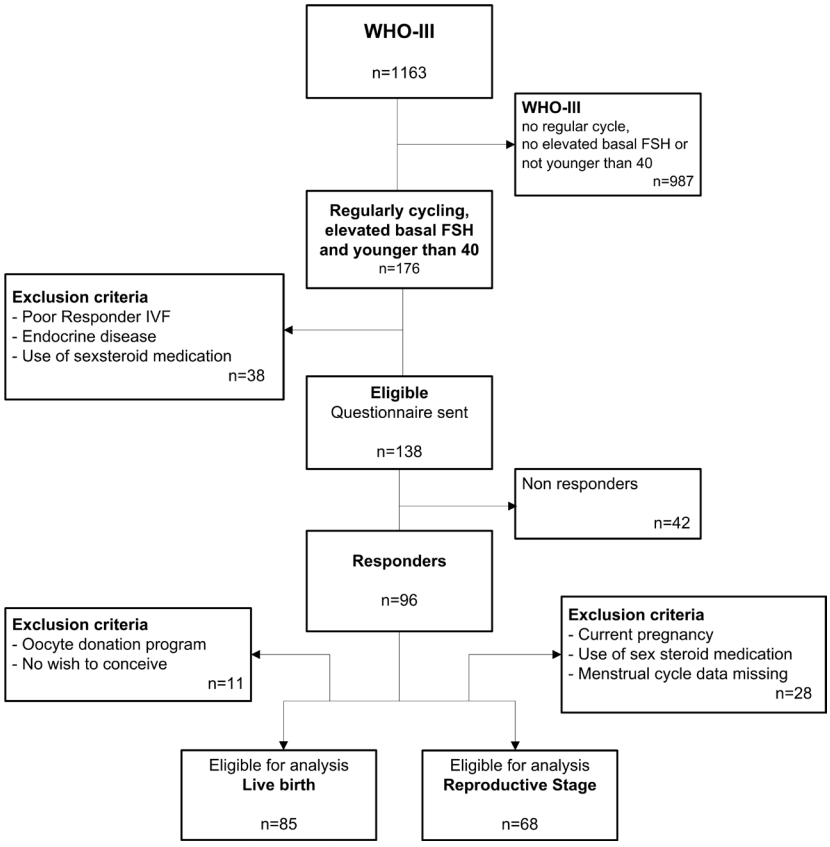


Figure 1. Search and selection of eligible subfertile women with elevated basal FSH levels from the COLA WHO III database of the UMC Utrecht until June 2009.

Fasting serum samples were drawn for endocrine markers. FSH concentrations were measured using a chemoluminescence-based immunometric assay (ADVIA Centaur/ Bayer Corp., Tarrytown, NY) up until December 2006. Inter- and intra-assay coefficients of variation for this assay system were less than 3.9 and 2.9%. From January 2007 onwards, there was an in-house change of immunoassay (Unicel DXI 800 Beckman Coulter; Inc.USA). 95% inter- and intra-assay coefficients of variation in this assay were less than 4.3 and 3.4%. In-house correlation was performed in our laboratory resulting in the following formula, which was consistent across the whole range of assay results: [FSH measured by DXI 800 BC] = "1.16 x [FSH measured by ADVIA Centaur] + 0.46 IU/L". FSH levels measured by ADVIA Centaur were converted to the DXI Assay. Increased baseline FSH was then defined as a level of serum FSH over 12.3 IU/L, which corresponds to 10.2 IU/liter measured

with the older ADVIA Centaur assay. The FSH cut-off of 10.2 IU/L that we used in this study was based on what the ADVIA Centaur assay considered to be the upper 95% reference value of the normal range. These references values were based on measures of FSH in the early follicular phase of healthy women without fertility problems.

Stored serum samples were used for measuring AMH levels using the sandwich ELISA (AMH Gen II ELISA, A79765, Beckman Coulter; Inc., USA) in one complete batch. The detection limit of the assay was 0.20 mcg/L, interassay coefficients of variation were 8.5 and 5.5% at 0.5 and 7.7mcg/L, respectively. None of the women had used any hormonal medication for at least 12 months prior to screening. Approval was obtained from the Institutional Review Board of the University Medical Center Utrecht.

Outcome Live Birth

Outcome parameters were obtained after a duration of follow-up of at least 12 months (1.1-5.2 years), when women filled out a standardized questionnaire concerning reproductive outcome. Women were asked whether they had been pregnant after the COLA visit and if they made use of assisted reproductive technology (ART), including intra-uterine insemination (IUI), in vitro fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI), or conceived spontaneously. Number of pregnancies and pregnancy outcomes were recorded, including foetal loss before 12 weeks or live birth.

Outcome Reproductive Stage

Reproductive stage was assessed at the same time as reproductive outcome and was categorised according to the STRAW classification (27) into: 1) regular menstrual cycles: average cycle length between 25 and 35 days; 2) menopausal transition: transformation to irregular cycles (> 35 days) or not able to predict the next menstrual bleed within 7 days precision or two or more skipped cycles or at least one intermenstrual interval of ≥ 60 days; 3) menopause: 12 consecutive months of amenorrhoea. Women were categorised as 'unknown' if data concerning menstrual cycle was missing, due to current pregnancy or use of sex steroid medication. Reproductive stage refers to the presence or absence of having a regular menstrual cycle at follow-up.

Statistical analysis

Descriptive parameters and patient characteristics were reported as mean ± SD or median [range] depending on the distribution. The Kaplan-Meier method was used to estimate the cumulative probability of live birth, with the period between COLA screening visit and live birth as the time variable. Women who did not achieve a pregnancy or had conceptions that resulted in a foetal loss before 12 weeks were censored at the date of filling out the questionnaire (t=1). The predictive value of

patient characteristics and ovarian reserve tests (ORTs) was analysed using a Cox proportional hazard model of the time to live birth. Results were expressed as a hazard ratio (HR). The log-rank test was used, P<0.05 was considered statistically significant. Subsequently, a multivariable Cox proportional hazard analysis was performed, using a forward stepwise selection method on all prognostic factors and female age. In case of an undetectable AMH level (<0.20 microgram/L), AMH values were arbitrarily assigned the level of 0.10 microgram/L. To detect a possible non-linear relationship between the predictive variables and outcome, a restricted cubic spline was used. For the second outcome, reproductive stage at follow-up, uni- and multivariate Cox proportional hazard models were used to investigate the predictive value of patient characteristics and ORTs.
Statistical analysis was performed using SPSS for Windows, version 20.0 (SPSS INC., Chicago, IL) and R version 2.15.1 (http://www.r-project.org).

RESULTS

A total of 138 eligible subfertile women with elevated basal FSH levels were identified from the COLA WHO III database and were sent the questionnaire (see flow chart in **Figure 1**). Forty-two women did not respond after repeated effort by telephone or email, resulting in 96 women who completed the questionnaire (response rate = 70%). The mean age at COLA screening was 35.0 (± 3.2) years (**Table 1**). In eleven women (11.5%), AMH in serum was undetectable. In case of a detectable AMH level, the median was 0.97 microgram/liter [range 0.20 – 4.50 mcg/L]. Antral follicle count ranged from 0 to 20 with a median count of 5 follicles. Responders and non-responders to the questionnaire did not differ in any of the baseline characteristics (data not shown). The median duration of follow-up was 3.3 years [1.1-5.2], with a mean age at end of follow up of 38.3 ± 3.2 years.

Outcome Live Birth

The statistical analysis of reproductive outcome was performed only in those women who wished to conceive after the COLA visit and did not start an oocyte donation program. This resulted in 85 women eligible for analysis. After the COLA visit for infertility screening, 57 women (67.1%) became pregnant. Thirty-six women (63.2%) became pregnant using ART (20 IVF, 8 IUI, 8 ICSI) and twenty-one women (36.8%) conceived spontaneously. The choice for ART or expectant management was made by the clinician and based on applying a prediction protocol for spontaneous pregnancy (128), the preference of the couple, and additional factors that would affect the couple's fertility such as semen quality and tubal function. Three pregnancies resulted in a foetal loss before 12 weeks; two spontaneous conceptions and one ART pregnancy. In total 63.5% of the women carried a pregnancy to a live birth. The cumulative live birth rate, estimated by the Kaplan Meier method, is

Table 1. Baseline characteristics of subfertile, regularly cycling women with elevated basal FSH levels (n=96)

Characteristic	Median	Range
Age at COLA screening (years)	35.4	24.5-39.6
Menstrual cycle minimum length (days)	26	21-30
Menstrual cycle maximum length (days)	29	21-35
Early follicular FSH (IU/L)	15.3	11-56
Undetectable AMH levels, n (%)	11	11.5%
AMH (mcg/L) if detectable	0.97	0.20 - 4.50
Antral follicle count (2-5mm)	5	0-20
Age at menarche (years)	13	9-17
Parity	0	0-2
Body Mass Index (kg/m^2)	22	17-40
Current smokers, n (%)	17	17.7%
Pack years if smoking	8.5	1-25
Duration infertility (years)	2.5	1-7.8

Results in median and range, except for undetectable AMH levels and smoking prevalence.
COLA= Cycle disorders, OLigo- and Amenorrhea.

shown in **Figure 2**. After a follow-up of 1 year, 22.4% of the women reported a live birth of a baby. The cumulative live birth rate after 2 years of follow up increased to 50.6%. The median time to reach a live birth was 14 months. No pregnancies occurred in case of an undetectable AMH level.

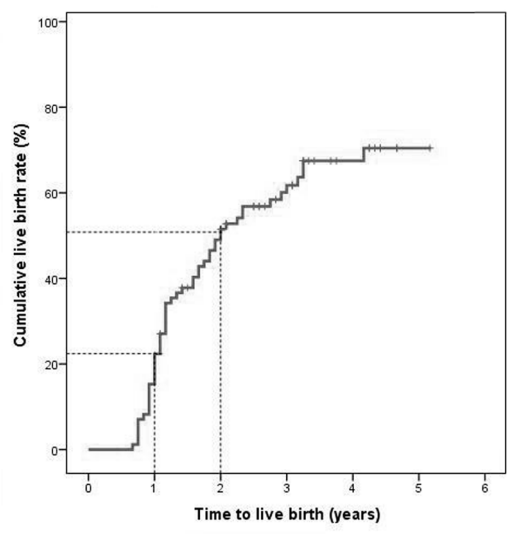


Figure 2. Cumulative live birth rate in subfertile women with elevated basal FSH levels, calculated by the Kaplan-Meier method. Time to live birth includes the duration of the pregnancy. The dotted lines demonstrate the live birth rate after one and two years follow-up respectively.

The results of the Cox proportional hazard models for the relationship between patient characteristics and ORTs on the one hand and live birth on the other are shown in **Table 2**. Univariate analysis showed serum AMH and FSH levels to be significant predictors of live birth, within the total follow up period of 5.2 years. In the multivariable analysis, including age at initial COLA screening, AMH and FSH level, only AMH level remained significant. Per unit (mcg/L) increase of AMH the probability of a live birth increased by 31% (HR 1.31, 95%CI 1.05, 1.63). Moreover, there was evidence from the spline analysis for a non-linear pattern of AMH levels with an increase in chances of live birth up till an AMH serum level of 1 microgram/L (**Figure 3a**). Up to 1 microgram/L an increased AMH was associated with higher live birth rates. Above 1 microgram/L however, further increases in AMH no longer resulted in significantly increased pregnancy rates resulting in a live birth. This non-linear effect was statistically significant (p=0.04). This is illustrated in **Figure 3b** where AMH is divided into two categories; AMH below 1 microgram/L (n=45) and AMH levels of at least 1 microgram/L (n=40). **Figure 3b** demonstrates the cumulative live birth rates for both AMH categories.

Table 2. Cox proportional hazard analysis for predictors of live birth in subfertile, regularly cycling women with elevated basal FSH levels (n=85)

	Pregnancy resulting in live birth			Univariate Hazard ratio
	Yes (n=54)	No (n=31)	*p*-value	(95% CI)
Age at COLA visit (years)	34.3 ± 3.2	35.8 ± 3.4	0.27	0.96 (0.90, 1.03)
Duration of infertility (years)	2.8 ± 1.6	3.0 ± 1.6	0.42	0.93 (0.78, 1.11)
Early follicular FSH (IU/L)	15.5 ± 3.9	19.3 ± 8.4	0.04	0.94 (0.88, 0.996)
Undetectable AMH levels (n)	0 (0)	8 (25.8)	0.07	0.04 (0.001, 1.24)
AMH (mcg/L)	1.50 ± 1.09	0.80 ± 0.87	0.02	1.31 (1.05, 1.63)
Antral follicle count 2-5 mm	6 ± 3	6 ± 4	0.39	1.03 (0.96, 1.10)
Menopausal transition or menopause (n)	1 (2.9)	4 (16.7)	0.17	0.25 (0.03, 1.81)
Pack years smoking (years)	3.3 ± 6.3	3.5 ± 5.1	0.87	1.00 (0.95, 1.05)
BMI (kg/m^2)	22.9 ± 4.0	22.9 ± 4.5	0.99	1.00 (0.94, 1.06)

Hazard ratio's for live birth with 95% Confidence Interval (CI). *p*-values were determined using the log-rank test. COLA= Cycle disorders, OLigo- and Amenorrhea.

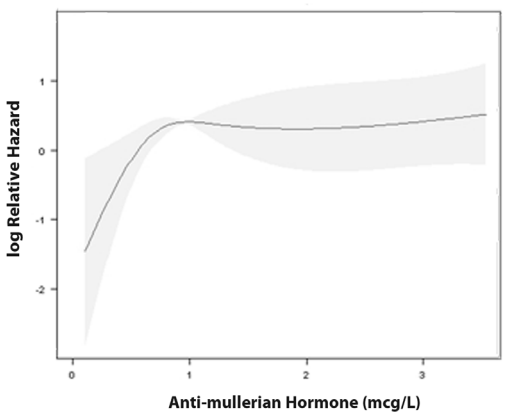


Figure 3a. Non-linear spline analysis between serum AMH level in microgram/L and live birth rate. Up to 1 microgram/L an increase in AMH was associated with higher live birth rates. Above 1 microgram/L, further increases in AMH no longer resulted in significantly increased live birth rates.

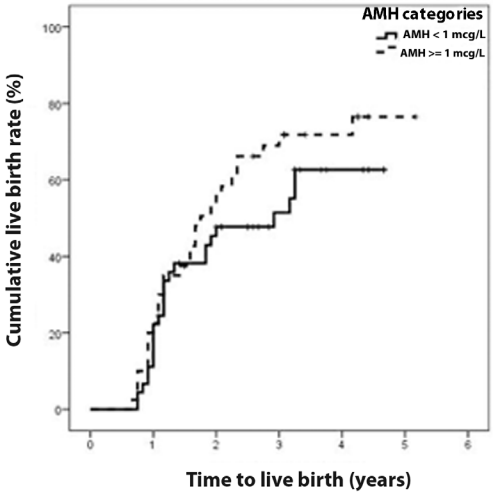


Figure 3b Kaplan-Meier curve demonstrating the cumulative live birth rate (%) in subfertile women with elevated basal FSH levels by category of AMH (AMH < 1 mcg/L (n=45) versus AMH ≥ 1 mcg/L (n=40)).

Outcome Reproductive Stage

At follow-up, 58 of the women (60.4%) still had regular menstrual cycles. Eight women (8.3%) developed irregular menses during follow-up and shifted towards menopausal transition, and two women (2.1%) reached (premature) menopause at the age of 37.5 and 38.4 years, respectively. Due to missing data, current pregnancy or hormone therapy at follow-up, reproductive stage remained unknown in 29.2% of the women. Predictors of reproductive stage are listed in **Table 3**. Sixty-eight women with a defined reproductive stage at follow-up were available for analysis: 58 regularly cycling women, 8 women who entered the menopausal transition and 2 postmenopausal women. Univariate Cox proportional hazard analysis showed a significant influence of FSH and AMH levels on reproductive stage (**Table 3**).

Table 3. Cox proportional hazard analysis for predictors of reproductive stage in subfertile, regularly cycling women with elevated basal FSH levels (n=68)

	Menopausal transition or menopause at follow-up		Univariate Hazard ratio	
	No (n=58)	Yes (n=10)	*p*-value	(95% CI)
Age at COLA visit (years)	35.0 ± 3.6	35.1 ± 3.1	0.62	1.05 (0.88, 1.25)
Early follicular FSH (IU/L)	16.3 ± 4.4	26.6 ± 14.3	0.002	1.08 (1.03, 1.14)
Undetectable AMH levels (n)	5 (8.6)	3 (30.0)	0.02	5.20 (1.26, 21.53)
AMH (mcg/L)	1.19 ± 0.93	0.45 ± 0.51	0.02	0.09 (0.01, 0.72)
Antral follicle count 2-5 mm	6 ± 4	4 ± 3	0.39	0.90 (0.70, 1.15)
Pack years smoking (years)	2.6 ± 4.7	4.1 ± 6.0	0.21	1.07 (0.96, 1.20)
BMI (kg/m²)	22.9 ± 3.8	21.2 ± 2.6	0.56	0.93 (0.72, 1.19)
Age at follow-up (years)	38.2 ± 3.5	38.7 ± 3.3	0.93	0.99 (0.85, 1.17)

Hazard ratio's for the occurrence of menopausal transition or menopause at follow-up (reproductive stage according to STRAW) with 95% Confidence Interval (CI). *p*-values were determined using the log-rank test. COLA= Cycle disorders, OLigo- and Amenorrhea.

In the multivariate analysis, including age at initial COLA screening, FSH and AMH levels, only early follicular FSH level remained a significant predictor of reproductive stage, with a hazard ratio of 1.08 and 95% confidence interval of 1.03-1.14.

DISCUSSION

This study demonstrates that serum AMH level is an independent predictor of pregnancy resulting in live birth in subfertile women with elevated basal FSH levels. Also, the present cohort of women with elevated basal FSH levels did not have a strikingly poor pregnancy prognosis, as 67.1% became pregnant, of which 36.8% were spontaneous conceptions. These results suggest a limited predictive value of elevated basal FSH in young women with regular menstrual cycles during infertility evaluation. To our best knowledge, this is the first study to demonstrate serum AMH level as a predictor of live birth in subfertile women with elevated early follicular FSH levels in a prospective study with the outcome live birth after both ART and spontaneous conceptions.

The reported pregnancy prospects are in line with previous publications concerning hypergonadotropic, regularly cycling, subfertile women (101;129;130). Van Rooij et al. found an ongoing pregnancy rate of 39% in regularly cycling, subfertile women with basal FSH levels between 15 - 20 IU/L (130). A prospective cohort study from the same group demonstrated an ongoing pregnancy rate per embryo transfer of 40% in women with elevated basal FSH levels (101). In another population of

young, regularly cycling, subfertile women with a FSH level > 10 IU/L a live birth rate of 42% was observed (129). These findings are in contrast to the well-known suggestions of basal FSH being a strong predictor of non-success in ART (131). However, in the studies on IVF patients cut off levels for FSH were often much higher and exposure to pregnancy was established in a single treatment cycle. It is therefore not unexpected that various studies have demonstrated much lower accuracy for basal FSH in predicting outcome pregnancy after IVF (132), especially if cumulative cycles were considered (39).

The present results do suggest that both AMH and FSH forecast the advent of menopausal transition and menopause at follow-up. Assessment of both of these ovarian reserve tests in a multivariate model reveals that FSH may be a stronger predictor for timing future cycle status than AMH in women with already elevated basal FSH levels. These results should be interpreted with caution as only a subgroup of 68 women was available for the analysis of reproductive stage at follow-up. Moreover, from the available subgroup only two women reached menopause and 8 women entered the menopausal transition.

In line with the endocrine changes accompanying the menopausal transition, FSH becomes increasingly elevated while AMH levels may have become very low or undetectable quite soon after cycle irregularity has become established (133). With this in mind it can be theorized that in this specific group of women, FSH is a better marker of the short term event menopausal transition, while AMH functions better as a long term predictor of the reproductive event menopause (as AMH levels start to decline before FSH elevations become evident).

Surprisingly, no statistically significant effect for female age was found in predicting live birth in subfertile women with elevated basal FSH levels. A possible explanation for this finding could be the relatively small size of this cohort. Also, the predictive capacity of female age may be flawed by an asymmetrical distribution of age across the cohort. However, the age at baseline ranged between 25 and 39 years, which is not an inadequate distribution. Also, there was no evidence for a non-linear distribution of age as the spline function analysis of age in relation to hazard of having a live birth was not significant (p=0.09). Aside from methodological explanations such as age distribution and sample size, it may be hypothesized that female age indeed is not predictive in this specific phenotype of diminished ovarian reserve. Perhaps the limited reserve is generally reflected by the elevated FSH and the true quantity of the remaining follicles is better represented by serum AMH. Since AMH levels decrease before there is a substantial rise in basal FSH, one would expect AMH levels to be very low once FSH is elevated. However, our findings demonstrate that there is a subgroup of women who have relatively high AMH levels (≥1 mcg/L), despite elevated FSH levels, with better pregnancy prospects than women with low AMH levels. The latter is supported by the observation that no

pregnancies occurred in women with undetectable AMH levels trying to conceive after the COLA visit (n=8). Taken together the expression of quantity is clearly better from AMH levels than basal FSH, a finding which corresponds to ovarian response studies in ART, where FSH has demonstrated to be less well related to outcome categories such as poor or excessive response (134). The observation that no pregnancies occurred in women with undetectable AMH levels is not surprising as our cohort represents women with already unfavourable pregnancy prospects due to an elevated FSH. However, based on the relatively small amount of women with undetectable AMH levels, this observation does not fully exclude the possibility of a pregnancy occurring. The discrepancies with other studies, where pregnancies have been reported in women with undetectable AMH (135;136) may stem from various sources, such as AMH assay failures or variation in storing and handling of the samples (137). Also, truly undetectable AMH levels may spuriously indicate a poor ovarian reserve if the sample has been taken during OC usage (138).

Ovarian reserve is currently defined as an interplay between the quantity and quality of the follicles left in the ovary and several proxy variables for pool size are well described in the literature. However, whether current ovarian reserve tests can predict pregnancy, which is often used as a proxy for oocyte quality, is still a matter of debate (131;139;140). Oocyte quality however, is thought to be predominantly affected by female age. An explanation for our finding that AMH can predict live birth in this cohort could be the fact that higher AMH values are associated with a higher oocyte yield in IVF treatment. This higher oocyte yield consequently results in higher chances of pregnancy. This notion has received support from recent literature (44;141). Since a large proportion of our cohort tried to conceive with ART, fecundity is highly dependent on the number of available follicles and this could be the driver of this phenomenon. The current follow-up study of subfertile women with elevated basal FSH levels was not designed to compare treatment methods, leaving this question unanswered. However, with regard to the time to live birth after the COLA screening, no differences were observed between the women who conceived naturally and those who underwent ART, 1.6 ± 0.99 years versus 1.5 ± 0.69 years respectively. Moreover, in the comparison of women who conceived quickly (live birth within 1 year of follow-up, n=13) and those who took longer to reach a live birth (≥ 2 years, n=13), the proportion of women who conceived naturally was similar to those who underwent ART. Also, no differences were observed in patient characteristics or ovarian reserve tests in women who conceived quickly or those who took longer to conceive.

The association between serum AMH and oocyte yield in IVF treatment could also explain the observation that an AMH level above 1 microgram/L no longer resulted in significantly increased live birth rates. Previous studies have demonstrated that there is an optimal range of oocyte numbers for achieving pregnancy (38;95;103;109).

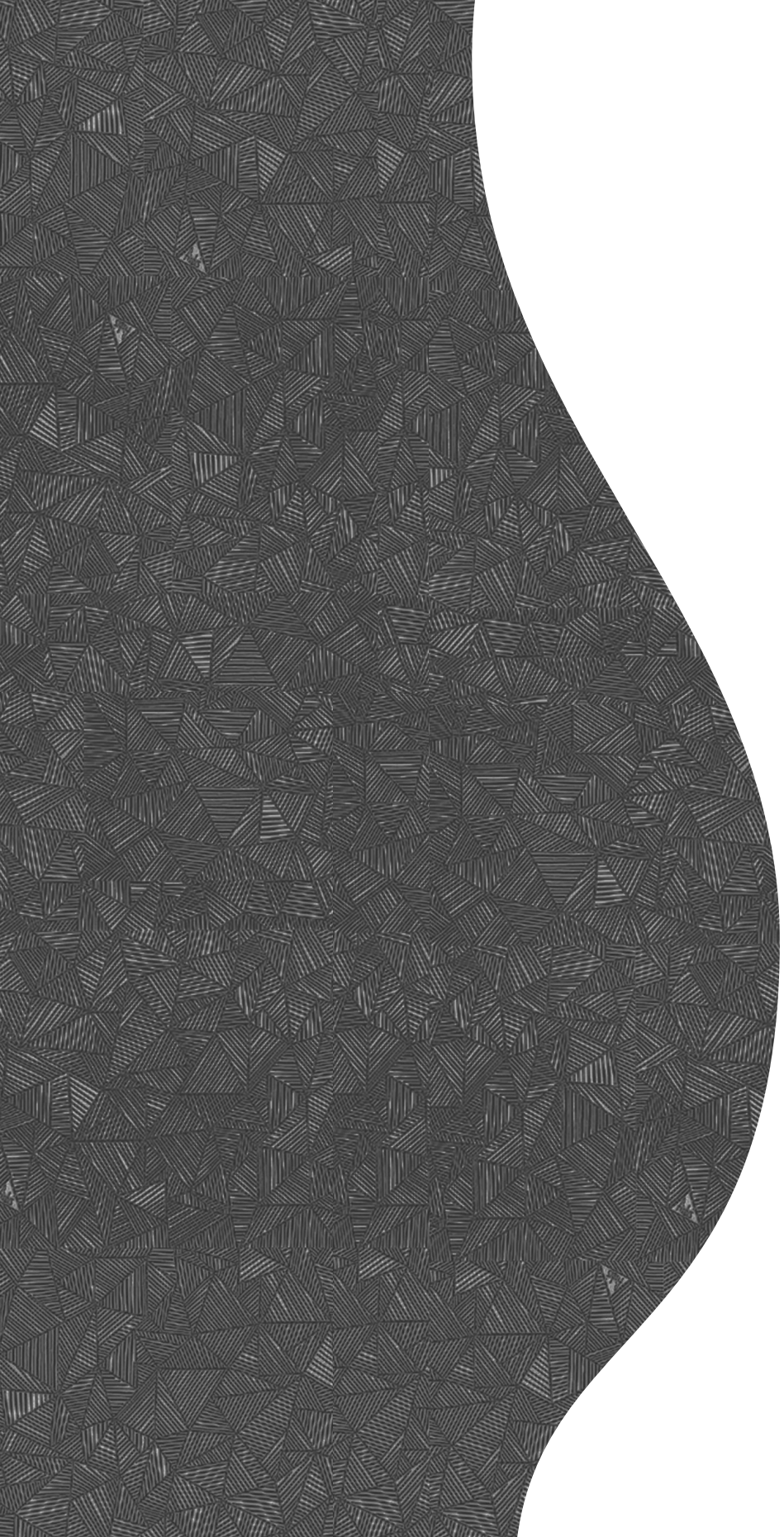
Only below a certain oocyte number, pregnancy prospects are clearly affected. Our demonstrated cut-off of 1 mcg/L might be the lower limit of this optimum and therefore women with an AMH above 1 mcg/L will have chances of pregnancy irrespective of the specific level of AMH. This has also been demonstrated in ART studies where AMH and female age were used to model prognosis categories (142). The strength of this study lies in the fact that this is a well-defined cohort of subfertile women with elevated early follicular FSH levels. We used strict inclusion criteria to ensure all women were regularly cycling and younger than 40 years of age with a basal FSH of 12.3 IU/L or higher. Within our well-defined cohort we observed a wide variation in the ovarian reserve parameters, suggesting that women with elevated FSH levels in fact constitute a heterogeneous group.

To prevent selection bias, women with a poor response in IVF treatment before the COLA screening were excluded, since these women represent a group with already unfavourable pregnancy prospects, expressed by their poor response to ovarian hyperstimulation. In fact, measuring basal FSH and additionally AMH, for prognosis assessment and possible adjustment of the treatment preferably takes place prior to starting ART. By adding poor responders with identification of elevated FSH post hoc this study group would become (too) heterogeneous. However, additional analysis including those women with a poor response to IVF treatment revealed that the effect of AMH on prediction of live birth remained the same. It should also be noted that a single measurement of early follicular FSH was used. Temporary normalization of FSH levels is known to occur (143;144). However, it has been shown that subfertile women with elevated basal FSH levels will always demonstrate some degree of diminished ovarian reserve, even if repeated measurements will yield normal FSH levels(145). A limitation of this study is that some information bias may have occurred. Women who failed to achieve a pregnancy or with unfavourable pregnancy outcomes might have been less inclined to respond to the questionnaires leading to an underrepresentation of unfavourable pregnancy outcomes and over optimistic pregnancy rates. However, even when a more pessimistic scenario is applied by assuming that all women who did not respond to our questionnaire did not become pregnant, a pregnancy rate of 41.3% instead of the observed pregnancy rate of 67.1% would still be calculated. With regard to other factors that may influence pregnancy rates, the responders and non-responders did not differ in any of the baseline characteristics. Finally, AMH measurements were carried out on samples that had been stored at -20 degrees Celsius. Currently, the debate on the effect of freezing and thawing on AMH measurements has not been fully completed(146). The fact that this procedure has been applied in all cases in the cohort excludes the possibility of creating a large source of bias.

The cut-off value for FSH of 12.3 IU/L should also be considered. Different studies, using different outcomes, have used different FSH cut-off levels (132). Our cut-off of 12.3 IU/L is based on the conversion of the upper limit (10.2 IU/L) of the normal range of the assay used in that time. As noted by the STRAW in 2001, most clinicians use a FSH of 10 IU/L as cut-off value (147).

The value of the present findings for clinical practice may be that subfertile women with elevated basal FSH levels may still have reasonable pregnancy prospects. Denial of treatment in these women therefore does not seem to be justified in the majority of cases. Further assessment using AMH or the response in a trial cycle of IVF may be the way to sort out those cases with still reasonable prognosis, and those who may better be referred to egg donation programs. Both early follicular FSH levels and serum AMH could be used as a guide to advise subfertile couples on their pregnancy prospects. However, serum AMH levels provide a more robust cut-off, since our study demonstrated that women with undetectable serum AMH levels were a subgroup with pregnancy prospects close to zero. The conclusion regarding the prediction of reproductive stage at follow-up are based on small numbers and should therefore not be extrapolated to clinical practice.

In summary, this is the first study to suggest Anti-Müllerian hormone as a single predictor for live birth in subfertile women with elevated basal FSH levels. These findings suggest that AMH may be applied to identify those women with very poor pregnancy prospects. Also, this study indicates that both AMH and FSH are predictors of the timing of reproductive stages to develop in this specific group of women with elevated FSH and subfertility.



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Serum AMH levels in women with a history of preeclampsia suggest a role for vascular factors in ovarian ageing

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ABSTRACT

Context
The association between early menopause and vascular disease as a possible causative factor has recently received attention. Preeclampsia (PE) is associated with future cardiovascular risk factors and this premature vascular ageing potentially modifies the ovarian ageing process.

Objective
The purpose of this study was to assess whether women with a history of PE have lower Anti-Müllerian hormone (AMH) levels than women with normotensive pregnancies.

Design
This was a retrospective cohort study.

Setting
The study was conducted in a tertiary referral center.

Patients
Clinical data and blood samples of participants of the Preeclampsia Risk EValuation in FEMales study were used (336 women with a history of PE and 329 women after a normotensive pregnancy).

Intervention(s)
There were no interventions.

Main outcome measures
The relative decrease in AMH level was assessed after a median follow up of 10.5 years.

Results
The mean AMH level was 2.00 ± 1.87 mcg/L in the PE group compared to 2.26 ± 2.56 mcg/L in the reference group. Linear regression analysis with censoring for undetectable AMH levels, adjusted for age, smoking and hormonal contraceptive use, showed a relative reduction in AMH level by 20.9% at any age (fold change 0.79, 95% CI 0.67, 0.94).

Conclusions
We demonstrate that women with a history of PE have significantly lower AMH levels compared to women with normotensive pregnancies. Calculations based on a reference population indicate advancement of reproductive age of approximately 1.5 years. As PE is considered as a manifestation of impaired vascular health, these results support the hypothesis that compromised vascular health could act as a causative mechanism in early ovarian ageing.

INTRODUCTION

Age-related female infertility is caused by a process referred to as ovarian ageing. It comprises the gradual decline in both quality and quantity of the ovarian follicle pool (4;5;11), resulting in the final cessation of menses at an average age of 51 years, defined as menopause. Hence, large differences in ovarian reserve are likely to exist in women of the same age.

Identification of the mechanisms dictating the ovarian ageing process may increase our understanding of the variation between individuals. The deposition of follicles during ovariogenesis, as well as the continuous process of follicle atresia thereafter (148), may both be subjected to variation caused by genetic, auto-immune, vascular and toxic factors (69;149).

The association between early menopause and vascular disease as a possible causative factor has recently received attention. It has been demonstrated that infertile women with reduced ovarian reserve, as expressed by a poor response to ovarian hyperstimulation for in vitro fertilization (IVF), appear to have an increased rate of vascular complications in a subsequent pregnancy (150-152). For women with premature menopause who become pregnant after oocyte donation, a similar pattern of vascular compromise in pregnancy has become obvious (153). Also, associations have been found between a variant of the APO-E gene, which is associated with longevity and atherosclerosis, and age at natural menopause (78;79). Finally, recent studies in women with a natural menopause have identified a number of loci associated with age at menopause in domains of genes related to pathways for DNA repair and cell death (67).

Preeclampsia (PE) is a major cause of maternal and foetal morbidity and mortality (154) and is clinically defined as the combination of hypertension and proteinuria after the 20th week of pregnancy in formerly normotensive women (155). Early-onset PE is commonly defined as preeclampsia requiring (iatrogenic) delivery before 34 completed weeks of gestation. Increasing evidence suggests that women with a history of PE are at increased risk for future cardiovascular disease (156-160). Preeclampsia and cardiovascular disease share common risk factors such as chronic hypertension, obesity and insulin resistance. The key pathophysiologic feature of PE is widespread endothelial dysfunction, resulting from generalized intravascular inflammation. These changes subside after pregnancy, but may re-emerge, mostly irreversibly, relatively early in life as atherosclerosis (161-163).

Pregnancy can therefore be considered a vascular stress test that may unmask a woman's tendency to develop cardiovascular disease later in life (159;164). We hypothesized that this premature vascular ageing also affects the ovarian ageing process.

Serum Anti-Müllerian hormone (AMH) as a measure of ovarian reserve is gaining importance in reproductive medicine. The cycle independent fashion (47;123) and strong correlation with the primordial and antral follicle pool make AMH a reliable and useful marker to reflect a woman's ovarian reserve status (44;46;165).
Studies regarding possible advancement in the ovarian ageing process after preeclampsia are lacking. To our best knowledge, this is the first study which aimed to compare ovarian ageing in women with a history of preeclampsia with women with normotensive pregnancies, using AMH as the marker for ovarian reserve status. Secondly, we aimed to study possible associations between vascular factors and ovarian reserve status.

METHODS

Study design

The current study, focusing on women with a history of PE in a follow up assessment on ovarian reserve status, was designed as a retrospective cohort study. The association between vascular factors and ovarian reserve status is assessed in a cross sectional design.

Study population

Clinical data and blood samples of participants included in the Preeclampsia Risk EValuation in FEMales (PREVFEM) study at the Isala klinieken in Zwolle, the Netherlands, were used. As described in our previous publication (157), a total number of 339 women with a history of PE and a reference group of 332 women after a normotensive pregnancy, registered in the obstetric database at the Isala Klinieken in Zwolle between 1991 and 2007, were included on average ten years after the index pregnancy.
The reference group consisted of normotensive women who gave birth in the hospital. They were selected from the obstetric database after selection based on age at delivery and date of delivery, and aiming for an equal distribution of these two variables (range ± 2yrs). For every two women with preeclampsia, three women with normotensive pregnancies were invited to participate in the study, expecting a lower response in the reference group. If a larger number of reference women was available, the women with the best match for age and delivery date were chosen. Exclusion criteria for the reference group were: complicated pregnancies defined as preterm birth, hypertensive pregnancy complications and/or placental problems. All participants gave written informed consent and approval for the study was obtained from the institutional review board of the Isala Klinieken in Zwolle.
Of six women stored serum for AMH measurement was lacking; consequently, 336 women with PE and 329 women with normotensive pregnancies were available for analysis. Preeclampsia was defined according to the International Society for

the Study of Hypertension in Pregnancy (ISSHP), i.e. diastolic blood pressure ≥ 90 mmHg with proteinuria (≥ 0,3 gram/ 24 h) diagnosed between 20 and 32 weeks of gestation in formerly normotensive women (166), almost always followed by premature delivery. All participants were invited for a cardiovascular screening program between April 2009 and May 2010. The screening was scheduled after a median period of 10 [range 1-27] years of follow up. In the screening schedule the women in the PE group were assessed first; screening of the reference group started at least six months after the first woman in the PE group was screened. Screening was omitted in case the participant appeared to be pregnant or breastfeeding (n=10).

Measurements at follow up

The cardiovascular screening included a questionnaire on medication use, lifestyle behaviour, obstetric history, family history and history of cardiovascular disease (CVD) or presence of cardiovascular risk factors. In addition, at a scheduled visit in the outpatient clinic of the department of Cardiology, a physical examination was performed by trained research nurses, consisting of measurements of length, weight, waist and hip circumferences and blood pressure. Lastly, fasting blood samples were collected for measurement of lipid profiles (Roche Modular P800), high-sensitivity C-reactive protein (CRP) (Roche Modular P800), glucose (Roche Modular P800) and HbA1c (Primus Ultra 2). All data were collected in an electronic case report form, provided by Diagram BV. Blood samples were stored at -80 degrees Celsius at the Durrer Center for Cardiogenetic Research in Amsterdam. To assess the ovarian reserve status, a serum sample from every participant was defrosted. Ovarian reserve status was assessed by measuring serum Anti-Müllerian hormone, using the Gen II enzyme-linked immunosorbent (ELISA) assay from DSL (Beckman-Coulter, Brea, CA, USA). The lower limit of quantitation of 0.16 µg/L was used as the detection limit, which is the functional sensitivity taking into account a 20% variation coefficient. The absolute detection limit of the AMH assay of 0.08 µg/L is accompanied with a more than 20% variation coefficient in this low range, making measurements at this level highly unreliable. As the study had a comparative design this is not likely to influence the findings and conclusions. Intra-assay coefficients of variation range from 3.5-5.5% (16 to 3 µg/L) and inter-assay coefficients of variation were 9.3% (at 0.5 µg/L) and 7.3% (at 7.6 µg/L).

Cardiovascular risk profile

Hypertension at follow up was defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90mmHg based on the mean value of three blood pressure measurements at the cardiovascular screening, or the use of antihypertensive medication. Total cholesterol ≥ 5.0 mmol/l or current use of statins were criteria for hypercholesterolemia. Both hypertension and hypercholesterolemia were analysed as dichotomous variables (presence or absence of the condition) and not as

continuous variables, as medication use will influence the objective measures such as blood pressure or serum cholesterol levels. Number of pack years smoking, body-mass index (BMI), waist circumference and CRP were considered to be vascular factors.

Statistical analysis

Descriptive parameters and population characteristics were reported as means ± SD and categorical data were expressed as percentages. Subsequently, the baseline characteristics of the index pregnancy as well as ovarian reserve status and vascular factors at follow up were compared between the PE group and reference group by applying Student's t-test or chi-square for respectively continuous or categorical data. The comparison of serum AMH levels between the PE group and the reference group with normotensive pregnancies was carried out by applying nonparametric testing. Variables identified as confounders were: age at screening, pack years smoking and hormonal contraceptive use. For the purpose of comparability, linear regression analysis with censoring using the Buckley James method on log transformed AMH was used to adjust for these confounders (167). In the case of an undetectable AMH level (<0.16 microgram/L), AMH values were censored.

Both the relation between PE and serum AMH levels and the association between serum AMH levels and vascular factors, i.e. hypertension (yes/no), BMI, CRP level, hypercholesterolemia (yes/no), waist circumference, positive family history for CVD (yes/no) were examined with the Buckley James linear regression model, adjusting for all the mentioned confounders. CRP levels exceeding 20mg/L were excluded from further analysis, as these values may represent a transient acute infection. To correctly model a possibly non-linear relationship between continuous variables (age at screening, pack years smoking, BMI, CRP level, waist circumference) and AMH level, a restricted cubic spline with three knots was used. Fold changes with 95% confidence intervals (95% CI) were calculated for variables with a linear relation. The fold changes describe the relative change in serum AMH level in the PE group compared to the reference group and the association between AMH level and vascular factors. If variables had a non-linear relationship with AMH, p-values were reported and the relationship was depicted graphically.

We then performed a series of sensitivity analyses to investigate whether the association between PE and AMH was stronger when a more severe phenotype of early-onset PE was selected according to gestational age: women with a delivery before 34 weeks and women with a delivery before 32 weeks of gestation. These analyses were restricted to the two subgroups of women with early-onset PE and all women whose index pregnancy was their first pregnancy. Additionally, the association between PE and serum AMH values was analysed after excluding women with an ovariectomy (n=9).

RESULTS

Baseline – index pregnancy

Baseline characteristics of the study population are summarised in **Table 1**. Mean age at index pregnancy differed significantly between the two groups (PE group 29.7 ± 3.8 years; reference group 28.6 ± 4.1 years). The duration of pregnancy was significantly shorter in the PE group compared to the reference group and resulted in offspring with lower birth weights (p<0.001). More women with PE were primigravida compared to the women with normotensive pregnancies (79.8% versus 69.9% respectively) at the time of the index pregnancy.

Table 1. Baseline - Index pregnancy

	PE	Reference group	
	n=336	n=329	*p*-value
Age at delivery in years (SD)	29.7 (3.8)	28.6 (4.1)	<0.001
Gestational age at delivery in weeks (SD)	31.0 (3.9)	39.6 (2.1)	<0.001
Birthweight in grams (SD)	1437 (793)	3408 (651)	<0.001
Primigravida (%)	268 (79.8)	230 (69.9)	0.003

Data are presented in mean (SD) or number (%). *p*-values are computed with *t*-tests or chi-square. PE = preeclampsia.

Follow up – ovarian reserve status and vascular factors

Reproductive characteristics of the two groups at follow up are displayed in **Table 2**. Mean duration of follow up was 9.1 ± 3.6 years for the PE group compared to 10.6 ± 3.0 years in the reference group. Women with a history of PE had a significantly lower number of pregnancies and a higher incidence of stillbirths compared to the women without PE. Moreover, a higher prevalence of hormonal contraceptive use was observed in the reference group. With regard to the ovarian reserve, non-detectable AMH levels were measured in 18.3% of women in the PE group and in 14.9% of women in the reference group (p=0.22). Mean serum AMH level was 2.00 ± 1.87 mcg/L in women with a history of PE compared to 2.26 ± 2.56 mcg/L in women with normotensive pregnancies. After adjustment for age at screening, pack years smoking and hormonal contraceptive use, women with a history of PE had significantly lower AMH levels (**Table 3**). A relative decrease of serum AMH by 20.9% was found for women with PE compared to women with normotensive pregnancies (**Figure 1**).

Table 2. Follow up - Ovarian reserve status and vascular factors

	PE	Reference group	
	n=336	n=329	*p*-value
Age at screening in years (SD)	38.8 (4.9)	39.3 (4.3)	0.26
Years post index-partus (SD)	9.1 (3.6)	10.6 (3.0)	<0.001
Ovarian reserve			
Non detectable AMH levels (%)	62 (18.3)	49 (14.9)	0.22
AMH levels if detectable in mcg/L (SD)	2.00 (1.87)	2.26 (2.56)	0.67**
Obstetric history			
Number of pregnancies (SD)	2.7 (1.4)	3.1 (1.4)	<0.001
Parity (SD)	2.1 (0.9)	2.6 (1.0)	<0.001
Pregnancy after ART (%)	28 (8.3)	17 (5.2)	0.10
Foetal loss before 24 weeks (%)	102 (30.4)	110 (33.4)	0.40
Stillbirth after 24 weeks (%)	49 (14.8)	13 (4.0)	<0.001
Gynaecologic history			
Age at menarche in years (SD)	13.0 (1.5)	13.0 (1.5)	0.82
Hysterectomy (%)	5 (1.5)	6 (1.8)	0.75
Ovariectomy (%)	5 (1.5)	4 (1.2)	0.76
Current use of hormonal contraceptives (%)	104 (31.0)	131 (39.8)	0.02
Cardiovascular risk factors or disease			
Body-mass index in kg/m^2 (SD)	26.9 (5.7)	26.2 (4.9)	0.13
Waist circumference in cm (SD)	86 (13)	83 (11)	0.002
High-sensitivity C-reactive protein in mg/L (SD)	3.02 (3.4)	3.0 (3.0)	0.56**
Current smoking (%)	54 (16.1)	59 (17.9)	0.52
Pack years smoking (SD)	4.2 (6.6)	5.3 (8.3)	0.04
Hypertension (%)	144 (42.9)	54 (16.4)	<0.001
Hypercholesterolemia (%)	135 (39.9)	139 (42.2)	0.55
Family history of pregnancy complications			
Gestational diabetes mellitus (%)	22 (7.1)	23 (7.6)	0.82
Pregnancy induced hypertension (%)	131 (45.6)	82 (29.3)	<0.001
Family history of cardiovascular risk and/or disease (%)	254 (77.0)	209 (64.7)	0.001

Data are presented in mean (SD) or number (%). p-values are computed with t-tests or chi-square. ** *p*-value computed with Mann-Whitney U test. AMH = Anti-Müllerian hormone, PE = preeclampsia, ART = assisted reproductive technology.

Table 3. Association between preeclampsia and serum AMH (n=665)

	Relative decrease in AMH in mcg/L	95% CI	*p*-value
Preeclampsia vs. Reference group	0.91	0.78, 1.08	0.26
Adjusted for age at screening	0.84	0.71, 0.99	0.036
Adjusted for age at screening and pack years smoking	0.81	0.68, 0.96	0.015
Adjusted for age at screening, pack years smoking and hormone use	0.79	0.67, 0.94	0.008

Regression coefficients with 95% confidence intervals (CI). AMH = Anti-Müllerian hormone.

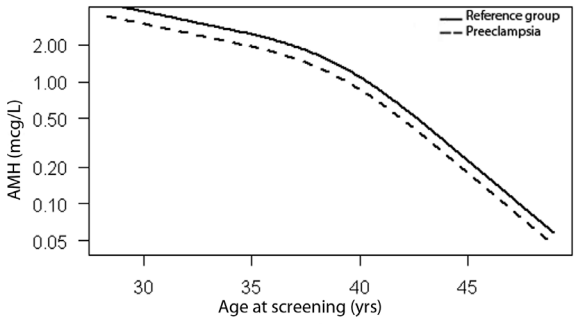


Figure 1. Age related AMH decline in women with PE compared with that of a reference group computed with linear regression using log transformed AMH. Serum AMH level is plotted on a logarithmic scale. In case of an undetectable AMH level (<0.16 microgram/L), AMH values are presented as 0.08 mcg/L.

At follow up women with PE had significantly more risk factors for CVD (**Table 2**), in particular hypertension and a positive family history. Moreover, a larger waist circumference was observed in the PE group as compared to the reference group. The number of pack years smoking was higher in the reference group compared to the PE group. Analysis with spline functions demonstrated a non-linear association between age at screening, pack years smoking, CRP level and serum AMH level, p-values for non-linearity were respectively p<0.0001, p=0.044 and p=0.015) (**Figure 2**).

Moreover, as demonstrated by **Table 4**, the presence of hypertension at follow up, adjusted for confounders, resulted in significantly lower AMH levels, with a relative decrease in AMH by 22.2%. No associations were found between hypercholesterolemia (yes/no), BMI, waist circumference, family history (yes/no) and serum AMH level, adjusted for age and smoking status.

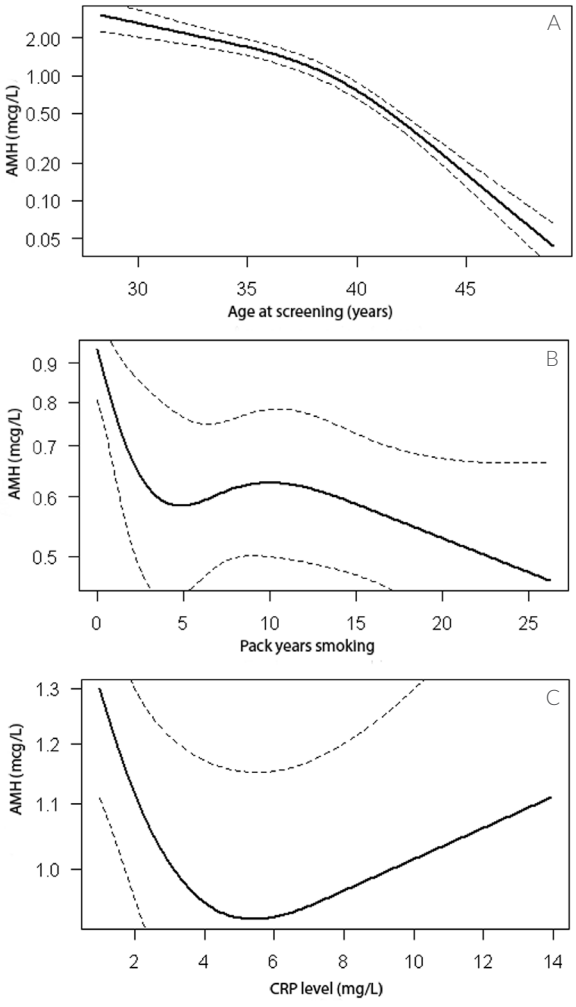


Figure 2. Non-linear spline analyses between age at screening, pack years smoking, CRP and serum AMH level. A) Serum AMH levels decline with increase age. B) An increase in pack years smoking was associated with a decline in AMH level until five pack years smoking. A further increase in pack years smoking no longer resulted in a significant decline in AMH. C) An increase in CRP levels until approximately 5 mg/L was associated with a decline in AMH levels. CRP levels exceeding 5 mg/L no longer resulted in significant lower AMH levels.

Table 4. Association between vascular factors and serum AMH (n=665)

Determinants	Relative decrease in AMH in mcg/L	95% CI	*p*-value
Hypertension (yes vs. no)	0.78	0.64, 0.94	0.009
Hypercholesterolemia (yes vs. no)	1.06	0.89, 1.26	0.51
Body-mass index (kg/m^2)	0.99	0.97, 1.01	0.20
Waist circumference (cm)	0.99	0.99, 1.00	0.09
Positive family history (yes vs. no)	0.97	0.91, 1.05	0.46

Regression coefficients with 95% confidence intervals (CI) adjusted for age, pack years smoking and hormone use. AMH = Anti-Müllerian Hormone.

Sensitivity analysis

When we restricted the analysis to all reference women whose index pregnancy was the first pregnancy (n=230) and to the women with early-onset PE (delivery before 34 (n=200) or before 32 weeks of gestation (n=159)) and who were also primigravida, a more prominent effect of PE on ovarian reserve status was observed in the subgroup with less than 32 weeks of gestation, with a relative decrease of serum AMH of 24.4% (fold change 0.76, 95%CI 0.61, 0.94). In women with early-onset PE with a delivery before 34 weeks of gestation, the relative decrease of serum AMH was consistent at a level of 20.4% (fold change 0.80, 95% CI 0.65, 0.97). After excluding the women with a reported ovariectomy, a relative decrease of serum AMH by 20.0% was found for women with PE compared to women with normotensive pregnancies (fold change 0.80, 95%CI 0.67, 0.95) after adjustment for confounders.

DISCUSSION

In this study we have demonstrated that women with a history of preeclampsia have a significantly lower ovarian reserve status, as indicated by AMH levels, after adjustment for age, smoking and hormonal contraceptive use, compared to women with normotensive pregnancies. Also, an association between the presence of hypertension at follow up and CRP levels on the one hand and serum AMH-based ovarian reserve status on the other hand was found. To the best of our knowledge, this is the first study to demonstrate that women with a history of PE have signs of advanced ovarian ageing. These results may support the hypothesis that vascular compromise acts as a driving factor in the ovarian ageing process, since PE and in particular early-onset PE, is to be considered as a manifestation of impaired vascular health.

The literature on this topic is limited. Woldringh et al. demonstrated in a case-control study that decreased ovarian reserve, reflected by a lower number of oocytes obtained during IVF treatment, was associated with an increased risk to develop PE in a subsequent pregnancy, where PE is considered a pregnancy complication indicating or even resulting from impaired vascular health (152).

With regard to the role of vascular health factors in the ovarian ageing process we are only aware of two other studies that have examined the association between AMH and CRP (168;169). Neither of these studies was able to detect an association. However, these studies examined quite different populations, namely adolescent females with an average age of 15 years (169) and a mixed population of Asian women with and without the polycystic ovary syndrome (168). A recent cross-sectional study of Bleil et al evaluated the association between reproductive ageing and cardiovascular disease risk and demonstrated that the number of cardiovascular risk factors was 52.1% higher among women with low versus high AMH levels (170).

Moreover, low AMH levels were associated with low HDL-levels, greater waist circumference and the presence of hypertension. It should be noted that these associations were attenuated when BMI was added to the model. In contrast to our study, AMH was analysed in categories and not as a continuous variable. Also, in our study BMI was not associated to the AMH level. Still, as BMI is highly associated with early vascular disease, these cross-correlations come as no surprise, although they may not be consistent as demonstrated in the present study.

As both hypertension and elevated CRP levels are expressions of impaired vascular quality and predictors of future CVD (171;172), the association with AMH suggests a role for vascular factors in the ovarian ageing process. Due to the cross-sectional design however, we are not able to establish causality or the direction of any association. For the increased prevalence of hypertension in women with a history of PE, it remains unclear whether impaired vascular health is the cause of PE rather than the result. We propose that a maternal vascular compromised status precedes a vascular complicated pregnancy and an advancement of the ovarian ageing process. The hypothesis that vascular compromise may act as a driving factor in the ovarian ageing process has been previously supported by Kok et al, who found an association between premenopausal cardiovascular risk factors and age at menopause (74). The increased risk of CVD in women with an early menopause could therefore be a reflection of an unfavourable atherosclerotic profile during a woman's premenopausal years. Also, a recent animal study demonstrated an inverse relationship between serum AMH and atherosclerotic conditions in female monkeys (75). Finally, a recent finding of 13 loci newly associated with age at menopause located in genetic domains associated with DNA repair, cell death and immune response (67) indicates that the variation in the ovarian ageing process may not be dictated by genes that affect ovarian paracrine functioning in early foetal or later adult life.

We may speculate about the mechanisms behind this vascular reproductive concept. Early atherosclerosis may elicit or reflect an accelerated biologic ageing process, where both an early menopause and increased risk for early CVD are expressions of this scenario.

Explanations could also lie in ovarian damage inflicted by impaired ovarian vascular flow. Since ovaries are highly vascularized organs, it is imaginable that an impaired ovarian blood flow induces ischemic damage leading to increased follicle demise at the primordial stages resulting in accelerated depletion of this pool.

Prospective studies where both cardiovascular status and ovarian reserve status are determined from early adulthood onwards may be justified to help elucidate the precise mechanism. If our hypothesis is correct and vascular compromise acts as a driving factor in the ovarian ageing process this may indicate that prevention or treatment of vascular compromise could lead to a longer reproductive lifespan. In

this way, cardiovascular health status could be used as a predictor for reproductive health.

A strength of our study lies in the well-phenotyped cohort of women with PE, who were all recruited in the same hospital, just as the women with normotensive pregnancies. Also, the use of standardised questionnaires and protocols provided a high precision of the estimated effects. Furthermore, all serum samples for AMH determination were analysed in a single batch, thus preventing the occurrence (and interference) of inter-batch variability.

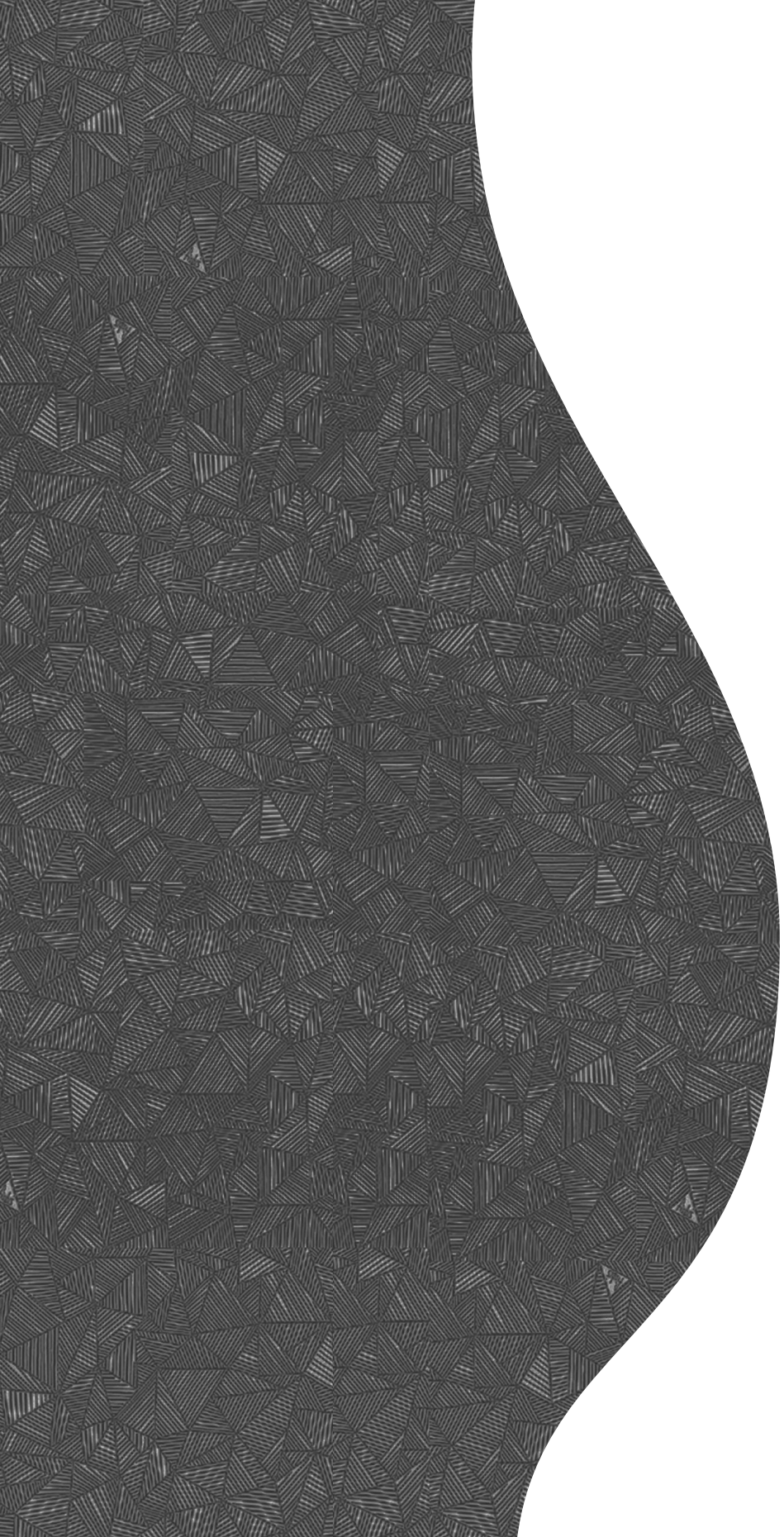
A limitation of this study is that no clinical data on blood pressure before the index pregnancy were available. A small portion of women reported hypertension before the index pregnancy.

Also, it should be noted that our reference group was selected from medium-risk women who delivered in the hospital, without any hypertensive pregnancy complications. However, we are not concerned that this has increased the difference in AMH level between the PE group and the reference group. With a low-risk reference population a larger rather than smaller difference in AMH level might be expected.

The direct clinical implications of a low age-adjusted AMH are largely unknown, but the reflection of an advanced reproductive ageing process is likely to result in an earlier menopause. Hence, data of premenopausal, regularly cycling women participating in the Doetinchem Cohort Study were used to interpret the relative decrease in serum AMH level (138). A detailed description of this ongoing multi-purpose prospective study has been published previously (173). Calculations based on this reference population indicate that a relative decrease in AMH level of 20.9% corresponds to an advancement in reproductive age of 1.5 years.

As this effect size remains well within the normal variation of reproductive ageing, no direct influence on reproductive performance of women with PE is expected. Still, in view of the relation between PE and future AMH levels and the possible advancement of the ovarian ageing process, effects on subsequent fertility may not emerge before a next pregnancy is attempted.

In conclusion, women with a history of preeclampsia not only show signs of impaired vascular health, but also advanced ovarian ageing a decade after pregnancy. The latter finding suggests a vascular mechanism for early menopause and may help to unravel the mechanism behind the individual variation of ovarian ageing.



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Association between vascular health and ovarian ageing in type 1 diabetes mellitus – the OVADIA study

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ABSTRACT

Context
The mechanism behind advanced ovarian ageing has not yet been elucidated. We hypothesize that vascular impairment precedes ovarian ageing. As type 1 diabetes mellitus (DM-1) is hallmarked by premature vascular complications this may consequently play a role in the rate of primordial follicle decline.

Objective
To study whether vascular health is associated with ovarian reserve status using DM-1 as a model for vascular compromise.

Design
Cross-sectional study.

Setting
University hospital.

Patients
150 premenopausal, regular cycling women with DM-1 included in the Ovarian Ageing in Type 1 Diabetes Mellitus (OVADIA) study (Clinicaltrials.gov identifier: NCT01665716).

Intervention(s)
In a single study visit an inventory of both ovarian reserve and vascular status was carried out. A transvaginal ultrasound to calculate the antral follicle count (AFC) and blood sampling for Anti-Müllerian hormone (AMH), lipids, C-reactive protein and HbA1c was performed. Furthermore, a vascular screening including measurements of blood pressure, flow-mediated dilation (FMD), peripheral arterial tonometry (Endo-PAT), pulse wave velocity (PWV), pulse wave analysis (PWA) and intima-media thickness (IMT) was carried out.

Main outcome measures
The association between vascular risk factors, vascular function tests and ovarian reserve, indexed by serum AMH and AFC.

Results
Systolic blood pressure was negatively correlated with both serum AMH (p=0.006) and AFC (p=0.004). A non-linear relationship between HDL-cholesterol and serum AMH was found (p=0.0001). No association was detected between other vascular risk factors or vascular function tests and serum AMH or AFC.

Conclusions
No conclusive evidence for the association between vascular health and ovarian ageing was found in women with DM-1. This may be the result of an insufficient vascular compromise in the relatively young, DM-1 group.

INTRODUCTION

Female reproductive ageing is the result of a gradual decrease in both quantity and quality of oocytes, referred to as ovarian reserve (11;174). Anti-Müllerian hormone (AMH) has emerged as an important reproductive biomarker; the strong correlation of serum AMH with the antral follicle count (AFC) as well as its role in cyclic recruitment of ovarian follicles has resulted in an increased use of AMH in clinical practice for both prognostic and diagnostic purposes (44;46;47;134;165;175).
Genetic factors play an important role in the process of and variation in ovarian ageing. Although the effect sizes may be small, several genes have been found to be associated with age at natural menopause (67;69). Other factors, such as environmental and life style factors, also contribute to the timing of depletion of the primordial follicle pool (70;71;176). Despite current knowledge on the contribution of these factors, the exact mechanisms behind the variation in the timing of menopause and ovarian ageing still remain to be elucidated.
The role of vascular factors in the ovarian ageing process has also recently received attention (74). This is supported by the negative correlation between atherosclerotic plaque sizes and ovarian reserve status observed in female monkeys (75). Moreover, in a multi-ethnic population of premenopausal women an association between a reduced ovarian reserve status and unfavourable cardio-metabolic profile was found (170). Finally, women with a history of preeclampsia, an expression of impaired vascular health, showed signs of advanced ovarian ageing a decade after pregnancy, when compared to women with normotensive pregnancies (177).
Type 1 diabetes mellitus (DM-1) is a disease hallmarked by premature vascular complications (178-181). It has been suggested that women with DM-1 experience an earlier or more rapid decline of the ovarian follicle pool, resulting in menopause at a younger age compared to women without diabetes (182). Moreover, microvascular complications (retinopathy and nephropathy) are suggested risk factors for an early age at menopause in women with DM-1 (183). Hence, vascular conditions may play a significant role in the process of ovarian ageing in women with DM-1.
The aim of the present study was to assess the possible associations between patient characteristics, vascular risk factors, vascular function tests and ovarian reserve status, indexed by AMH levels and AFC, in women with DM-1. In addition, we aimed to confirm DM-1 as a determinant of advanced ovarian ageing, by comparing serum AMH levels in women with DM-1 to those from a healthy, normal fertile, reference population.

METHODS

Study design

We performed a cross-sectional study to investigate the association between vascular risk factors, vascular function tests and ovarian reserve status, indexed by

AMH and AFC, in women with DM-1: the OVarian Ageing in type 1 DIAbetes mellitus (OVADIA) study. Subsequently, we evaluated the prevalence of advanced ovarian ageing in the DM-1 group compared to the Scheffer, Van Rooij, De Vet (SRV) cohort of normal fertile Caucasian women as a historical reference group (44;47;184). Approval was obtained from the Institutional Review Board of the University Medical Center Utrecht (UMCU) and all participants gave their written informed consent. This study was registered at http://www.clinicaltrials.gov (NCT01665716).

Study population

Women with DM-1 were recruited from the department of Internal Medicine of several Dutch Hospitals and through the Dutch Diabetes Society (by using website and newsletter). The OVADIA study population consisted of Caucasian women above 18 years of age with DM-1 and regular menstrual cycles. A regular menstrual cycle was defined as a mean cycle length between 25 and 35 days, with a minimum of 21 and a maximum of 35 days as well as a variation in cycle length not exceeding 7 days.

DM-1 was defined according to the criteria established by the American Diabetes Association (185). A total of 419 women were identified as possible eligible participants and were sent an invitation letter including the study information. In total, 390 women (93%) responded from whom 58% were willing to consider participation. These women were screened for in- and exclusion criteria via a telephone interview. In total, 76 women (34%) were excluded due to (a history of) an irregular menstrual cycle (21%), use of hormonal contraceptives with a previous indefinite regular cycle (5%), pregnancy (3%), use of hormonal injections (2%). Furthermore, women who were not Caucasian (1%) or those with (risk of) an induced menopause, i.e. ovarian surgery, hysterectomy, chemotherapy or endometrial ablation (2%) were also excluded. This resulted in 150 participants.

Study parameters

After inclusion, all women with DM-1 completed a questionnaire regarding their reproductive history, cycle regularity, cardiovascular health and family history. Subsequently, all participants were invited for a non-invasive screening to assess both vascular endothelial condition and ovarian reserve status.

The screening was performed in the morning during a single study visit after an overnight fast. All vascular measurements were conducted by two trained research nurses in a quiet, temperature-controlled room (21-24 °C).

Since endothelial function tests were influenced by hormonal fluctuations during the menstrual cycle, all measurements were performed at a prefixed moment of the menstrual cycle. Women without hormonal contraceptive use were examined in the early follicular phase (cycle day 1-5). Women using oral contraceptives were examined at day five, six or seven during their pill free week, provided that they had a normal withdrawal bleeding. Finally, those women who no longer had a spontaneous menstrual cycle because of hormonal intrauterine devices-use were screened randomly.

Vascular risk factors

Venous blood samples were drawn after overnight fasting. Triglycerides, total cholesterol and HDL- cholesterol were analysed in heparin plasma on an AU5811 routine chemistry analyser (Beckman Coulter, Brea, California). LDL-cholesterol was calculated, using the Friedewald formula (186). HbA1c was measured on the Menarini HA8180 HPLC after ion exchange separation of the haemoglobin forms (Menarini Diagnostics, Florence, Italy). C-reactive protein (hs-CRP) levels were measured using a highly sensitive CRP assay on the BN ProsPec nephelometer (Siemens Healthcare, Breda, the Netherlands). The coefficient of variation for hs-CRP at 1.46 mg/L was 3.6%. The coefficients of variation for HbA1c at 42 and 90 mmol/mol, were 1.5 and 1.0% respectively.

Hyperlipidaemia was defined as having total serum cholesterol above 5.0 mmol/L or the use of cholesterol-lowering medication. Blood pressure was measured three times in sitting position. The mean systolic and diastolic pressure was calculated using the second and third blood pressure measurement. Hypertension, high blood pressure, was defined as having a mean systolic blood pressure of at least 140 mmHg and/or a mean diastolic blood pressure of at least 90 mmHg or when the use of antihypertensive medication was reported.

Vascular function tests

FMD was measured by ultrasonography (Ultrasonix SP machine with a 5-14 MHz transducer) of the brachial artery. After five minutes of occlusion with a blood pressure cuff, reactive hyperaemia was induced by rapidly deflating the cuff. The dilation of the brachial artery was continuously monitored. FMD was defined as (maximum baseline diameter / baseline diameter) x 100%. All FMD scans were assessed by two independent investigators and a maximum difference of 2% was considered as good agreement. Discrepancies of more than 2% were discussed and agreed upon by a third calculation performed by both investigators together.

Simultaneously with the FMD measurement, an Endo-PAT (peripheral arterial tonometry) was measured, reflecting peripheral endothelial function via finger plethysmography (EndoPAT2000, Itamar Medical Ltd., Caesarea, Israel). The resulting reactive hyperaemia index (RHI) represents microvascular endothelium-dependent vasodilation.

Vascular stiffness was reflected by carotid-radial Pulse Wave Velocity (PWV) and augmentation index was measured by pulse wave analysis (PWA). Finally, ultrasonography was performed to determine the intima-media thickness (IMT) as marker of subclinical atherosclerosis. Mean IMT was calculated based on six measurements of the far-wall of the left and right common carotid arteries, the carotid bifurcation and the internal carotid artery.

Ovarian reserve status

Transvaginal ultrasound was performed by one trained investigator to assess the antral follicle count (AFC) using the 7.5-MHz transvaginal probe on a Aloka SSD-4000 (Hitachi-Aloka Medical, Japan). AFC was calculated by adding up the follicles with a diameter of 2 to 10 mm from both ovaries, using a standard systematic approach (127).

AMH was determined by using the Gen II enzyme-linked immunosorbent (ELISA) assay from DSL (Beckman-Coulter, Brea, CA, USA) and carried out in the laboratory of the UMC Utrecht. The assay detection limit was 0.16 ng/ml; intra-assay coefficients of variation ranged from 3.5-5.5% (16 to 3 ng/ml) and inter-assay coefficients of variation were 9.3% (at 0.5 ng/ml) and 7.3% (at 7.6 ng/ml).

Reference population

We used the SRV cohort of healthy, normal fertile, Caucasian women as a reference group for the women with DM-1 (44;47;184). The SRV cohort is a combined population from three highly comparable, prospective longitudinal studies on ovarian function from the Netherlands. Details on the SRV cohort were described previously by Broer et al. (165). We used the data from 176 women with these characteristics: regular menstrual cycles varying from 21 to 35 days, no history of ovarian surgery, hormonal contraception stopped at least three months before AMH measurements and no missing data in outcome variables. Blood withdrawal for AMH measurement was performed during the early follicular phase of the menstrual cycle. Serum was stored at -20 C until further processing.

The AMH measurements in the SRV cohort were performed in the laboratory of the Erasmus MC, according to the same analytical methods, ensuring the comparability with the assay results in the DM-1 group. In one of the three studies contributing to the SRV cohort, AMH levels were measured by an enzyme-immunometric assay of Diagnostic Systems Laboratories (DSL, Inc, Webster, TX) and in the other two studies with the ultra-sensitive immunoenzymometric assay (Immunotech-Coulter (IC), Marseille, France (187). The inter- and intra-assay coefficients of variation of the DSL assay were less than 5% at the level of 3.0 ng/ml and less than 11% at the level of 13.0 ng/ml. The detection limit of the DSL assay was 0.026 ng/ml. The IC assay inter- and intra-assay coefficients of variation were less than 8% and less than 5% respectively. The detection limit of the IC assay was 0.05 ng/ml. As previously described, the AMH results measured with the DSL assay were corrected by a factor of 2.0 to ensure a more accurate comparison and pooling of the AMH results measured with the IC assay (165;188).

To compare the AMH values of the DM-1 group with the SRV cohort, AMH values of the SRV cohort were converted into values representative of the Gen II assay by applying a conversion factor. This lab-specific conversion factor was determined and amounted to “SRV (IC) * 1.564 = Gen II”, as previously described (189;190).

Statistical analysis

Descriptive parameters and population characteristics were reported as means ± SD and categorical data were expressed as percentages. Linear regression analyses were used to detect a possible association between patient characteristics, vascular risk factors, vascular function tests and serum AMH levels within the DM-1 group, while censoring for undetectable AMH levels according to the Buckley James method on log-transformed AMH (167). In the case of an undetectable AMH level (<0.16 ng/ml), AMH values were censored. Patient characteristics included age, current smoking, body mass index (BMI), hormonal contraceptive use, parity, glycaemic control (HbA1c) and duration of DM-1. Lipids (HDL- and LDL-cholesterol), systolic and diastolic blood pressure and hs-CRP were considered vascular risk factors. Finally, vascular function tests comprised FMD, Endo-PAT, PWA, PWV and IMT measurements.

Next, linear regression analyses on log transformed AFC were performed to detect a possible association between patient characteristics, vascular risk factors, vascular function tests and the AFC in women with DM-1.

For the comparison of population characteristics and ovarian reserve status between the DM-1 group and reference group, Student's t-test or chi-square were applied for respectively continuous or categorical data. Subsequently, similar Buckley James regression analyses were performed to investigate the difference in serum AMH levels between the DM-1 group and the reference group. Female age at sampling, smoking, BMI and hormonal contraceptive use were considered as confounders in advance.

To correctly model a potentially non-linear relationship between continuous variables and serum AMH level or AFC, a restricted cubic spline with three knots was used. In case of a significant non-linear relationship, the p-value for non-linearity was reported and the relationship was depicted graphically. Fold changes with 95% confidence intervals (95% CI) were calculated for variables with a linear relationship describing the relative change in serum AMH level.

Power calculations were based on correlation and association analyses between the degree of ovarian reserve and vascular status. In order to detect a correlation of 0.22 with $\alpha<0.05$ and $\beta= 0.80$, a number of 150 women with DM-1 were needed. A total of 150 women with DM-1 and 150 references would be sufficient to detect a difference in AMH of 0.36 ng/ml (SD ± 1.13 ng/ml), indicating a difference in biological age of approximately 2 years (47).

Data were analysed using SPSS for Windows, version 20.0 (SPSS INC., Chicago, IL) and R version 2.10.0 (http://www.r-project.org).

RESULTS

The mean age of the DM-1 group was 33.8 ± 8.4 years with an average duration of disease of 16.6 ± 10.3 years and HbA1c 65 ± 13 mmol/mol. The majority of women with DM-1 never gave birth (61%) and used hormonal contraceptives (62%). Mean AMH levels were 2.5 ± 1.9 ng/ml and AFC ranged from 2 to 67 with a median count of 17 follicles in both ovaries.
Both the population characteristics, vascular risk factors and vascular function tests are shown in **Table 1**. In a quarter of the women with DM-1 hyperlipidaemia was present and one in five cases fulfilled our criteria of hypertension. A carotid plaque was observed in 7 women (5%).

Table 1. Population characteristics and vascular factors of women with DM-1 (n=150)

Population characteristics	
Age at screening (years)	33.8 ± 8.4
Current smokers	27 (18%)
Body mass index (kg/m^2)	25 ± 5
HbA1c (mmol/mol)	65 ± 13
Duration of diabetes mellitus (years)	16.6 ± 10.3
Vascular risk factors	
Hypertension, n (%)	31 (21%)
Systolic blood pressure (mm/Hg)	123 ± 13
Diastolic blood pressure (mm/Hg)	75 ± 9
Hyperlipidaemia, n (%)	37 (25%)
Total cholesterol (mmol/L)	4.86 ± 0.90
LDL-cholesterol (mmol/L)	2.82 ± 0.75
HDL-cholesterol (mmol/L)	1.64 ± 0.35
Triglyceride (mmol/L)	0.86 ± 0.46
hs-CRP (mmol/L)	2.9 ± 3.1
Vascular function tests	
Percentage of dilatation FMD (%)	6.7 ± 3.0
Reactive hyperaemia index	2.2 ± 0.7
Pulse Wave Velocity (m/s)	8 ± 2.2
Augmentation index (%)	13 ± 13
Carotid Intima Media Thickness (mm)	0.55 ± 0.10
plaque present (yes vs. no)	7 (5%)

Data are presented in mean ± SD or number (%).

Factors associated with ovarian reserve status in DM-1

The use of hormonal contraceptives was associated with significantly lower values for AMH, whereas AMH levels were not related to smoking, BMI or degree of glycaemic control (**Table 2**). Serum AMH levels were negatively correlated with systolic blood pressure (relative decrease 0.84, 95% CI 0.75-0.95), with an age-adjusted decrease of 16% in AMH for a systolic blood pressure increase of 10 mmHg. Analyses with spline functions demonstrated a non-linear relationship between HDL and AMH levels (p-value for non-linearity p=0.0001), as depicted in **Figure 1a**. Given the small variation in AMH due to HDL this association was considered a weak one.

No associations were detected between diastolic blood pressure, LDL-cholesterol, hs-CRP and serum AMH. As summarized in **Table 2**, vascular function tests, reflecting endothelial function and vascular stiffness, were not related to AMH in women with DM-1.

With respect to the AFC, we also detected a negative correlation with the use of hormonal contraceptives but found no relation between AFC and smoking or BMI (**Table 2**). Moreover, similar to AMH, an association was observed between systolic blood pressure and AFC with a non-linear relationship, (p-value for non-linearity p=0.004). This is depicted in **Figure 1b**. Also no association with vascular function tests and AFC was found.

Overall, hormonal contraceptive use was associated to both lower age-specific AMH levels and a lower AFC. Out of the vascular factors only systolic blood pressure and HDL levels were associated to ovarian reserve status.

Ovarian reserve in women with DM-1 compared to references

Population characteristics and reproductive parameters of the DM-1 group as well as the reference group are presented in **Table 3**. Women with DM-1 were younger (p=0.002) and had a higher BMI (p=0.001) compared to the references. Age at menarche was similar in both groups. The majority of the DM-1 group was using hormonal contraceptives, while the reference group did not use any hormonal contraceptives for at least three months, according to the assessment protocol (p<0.001).

With respect to the ovarian reserve, similar proportions of undetectable AMH levels were observed between the DM-1 group and reference group (11% versus 7%). Mean serum AMH level was 2.5 ± 1.9 ng/ml in the DM-1 group compared to 3.0 ± 2.8 ng/ml in reference group.

Analyses with spline functions demonstrated a non-linear association between age at screening and serum AMH level (p-value for non-linearity p<0.0001). As demonstrated in **Table 4**, after adjustment for age at screening, smoking behaviour and BMI, women with DM-1 had significantly lower AMH levels.

Table 2. Association between patient characteristics, vascular risk factors and vascular function tests with serum AMH levels and AFC in women with DM-1

	AMH (ng/ml)		AFC (2-10mm)	
Determinants	relative change	95% CI	relative change	95% CI
Patient characteristics				
Current smoking (yes vs. no)				
unadjusted	0.97	0.65, 1.46	1.01	0.76, 1.34
adjusted for age	0.92	0.61, 1.40	0.95	0.77, 1.17
Hormonal contraceptive use (yes vs. no)				
unadjusted	1.74	1.24, 2.44	1.34	1.07, 1.66
adjusted for age	0.64	0.44, 0.93	0.82	0.68, 0.98
Nulliparity (yes vs. no)				
unadjusted	1.44	1.05, 1.98	1.23	0.99, 1.53
adjusted for age	0.77	0.53, 1.13	0.91	0.76, 1.09
Body mass index (kg/m²)				
unadjusted	0.94	0.91, 0.97	0.98	0.96, 1.00
adjusted for age	0.97	0.94, 1.00	1.00	0.98, 1.01
HbA1c (mmol/mol)				
unadjusted	1.01	1.00, 1.02	1.00	1.00, 1.01
adjusted for age	1.01	0.99, 1.02	1.00	1.00, 1.01
Duration of diabetes mellitus (years)				
unadjusted	0.96	0.94, 0.97	0.99	0.98, 1.00
adjusted for age	1.00	0.98, 1.02	1.01	1.00, 1.02
Vascular risk factors				
Systolic blood pressure (per 10 mm/Hg)				
unadjusted	0.75	0.66, 0.85	*	p=0.024
adjusted for age	0.84	0.75, 0.95	*	p=0.004
Diastolic blood pressure (per 10 mm/Hg)				
unadjusted	1.02	0.86, 1.21	1.00	0.89, 1.13
adjusted for age	0.89	0.75, 1.07	0.95	0.87, 1.04
LDL-cholesterol (mmol/L)				
unadjusted	1.38	1.12, 1.70	1.17	1.02, 1.35
adjusted for age	1.07	0.86, 1.32	1.04	0.93, 1.16
HDL-cholesterol (mmol/L)				
unadjusted	*	p=0.0035	0.77	0.57, 1.05
adjusted for age	*	p=0.0001	0.97	0.77, 1.23
hs-CRP (mg/L)				
unadjusted	1.00	0.95, 1.05	1.01	0.98, 1.05
adjusted for age	0.97	0.92, 1.02	1.00	0.97, 1.03

Table 2. Continued

	AMH (ng/ml)		AFC (2-10mm)	
Determinants	relative change	95% CI	relative change	95% CI
Vascular function tests				
FMD: % dilatation				
unadjusted	1.01	0.96, 1.07	1.00	0.96, 1.04
adjusted for age	1.03	0.97, 1.08	1.01	0.98, 1.04
Reactive hyperaemia index				
unadjusted	0.66	0.51, 0.86	0.85	0.72, 1.01
adjusted for age	0.95	0.73, 1.25	1.02	0.90, 1.16
Pulse Wave Velocity (m/s)				
unadjusted	0.82	0.76, 0.89	0.94	0.90, 0.99
adjusted for age	0.96	0.88, 1.05	1.01	0.97, 1.05
Augmentation index (%)				
unadjusted	0.96	0.94, 0.97	0.98	0.97, 0.98
adjusted for age	0.99	0.98, 1.01	0.99	0.99, 1.00
IMT (per mm)				
unadjusted	0.59	0.50, 0.70	0.80	0.72, 0.89
adjusted for age	0.97	0.80, 1.19	1.00	0.91, 1.09

Linear regression analyses with censoring for undetectable AMH levels. Fold changes with 95% confidence intervals (CI). * Statistically significant non-linear association. FMD = Flow Mediated Vasodilatation. IMT = carotid Intima Media Thickness.

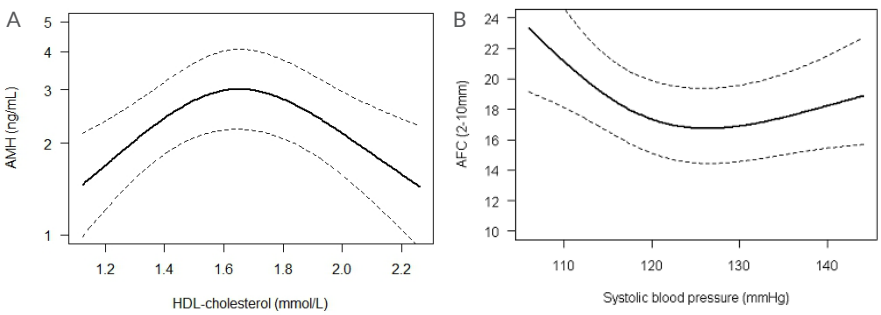

Figure 1. A) Non-linear relationship between HDL-cholesterol and AMH levels. An increase in HDL-cholesterol until approximately 1.6 mmol/L was associated with an increase in AMH until 3.0 ng/ml, whereas HDL-cholesterol levels exceeding 1.6 mmol/L were associated with a decline in AMH levels. B). Non-linear relationship between systolic blood pressure and AFC. An increase in blood pressure until approximately 125 mmHg was associated with a decrease in AFC. Blood pressure levels exceeding 125 mmHg were no longer associated with AFC.

Table 3. Population characteristics and ovarian reserve parameters of women with DM-1 compared to references

	DM-1 group n=150	Reference group n=177	p-value
Population characteristics			
Age at screening (years)	33.8 ± 8.4	36.4 ± 5.9	0.002
Body mass index (kg/m^2)	25 ± 5	24 ± 4	0.001
Current smokers	27 (18%)	34 (19%)	0.29
Reproductive parameters			
Age at menarche (years)	13.1 ± 1.6	13.2 ± 1.4	0.65
Nulliparity	91 (61%)	63 (36%)	<0.001
Use of hormonal contraceptives	93 (62%)	0 (0%)	<0.001
Cycle length (days)	28.4 ± 2.3	27.7 ± 2.3	0.03
Outcome parameters			
Undetectable AMH levels	16 (11%)	12 (7%)	0.22
AMH (mcg/L) if detectable	2.5 ± 1.9	3.0 ± 2.8	0.03

Data are presented in mean ± SD or number (%). p-values are computed with t-tests or chi-square. AMH = Anti-Müllerian hormone. Cycle length is only reported for women without hormonal contraceptive use.

Table 4. Association between DM-1 and serum AMH levels

	Relative change in AMH (ng/mL)	95% CI
DM-1 vs. reference group		
Crude model	0.77	0.62, 0.96
Adjusted for age	0.73	0.59, 0.90
Adjusted for age, smoking	0.73	0.59, 0.90
Adjusted for age, smoking, BMI	0.75	0.60, 0.94
Adjusted for age, smoking, BMI, hormonal contraceptive use	0.92	0.68, 1.23

Linear regression with censoring for undetectable AMH levels. Regression coefficients and 95% confidence intervals (CI), adjusted for age, smoking, BMI and hormonal contraceptive use. AMH = Anti-Müllerian hormone. DM-1 = type 1 diabetes mellitus

However, the relative decrease of serum AMH by 25% was attenuated by additional adjustment for hormonal contraceptive use, resulting in a non-significant difference in AMH levels between women with DM-1 compared to references (fold change 0.92, 95%CI 0.68-1.23) as graphically depicted in **Figure 2**.

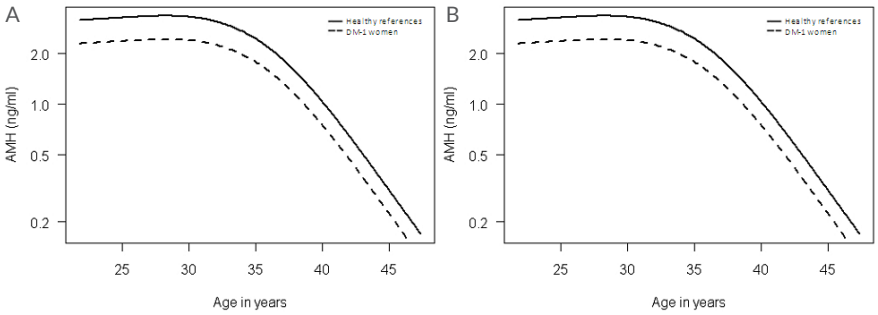

Figure 2. Age related AMH decline as plotted on a logarithmic scale in women with DM-1 (dotted line) compared to references (solid line), as computed with linear regression using log transformed AMH. A) Model adjustment for age and smoking. B) Model adjusted for age, smoking, BMI and hormonal contraceptive use.

DISCUSSION

In this study, vascular health status was not related to markers of ovarian reserve in women with DM-1. Out of all vascular factors, only systolic blood pressure was negatively correlated with ovarian reserve status estimated by serum AMH levels and AFC. A weak, non-linear relationship was observed between HDL-cholesterol and AMH. These current results do not support our hypothesis that vascular health is associated with ovarian reserve status.

The literature is limited with regard to our vascular hypothesis of ovarian ageing; we are only aware of a few studies indicating an association between cardiometabolic factors and ovarian reserve status. In female monkeys, a negative correlation between atherosclerotic plaque sizes and AMH level was observed (75). Also, the presence of hypertension has been associated with lower AMH levels in a mixed cohort of women with and without a history of preeclampsia (177). The mechanism behind advanced depletion of the primordial follicle pool could lie in the deterioration of the ovarian vascular blood supply. This was supported by the study finding that the presence of microvascular complications, i.e. retinopathy and nephropathy in women with DM-1, was negatively correlated with menopausal age (183). Furthermore, surgeries affecting the vascular supply of the reproductive organs, such as salpingectomy, hysterectomy and uterine artery embolization, were associated with a reduced ovarian reserve status (191;192).

Possible explanations for the contrast between the present findings and existing literature on the association between vascular health and ovarian reserve status should be considered. The women with DM-1 in the present study were not as “vascularly compromised” as we had assumed according to their duration of DM-1(193). Lipids were well within the normal ranges and the prevalence of smokers was

below the Dutch average (194). Furthermore, measurements of endothelial function and subclinical atherosclerosis were not much different from healthy women of the same age (195;196). Due to their relatively young age, a longer duration of the disease may be necessary for sufficient vascular compromise to develop in such a way to affect the ovarian reserve (183).
Subsequently, this may explain why we were unable to detect a reduced ovarian reserve status in the DM-1 group compared to the reference group.
In view of the current literature, only one study was comparable with the present one, although smaller in size (197). In this cross-sectional study by Soto et al., ovarian reserve markers of 66 women with DM-1 were compared to 58 healthy, age- and BMI-matched controls. The ages in both groups were under 45 years and none of the participants used hormonal contraceptives for at least six months. There was no observed difference in AMH level between both groups. After applying an age limit of 33 years, , no difference in AMH levels was detected between women with DM-1 and controls below the age limit. However, above the age of 33 years, significantly lower AMH levels were observed in women with DM-1 compared to the control group, suggesting an earlier or faster decline in the size of the ovarian follicle pool. Explanations for these findings, however, could not be evaluated as having a vascular origin as women with DM-1 with micro- or macrovascular complications were excluded. On the other hand, the exclusion of vascular compromised cases may have also attenuated the observed difference in AMH level, if indeed a relation would exist between vascular compromise and the rate of ovarian ageing. Sub-analysis of the current study data, through application of the age limits in analogy to the study by Soto et al., did not change the results (data not shown).
Other possible explanations for the absence of an association between DM-1 and ovarian reserve could lie in glycaemic control, which may have been too optimal in the current study population. This has been suggested by the observation that HbA1c levels were negatively correlated with ovarian reserve status, indexed by the antral follicle count, in women with type 2 diabetes mellitus (198).
The main strength of the current study lies in the structured assessment of all study parameters. Both vascular screening and ovarian reserve screening were performed by trained personnel, providing a high precision of the estimated effects. Limitations of the study were the use of different assays to measure AMH and the cross-sectional design. The DM-1 group and reference group were sampled from different populations, requiring adjustment for confounders to ensure comparability. Specifically, the reference group refrained from hormonal contraceptive use for at least three months before entering the study. As a result, there was not enough power to detect between group differences in subgroups only comprising of women without any hormonal contraceptive use.

Clinical implications that can be drawn from this study involve reassurance with regard to the reproductive lifespan of women with DM-1. Instead of the earlier suggestions that women with DM-1 may experience a six-year reduction of reproductive lifespan (182), we did not find evidence for advanced ovarian ageing.
Future research is needed to elucidate the association between vascular health and ovarian reserve status. Preferably, longitudinal studies from adolescence onwards with measurements of both vascular and reproductive health status, or animal studies using models with induced vascular health problems are needed to investigate reproductive ageing. If DM-1 will be used as a model for vascular dysfunction in future studies, more advanced cases and cases with poor glycaemic control should be included.
In conclusion, we found no evidence for an association between vascular health and the timing of reproductive ageing, using DM-1 as a model for early-onset vascular compromise.

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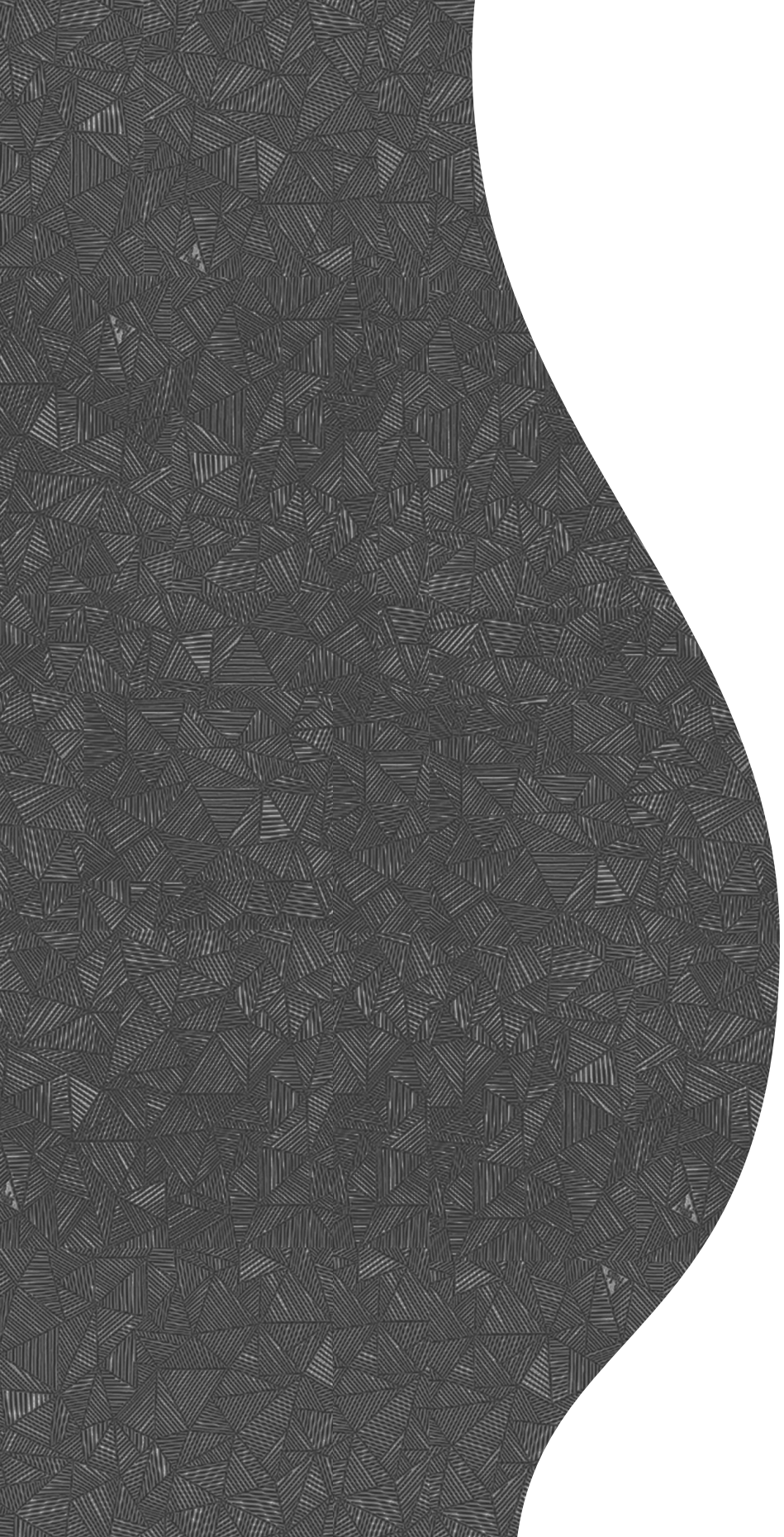

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Prenatal famine, birth weight, reproductive performance and age at menopause: the Dutch Hunger Winter Families Study

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ABSTRACT

Study question
Is there an association between acute prenatal famine exposure or birth weight and subsequent reproductive performance and age at menopause?

Summary answer
No association was found between intra-uterine famine exposure and reproductive performance, but survival analysis showed that women exposed in utero were 24% more likely to experience menopause at any age.

What is known already
Associations between prenatal famine and subsequent reproductive performance have been examined previously with inconsistent results. Evidence for the effects of famine exposure on age at natural menopause is limited to one study of postnatal exposure.

Study design, size, duration
Cohort study of men and women born around the time of the Dutch Famine of 1944-1945 and their siblings. Study participants (n=1,070) underwent standardized interviews on reproductive parameters at a mean age of 59 years.

Participants/materials, setting, methods
Men and women with prenatal famine exposure (n=407), born before or after the famine period (time controls, n=344), and same-sex siblings (family controls, n=319). Associations of famine exposure with reproductive performance and menopause were analysed using logistic regression and survival analysis with competing risk, both after controlling for family clustering.

Main results and the role of chance
Gestational famine exposure was not associated with nulliparity, age at birth of first child, difficulties conceiving or pregnancy outcome (all $p>0.05$) in men or women. At any given age, women were more likely to experience menopause after gestational exposure to famine (Hazard Ratio 1.24; 95% CI 1.03, 1.51). The association was not attenuated with additional control for a woman's birth weight. In this study, there was no association between birth weight and age at menopause after adjustment for gestational famine exposure.

Limitations, reason for caution
Age at menopause was self-reported and assessed retrospectively. Study power to examine associations with specific gestational periods of famine exposure and reproductive function was limited.

Wider implications of the findings

Our findings support previous results that prenatal famine exposure is not related to reproductive performance in adult life. Natural menopause occurs earlier after prenatal famine exposure however, suggesting that early life events can affect organ function even at the ovarian level.

INTRODUCTION

Female reproductive ageing represents the decline with increasing age of both quantity and quality of the ovarian follicle pool (11). Demographic studies have shown that women experience optimal fertility before the age of 30-31 years (4;5). Thereafter, a gradual decline in monthly fecundity rate is observed, with an acceleration from 36 years onwards. The age-related decrease in follicle numbers dictates the onset of cycle irregularity and menopause, the final cessation of menses, which marks the end of female reproductive function (105). In general, women experience no signs of this reproductive ageing process, except for the occurrence of subfertility and involuntary childlessness.

Several factors contributing to the rate of the reproductive ageing process have been identified. Smoking and nulliparity have been associated with an early age at menopause (70;71); other identified predictors are lower socioeconomic status, early menarche and a low body mass index (70;176;199). Next to environmental and life-style factors, multiple genetic factors have been claimed to influence menopausal timing (67;69). However, the variation in age at menopause can only partly be explained by these characteristics.

An adverse environment in utero is thought to permanently change the physiology, metabolism and organ structure of the developing foetus and thereby affect health in later life (80). As a complex mechanism of hormonal and physical events is necessary for normal reproductive function, it has been hypothesized that caloric restriction during pregnancy might also affect in utero development of the organs responsible for reproductive function, and as such may affect fertility and age at menopause (82).

Growth-retarded foetuses have impairment of ovarian development, which may also have implications for the timing of menopause (200). Furthermore, low birth weight infants with prematurity or growth retardation tend to have fewer offspring (201;202), and decreased age at menopause has been reported following exposure to famine in early childhood (83).

Two previous studies of prenatal famine exposure and subsequent reproductive performance have been reported, with inconsistent results. Lumey and Stein (1997) found no adverse impact of famine exposure on a range of measures of female fertility ascertained at age 43 years, while Painter et al (2008), who interviewed the same sample of exposed women at mean age 50 years, but with a different sample of controls, found a small but significant decrease in the prevalence of nulliparity.

We therefore conducted a study in an independent sample, at an age when the study population would have been expected to be postmenopausal. The aim of the present study was to assess the effect of gestational exposure to famine on measures of reproductive function in both men and women. We also examined whether there is a relation between famine exposure, birth weight and age at menopause.

MATERIALS AND METHODS

Historical background: The Dutch Hunger Winter

The Dutch famine, during the winter of 1944–1945, provides a unique opportunity to study the effects of maternal undernutrition at different stages of gestation on adult health. The famine resulted from a transport embargo on food enforced by the German military initiatives and was clearly defined in place (limited to the western Netherlands) and time (October 1944–May 1945). Widespread starvation was seen in the western Netherlands and the severity of the famine has been fully documented (203-205). Official rations, which were generally adequate before the onset of famine (206), fell below 900 kcal/d by 26 November 1944 consisting mainly of bread and potatoes, and eventually lowered to 500 kcal/d by April 1945. The famine ceased immediately at liberation in May 1945, after which Allied food supplies were rapidly restored and distributed across the country. Widespread effects of famine regarding mortality, especially in the youngest and oldest age categories, fertility, pregnancy weight gain, and infant size at birth have been documented (204;207-211).

Study population

As described in greater detail elsewhere (212), a birth cohort of 3,307 live-born singleton births was identified at three institutions in the western Netherlands which experienced famine (the midwifery training schools in Amsterdam and Rotterdam and the university hospital in Leiden). We selected all 2,417 infants born between 1 February 1945 and 31 March 1946, whose mothers experienced exposure to famine during or immediately preceding that pregnancy. Moreover, a sample of 890 births from 1943 and 1947 was selected of infants whose mothers did not experience any famine exposure during this pregnancy, whom we designated as hospital time controls. The sample of time controls consisted of an equal number of births for each month and was allocated across the three institutions according to their size.

Tracing to current address

To trace the 3,307 infants to their current address, we filed a request to the Population Register in the municipality of birth providing names and addresses at birth. The Population Register in Rotterdam declined to trace 130 (4%) individuals born out of wedlock, 308 (9%) were reported to have died in the Netherlands, 275 (8%) were reported to have emigrated, and current address could not be located for 294 subjects (9%). As a result, address information was obtained for 2,300 individuals (70% of the birth cohort).

Enrollments and examinations

These 2,300 individuals were sent a letter of invitation signed by the current director of the institution in which they were born, enclosed a brochure describing the study and a response card. One reminder letter was sent to non-responders. Subsequently, all individuals with a same-sex sibling were asked to contact this sibling for study enrollment. For the siblings, there was no information available from prenatal or delivery records, as they were not members of the birth series in the three institutions and were generally delivered elsewhere. Initially, our study design aimed at recruiting same-sex sibling pairs only and the lack of an available sibling was a reason for ineligibility. Later, all individuals from the birth series were contacted once more and invited for the study irrespective of sibling availability.
We completed 1,070 telephone interviews. All study protocols were approved by the Human Subjects (Medical ethics) committees of the participating institutions. All participants provided verbal consent at the start of the telephone interview.

Famine exposure during gestation

The start of gestation was defined by the date of last menstrual period (LMP) as noted in the hospital records unless it was missing or implausible (12%). In case the LMP was missing, we derived the date from relevant annotations on the birth record and estimated gestational age from birth weight and date of birth, using cut-offs from tables of sex-, parity-, and birth weight-specific gestational ages from the combined birth records of the Amsterdam midwifery school (1948–1957) and the University of Amsterdam Obstetrics Department (1931–1965) (213). Subsequently, the most consistent and plausible estimation of gestational age was selected for each infant and used together with date of birth to derive the date of LMP. Gestational famine exposure was characterized by determining the gestational weeks during which the mother was exposed to an official ration of <900 kcal/d between 26 November 1944 and 12 May 1945. We considered the mother exposed to famine in gestational weeks 1–10, 11–20, 21–30, or 31 to delivery if these gestational time windows were entirely included in this period. By this means, all pregnancies with a LMP date between 26 November 1944 and 4 March 1945 were considered exposed in weeks 1–10, between 18 September 1944 and 24 December 1944 exposed in weeks 11–20, between 10 July 1944 and 15 October 1944 exposed in weeks 21–30, and between 2 May 1944 and 24 August 1944 exposed in week 31 through delivery. These definitions could have let any woman to be exposed to famine during at most two adjacent 10-wk periods. We characterized any prenatal famine exposure if infants were exposed in ≥1 of the 10-wk periods. In **Figure 1** the resulting sample size in the males and females by (overlapping) periods of exposure for gestational weeks 1-10, 11-20, 21-30 and 31 to delivery are depicted.

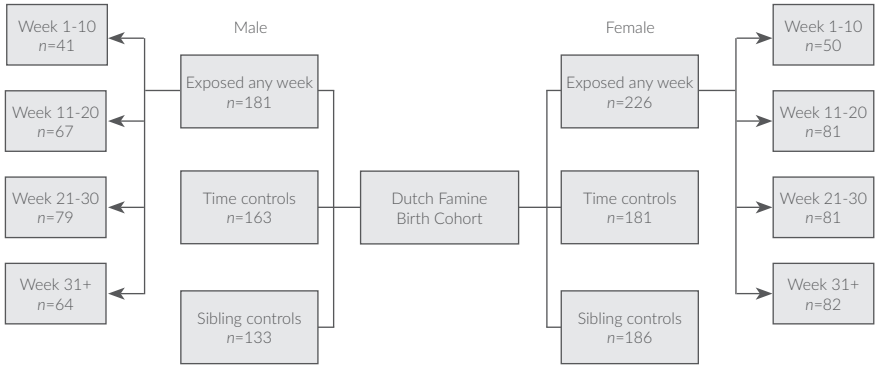


Figure 1. Flow chart of the study population. Exposed any week: all births between 1 February 1945 and 31 March 1946 and divided in gestational weeks of exposure to an official ration of <900 kcal/day. Time controls: births from 1943 to 1947. Sibling controls: same sex siblings unexposed to famine.

Study parameters

The main measures to express reproductive performance included (1) never gave birth or having fathered a child (nulliparity) and (2) attempting to conceive for at least 12 months without success (infertility). Natural menopause was defined as cessation of menstrual periods for twelve consecutive months in the absence of any other known cause of amenorrhea and this criterion was used to classify women as premenopausal or postmenopausal. Women were further categorized as having undergone induced menopause (by ovariectomy, hysterectomy, chemo- or radiotherapy) or having an unknown menopausal status due to exogenous hormone use or missing data. The 13 women (3 famine-exposed) who reported cessation of menses less than 12 months prior to the interview were considered to be postmenopausal. In sensitivity analyses we further considered them as still being premenopausal.

Socioeconomic status was categorized according to education level in low, medium and high. Current smoking status was categorized as non-smoker, former smoker or current smoker. We calculated pack years of smoking from the reported ages of starting smoking, quitting smoking (if applicable) and current age if still smoking, together with the average number of cigarettes smoked per day. This yields the total number of years of smoking the equivalent of 20 cigarettes a day. Use of other tobacco products was converted by equating 1 g of loose tobacco to one cigarette; 1 can or pack of pipe tobacco to 50 g of loose tobacco and one cigar to 5 g of tobacco.

Statistical analysis

Descriptive parameters and population characteristics were reported as mean ± SD and categorical data were expressed as percentages. Comparison of population characteristics and measures of reproductive function between the exposure categories was done using ANOVA and chi-square. We built regression models to assess the association of famine exposure for exposure during any week of gestation as well as exposure during gestational weeks 1-10, 11-20, 21-30 or 31 to delivery with reproductive performance and age at natural menopause. As a respondent could be exposed to two adjacent gestational time windows, we entered each specific time window independently into the regression model, to estimate the independent effect of each gestational time period (adjusted for the effect of the other).

We used logistic regression models with control for family clustering to compare nulliparity and infertility across exposure categories. Separate models were generated for men and for women. We used a survival analysis model with competing risks and control for family clustering, to investigate the association between famine exposure and age at natural menopause (214). Induced menopause was treated as a competing risk of natural menopause where the age at the last menses before operation or cancer therapy, was taken as the endpoint. Follow up time was in years since birth until age at natural menopause or age at last menses in women with an induced menopause. Premenopausal women were censored at the age of interview. Hazard ratios (HR) with 95% confidence intervals (CI) were computed. We adjusted for smoking history.

To determine if any association of famine exposure with age at menopause is related to birth weight, we developed survival models with competing risk in which famine exposure and birth weight were entered jointly. Moreover, a possible interaction between birth weight and famine exposure was examined. As birth weight was not available for the sibling controls, these analyses were restricted to the hospital series. We used SPSS for Windows, version 20.0 (SPSS INC., Chicago, IL) and STATA, version 11.1 (STATA Corporation, TX, USA) for all analyses.

RESULTS

We conducted interviews with 1,070 participants (477 males and 593 females). Both male and female unexposed siblings were younger than the exposed participants (**Table 1**). Birth weight was lower among both male and female exposed participants than among the time controls. There were no differences in smoking status by famine exposure category among either males or females.

Table 1. Selected characteristics of 1070 men and women who participated in the telephone interview of the Hunger Winter Family Study, by recruitment category and gender.

	Females				Males			
	Famine exposed (*n*=226)	Time control (*n*=181)	Sibling control (*n*=186)	*p*-value	Famine exposed (*n*=181)	Time control (*n*=163)	Sibling control (*n*=133)	*p*-value
Age at interview, years ± SD	58.7 ± 0.44	58.7 ± 1.6	56.9 ± 6.4	<0.01	58.8 ± 0.42	58.6 ± 1.5	57.4 ± 6.3	<0.01
Birth weight, grams ± SD	3256 ± 514	3391 ± 505	-	0.01	3357 ± 500	3537 ± 491	-	<0.01
Age at menarche, years ± SD	13.0 ± 1.7	13.0 ± 1.8	13.2 ± 1.6	0.27	na	na	na	
Socio-economic status				0.06				0.53
Low (%)	101 (45)	59 (33)	76 (41)		46 (25)	37 (23)	27 (20)	
Medium (%)	90 (40)	87 (48)	88 (47)		77 (43)	78 (48)	56 (42)	
High (%)	35 (16)	35 (19)	22 (12)		58 (32)	48 (29)	50 (38)	
Never married (%)	6 (3)	6 (3)	6 (3)	0.91	10 (6)	7 (4)	4 (3)	0.56
Ever smoked (%)	139 (62)	101 (56)	120 (65)	0.22	137 (76)	115 (71)	103 (77)	0.36
Pack years if ever smoking ± SD	25.7 ± 22.0	23.8 ± 18.4	22.3 ± 18.9	0.38	29.8 ± 23.2	28.3 ± 26.3	23.8 ± 21.0	0.14

Data are shown in mean ± SD or n (%). Birth weight is not available for the siblings. na = not applicable

Reproductive performance

Most women reported at least one pregnancy (**Table 2**). Almost one fifth of the women reported difficulties conceiving, with no differences across recruitment categories. The mean age at first birth was higher ($p<0.05$) in the same-sex siblings compared to the exposed females. The other reproductive characteristics did not differ among the three groups. In logistic regression models (**Table 3**), there were no significant differences between exposed and unexposed females.

Among males, neither increased prevalence of not having any children nor a higher prevalence of infertility problems was observed following famine exposure. In logistic regression analyses, neither exposure to famine in any week during gestation nor exposure during any specific period of gestation had any significant association with any of the reproductive performance parameters (data not shown). Analyses restricted to only married males and females did not alter these results (data not shown).

Age at natural menopause

Among the 593 women, 390 (66%) had reached natural menopause. The median [range] age of natural menopause was 50 [35-59] years. The median age at natural menopause in the exposed group, the time controls and the same-sex siblings was 50 [35 – 58], 52 [37-59] and 50 [38-58], respectively (**Table 2**). Reflecting the overall younger age of the sibling controls, more premenopausal women were observed in this group. The cumulative incidence of natural menopause as a function of age is presented graphically in **Figure 2**, according to the survival analysis with competing risks. Women exposed to famine in utero had a 24% increase in hazard of natural menopause (95% CI 1.03, 1.51), across the life course, compared to controls after adjustment for smoking (**Table 4**). When the relation between famine exposure and age at menopause was analysed according to the four specific periods of gestational exposure to famine, the associations were consistent across periods, without reaching statistical significance (**Table 4**).

When the 13 women who reported cessation of menses less than 12 months were considered to be premenopausal, a slightly stronger association between famine exposure and age at natural menopause was observed (HR 1.27 95%CI 1.05, 1.54 after adjustment for smoking status). When these 13 women were excluded from the analysis altogether, the results were similar.

When exposure to famine was defined by trimester rather than 10-week period, an association between famine exposure in each trimester and an earlier age at menopause was also observed. The association was statistically significant for third trimester exposure (HR 1.46, 95%CI 1.10,1.94 after adjustment for smoking status). This association was not attenuated by additional control for birth weight (HR 1.39, 95%CI 1.04,1.85).

Table 2. Selected measures of reproductive function among 1070 men and women who participated in the telephone interview of the Hunger Winter Family Study, by recruitment category and gender

	Females				Males			
	Famine exposed (*n*=226)	Time control (*n*=181)	Sibling control (*n*=186)	*p*-value	Famine exposed (*n*=181)	Time control (*n*=163)	Sibling control (*n*=133)	*p*-value
Nulliparity (%)	21 (9)	17 (9)	24 (13)	0.42	20 (11)	24 (15)	18 (14)	0.59
Difficulties conceiving (%)	42 (19)	37 (20)	41 (22)	0.68	29 (16.0)	27 (17)	20 (15)	0.95
Menopausal status								
Premenopausal (%) vs. all others	1 (0)	0 (0)	33 (18)	<0.01				
Postmenopausal (%) vs. all others				<0.01				
Natural menopause (%)	167 (74)	110 (61)	113 (61)					
Induced menopause (%)	45 (20)	55 (30)	34 (18)					
Perimenopausal use of hormones (%) vs. all others	11 (5)	16 (9)	4 (2)	0.02				
Missing (%)	2 (1)	0 (0)	2 (1)	0.40				
Median age at natural menopause, years [range]	50 [35 - 57]	51 [37 - 58]	50 [38 - 58]	0.19				
Miscarriage or abortion (%)	56 (25)	44 (24)	33 (18)	0.18	32 (18)[a]	22 (14)[a]	16 (12)[a]	0.33[a]
Preterm birth in offspring (%)	19 (8)	19 (11)	20 (11)	0.67	14 (8)	14 (9)	13 (10)	0.82
Perinatal death in offspring (%)	8 (4)	3 (2)	11 (6)	0.10	6 (3)	3 (2)	2 (2)	0.51
Age at first birth, years ± SD	23.7 ± 4.0	23.6 ± 4.4	25.0 ± 4.9	<0.01	26.7 ± 4.6	27.4 ± 5.1	27.1 ± 4.5	0.37
Interval between first and second birth, years ± SD[b]	3.1 ± 2.5	3.1 ± 3.0	2.9 ± 2.2	0.79	2.9 ± 1.7	2.8 ± 2.1	3.5 ± 2.4	0.03
Total number of children, mean ± SD	2.2 ± 1.2	2.2 ± 1.2	2.1 ± 1.3	0.82	2.1 ± 1.2	1.9 ± 1.2	2.1 ± 1.3	0.40

Data are shown in mean ± SD, median [range] or *n* (%). [a]Males were asked questions that parallel the questions routinely asked of women. [b]Only calculated for women with two or more live births (*n*=176, 142, 139 for famine exposed, time control, sibling control, respectively) and for men who have fathered two or more children (*n*=139, 115, 100 for famine exposed, time control, sibling control, respectively). Menopausal status: percentages may not add to 100 due to rounding, induced menopause includes ovariectomy, hysterectomy, chemo- or radiotherapy.

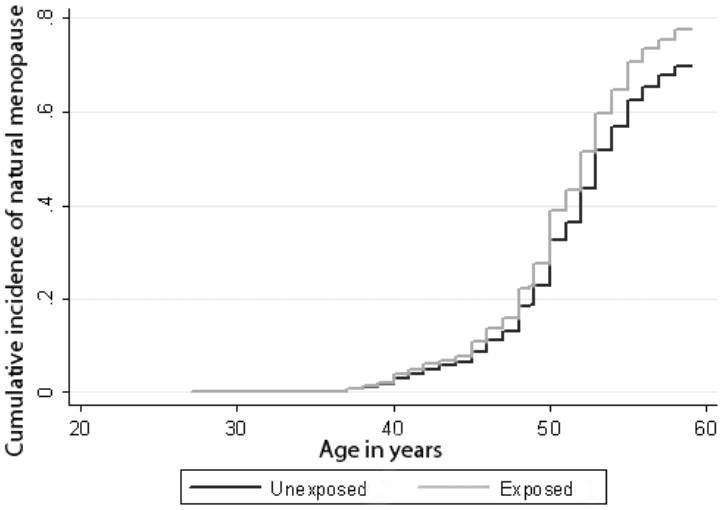


Figure 2. The cumulative incidence of natural menopause, as a function of age, with induced menopause as a competing risk. As a proportion of women do not reach natural menopause due to an induced menopause, the cumulative incidence of reaching menopause will not closely approach 1. Computed with competing-risks regression based on Fine and Gray's proportional subhazards model, by exposure to acute famine in utero (grey line) compared with no exposure (black line).

The relation between famine exposure, birth weight and age at menopause

We investigated whether the relation between famine exposure and age at natural menopause was related to birth weight (**Table 5**). As birth weights of the sibling controls were not known we restricted this analysis to the exposed women and their time controls with a known menopausal status (n=376). Exposure to famine was associated with a 36% increase in the hazard of natural menopause (HR 1.36; 95% CI 1.08, 1.71), compared to controls (adjusted for smoking status). Additional adjustment for birth weight made little change in this estimate (HR1.32; 95% CI 1.05, 1.66). Each kilogram increase in birth weight was associated with a 22% decrease in the hazard of natural menopause (HR 0.78; 95% CI 0.62, 0.98) and adjustment for smoking did not change this estimate. When famine exposure was added to the model, the relation with birth weight showed little change (HR 0.81) but was no longer statistically significant (95% CI 0.64, 1.03). In these models, birth weight and exposure to famine did not show a significant interaction (p=0.33).

Table 3. Association of prenatal famine exposure in females with reproductive performance compared to unexposed controls (n=593)

	Period of gestational exposure											
	Exposed any week			Weeks 1-10		Weeks 11-20		Weeks 21-30		Weeks 31 to delivery		
	OR	95% CI	*p*-value	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	*p*-value
Nulliparity												
Unadjusted	**0.81**	0.47, 1.42	0.47	**0.63**	0.19, 2.16	**0.53**	0.20, 1.43	**1.33**	0.62, 2.86	**1.03**	0.48, 2.19	0.57
Adjusted	**0.95**	0.53, 1.69	0.85	**0.71**	0.21, 2.44	**0.58**	0.21, 1.59	**1.45**	0.66, 3.17	**1.16**	0.53, 2.52	0.57
Difficulties conceiving												
Unadjusted	**0.79**	0.48, 1.29	0.35	**0.57**	0.21, 1.53	**0.84**	0.40, 1.77	**1.69**	0.84, 3.40	**0.55**	0.26, 1.20	0.29
Adjusted	**0.78**	0.47, 1.28	0.32	**0.57**	0.21, 1.56	**0.81**	0.38, 1.73	**1.72**	0.85, 3.49	**0.54**	0.25, 1.18	0.27

Odds ratio (OR) with 95% confidence intervals (CI) were obtained by logistic regression with correction for clustering of sibships. Adjusted for age and smoking status. P-value for test of association for all four ten-week exposure periods considered as a group (Wald test, four degrees of freedom)

Table 4. Survival analysis with competing risk for age at menopause in females exposed to famine compared to unexposed controls (n=558)

	Period of gestational exposure											
	Exposed any week			Weeks 1-10		Weeks 11-20		Weeks 21-30		Weeks 31 to delivery		
	HR	95% CI	*p*-value	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	*p*-value
Natural menopause												
Unadjusted	1.25	1.03, 1.51	0.02	1.02	0.76, 1.37	1.18	0.89, 1.56	1.22	0.91, 1.62	1.12	0.84, 1.49	0.28
Adjusted	1.24	1.03, 1.51	0.03	1.01	0.75, 1.37	1.17	0.88, 1.55	1.23	0.92, 1.63	1.11	0.83, 1.48	0.29

Hazard ratio's (HR) for the risk per year of becoming postmenopausal with 95% Confidence Interval (CI) with correction for clustering of sibships. Adjusted for smoking status. P-value for test of association for all four ten-week exposure periods considered as a group (Wald test, four degrees of freedom).

Table 5. Hazard ratios for age at menopause according to birth weight and famine exposure, using survival analysis with competing risk (n=376)

	Age at menopause		
	HR	95% CI	*p*-value
Birth weight (kg)			
Unadjusted	0.78	0.62, 0.98	0.04
Adjusted for smoking	0.78	0.62, 0.99	0.04
Adjusted for smoking and famine exposure	0.81	0.64, 1.03	0.09
Famine exposure: any week			
Unadjusted	1.38	1.09, 1.73	0.01
Adjusted for smoking	1.36	1.08, 1.71	0.01
Adjusted for smoking and birth weight	1.32	1.05, 1.66	0.02

Hazard ratio's (HR) for the risk per year of becoming postmenopausal with 95% Confidence Interval (CI).

DISCUSSION

This large population-based study demonstrates that prenatal famine exposure is not associated with later characteristics of reproductive performance in men or women.

Famine exposed women were 24% more likely to experience natural menopause at any age (95% CI 1.03, 1.51; p=0.03) as estimated from survival models with induced menopause as competing risk. This suggests a direct relationship between prenatal famine exposure and the age of menopause. The association was not attenuated by additional control for birth weight.

Little is known about the influence of the environment encountered during foetal life on the reproductive function in human adult life. Our findings on measures of reproductive performance confirm the findings by Lumey and Stein (82). Our results are in contrast however with the findings of Painter et al. who reported an increase in reproductive success in women exposed to famine (215). These inconsistent findings are based on largely the same famine exposed females born in the Wilhelmina Gasthuis Hospital in Amsterdam, but the two studies utilized different reference populations. As the estimates from the two studies have overlapping confidence intervals, the inconsistencies may also reflect chance variation around an overall weak association.

In the animal kingdom, the importance of prenatal nutrition for reproductive function is well recognized. A reduction of lifetime reproductive capacity after

prenatal undernutrition has been reported in mice (216) and sheep (217). Another study recently found evidence that prenatal dietary restriction influences the ovarian reserve in the bovine model (218); first trimester caloric restriction resulted in offspring with diminished ovarian reserve, as assessed by higher follicle-stimulating hormone levels, lower Anti-Müllerian hormone levels and a reduction in the antral follicle count, compared to offspring from adequately fed mothers.

The literature on the relation between undernutrition in utero and age at menopause is limited. Elias et al. reported a decrease of 0.36 years in age at natural menopause following famine exposure during early childhood (83). More frequently reported are studies that assess the association between birth weight, taken as a proxy for intrauterine nutritional status, and subsequent age at menopause. Steiner et al. reported a weak association between birth weight and age at menopause (HR 1.09; 95% CI 0.99, 1.20) (219). In our study this relation was attenuated after adjustment for gestational exposure to famine, suggesting that exposure to prenatal famine may affect the age at menopause through its impact on birth weight.

The association between birth weight and subsequent age at menopause has not been observed unanimously, however, as Treloar et al. did not find any association of birth weight with subsequent age at menopause in twins (220). Two other studies also failed to show an association for birth weight, but did find menopause to occur earlier in women with a low weight at the age of 1 (221) or 2 years (222).

Literature on the effects of famine on male reproductive performance is scarce. The analyses concerning gestational famine exposure and male reproductive performance were therefore primarily hypothesis-generating. Developmental problems of the testis such as cryptorchidism are associated with reduced fertility in adult life. The exact mechanisms that regulate the testicular descent are unknown, but may involve endocrine, genetic and environmental factors. Conditions as low birth weight, prematurity and small for gestational age are associated with a higher prevalence of cryptorchidism (223-225). In our study, no evidence for a possible association of maternal undernutrition with male reproductive performance was found.

A strength of our study is the population-based design. Individuals were recruited from institutional birth records on the basis of their place and date of birth, irrespective of their health status. The timing of exposure was based on the gestational age relative to the last menstrual period. Another strength is the use of same-sex siblings as controls, as they can be used to correct for any genetic predisposition.

With regard to the response rate of eligible participants, 9% of the 3,307 individuals selected for follow up at the birth clinics were no longer alive, 8% had emigrated, and 13% could not be located at age 58 years. All others were invited by mail to join the study. There was no association between famine exposure and follow up status at age 58 years. We found no differences in birth characteristics or demographic

characteristics by follow up status at age 58 or by comparing responders and non-responders to our invitation letter (212). Therefore we do not think that selection bias related to early mortality or to other reasons for non-response could explain our study results.

Although several studies have been published that find evidence for lifestyle factors besides smoking in relation to age at natural menopause (70;176;199), we considered only smoking as a possible confounder. As women were interviewed at the mean age of 58 years, all life-style factors were measured after menopause had already occurred. As menopause may lead to changes in life-style and behaviour, it would not be appropriate to control for these factors. Smoking is an exception, because virtually all smokers start smoking as young adults, and smoking status has been consistently associated with menopause (70;71).

A limitation is that information on reproductive performance was obtained from the participants themselves. Prolonged time to pregnancy is a commonly used measure for subfertility (226-229). As exact information regarding time to pregnancy was not available, we used 'difficulties conceiving exceeding 12 months duration' as a proxy. The use of nulliparity as a marker of reproductive performance combines both physiological incapacity and intention and we did not ascertain voluntary childlessness or use of contraception. To the extent that reproductive choices are not influenced by exposure to famine, this will not have biased our results.

Fertility selection might be a factor. During the famine, conception rates went down and those women who did conceive were possibly more fertile, creating offspring who are themselves more fertile. Use of sibling controls is likely to adjust for this factor.

We asked women to report their age (in completed years) at menopause, and coded the information accordingly. Use of a woman's recall of age at natural menopause is a widely-accepted method, but this measure does not have perfect reliability and validity (230-234). Recall bias might lead to inaccuracy in age at menopause at the individual level, but the bias is unlikely to be differential across exposure groups, and as such it would only attenuate measures of association with age at menopause but not explain the present finding of differences in age at menopause between exposure groups.

That we did not find a difference in median age at menopause, but did find that famine exposed women were more likely to be postmenopausal at any given age could be explained by the use of a survival analysis with competing risk, which accounts for imbalances between the exposure categories in terms of an induced menopause and the proportion of women who had not reached menopause yet.

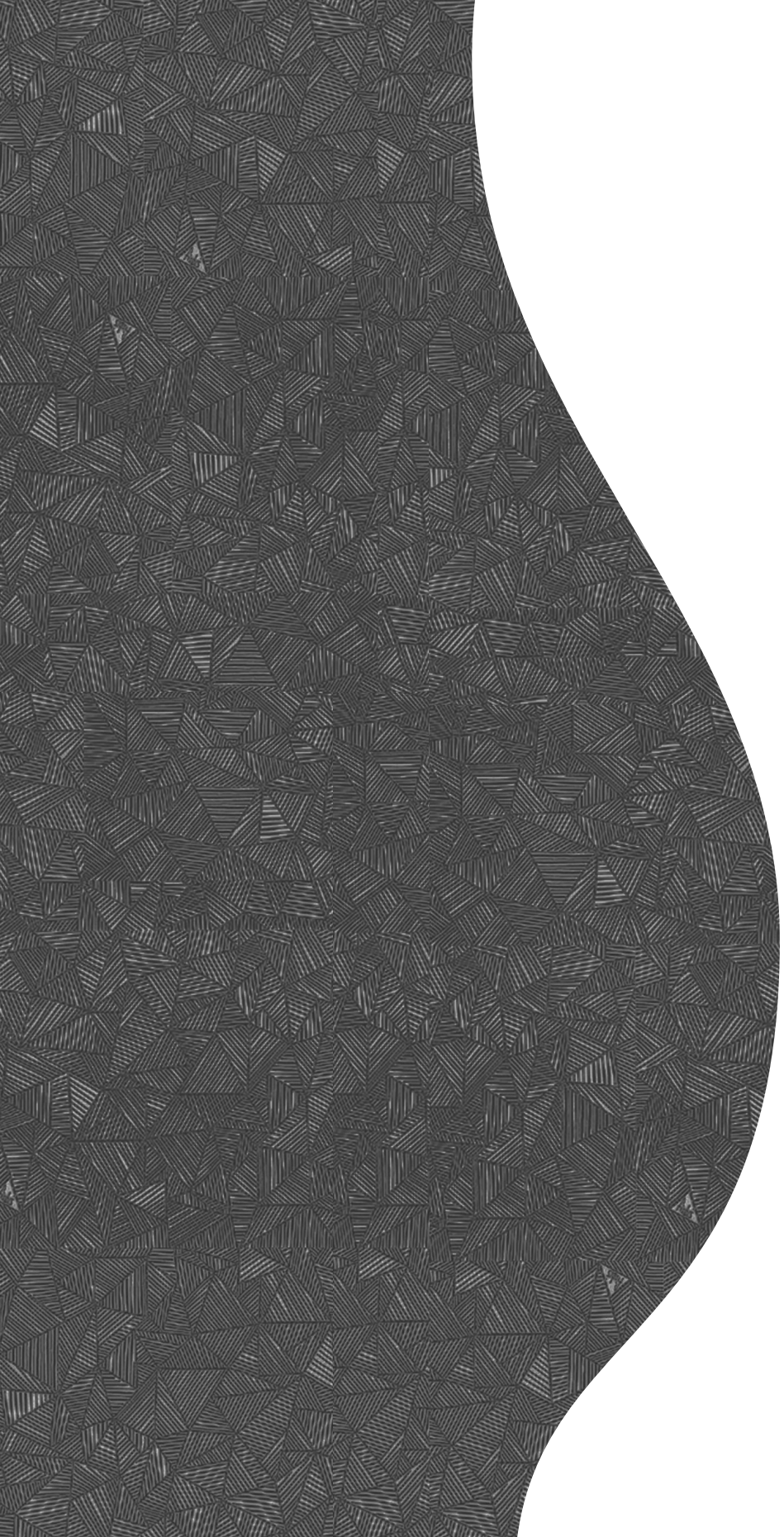
Female reproduction requires both quality and quantity of the oocytes residing within the ovarian follicles. A woman receives her endowment of oocytes during foetal development and during the reproductive years the quantity of the follicle

pool declines. Next to the decrease in quantity, the oocyte quality demonstrates changes with increasing age, which becomes apparent in increased aneuploidy rates leading to a higher prevalence of miscarriage and infertility observed at older age (12).

Our study population comprises a generation of women who were relatively young while giving birth to their first child and therefore might not experience the detrimental effects of reproductive ageing in terms of oocyte quality. Another possibility is that prenatal caloric restriction impairs the endowment of primordial follicles and in that way results in already a smaller foetal ovarian reserve. If we look at the phenomenon menopause, when fewer than 1,000 follicles are left, the final cessation of menses will occur and is therefore primarily dictated by the quantity of the ovarian follicles (235). This could explain our observation that menopause occurs earlier in famine exposed women compared to unexposed women. And this observation suggests that next to postnatal factors as smoking, there is room for prenatal factors in the understanding of the female reproductive ageing process.

The reported menopausal ages are well within the normal range, 40 to 60 years, with a median age of 51 years (11;174). No differences were observed between exposed and unexposed women in the incidence of premature (before 40 years) or early (between 40-45 years) menopause. However, the observation that menopause occurs earlier in famine exposed women compared to unexposed women, does support the theory that prenatal factors can influence reproductive lifespan in later life. The exact knowledge of the process through which maternal undernutrition affects reproductive ageing in the offspring remains very limited and justifies further studies on this subject.

In conclusion, we did not find clinical evidence that prenatal famine exposure affects a range of measures of reproductive performance in males or females. However, evidence for an earlier menopause in women exposed to famine in utero was obtained, suggesting that environmental circumstances early in life might influence the pattern of endowment or the rate of decline of the ovarian follicle pool.



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Age at menopause in women with type 1 diabetes mellitus – the OVADIA study

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ABSTRACT

Objective

We examined whether women with type 1 diabetes mellitus express a more advanced pattern of ovarian ageing, resulting in an early age at natural menopause.

Research design and methods

A cross-sectional study was performed in 140 postmenopausal women with type 1 diabetes included in the OVarian Ageing in type 1 DIAbetes mellitus (OVADIA) study. 5426 Dutch naturally postmenopausal women without diabetes from the Prospect-Epic cohort served as reference population. Study participants filled out a standardized questionnaire including report of their age at last menstrual period. Differences in menopausal age were analysed using linear regression analyses, with adjustment for possible confounders.

Results

Mean age at natural menopause was 49.8 ± 4.7 years in women with type 1 diabetes and 49.8 ± 4.1 in women without diabetes. Linear regression analyses showed that type 1 diabetes was not associated with an earlier menopause compared to the reference group without diabetes, after adjustment for age, smoking history and parity (difference in age at menopause between women with type 1 diabetes and reference group 0.34 years, 95% CI -0.34, 1.01).

Conclusions

In the present study no clear evidence was provided that type 1 diabetes is associated with the occurrence of early menopause.

INTRODUCTION

Menopause is defined as the permanent cessation of menstrual cyclicity resulting from follicle pool depletion in the ovaries. This clinical diagnosis is made after twelve consecutive months of amenorrhea and occurs at a mean age of 51 years, although age at menopause varies widely between 40 and 60 years (149).

Timing of menopause has substantial implications for women's health and fertility. Early menopause has been associated, among others, with a moderately increased risk of cardiovascular disease (57-61) and type 2 diabetes (236). However, the prevailing theory that oestrogen depletion causes this advanced vascular ageing has been challenged based on observations that oestrogen replacement therapy does not prevent and possibly even enhances the development of cardiovascular disease in postmenopausal women (73). These findings have led to the reverse hypothesis that it is not the lack of oestrogen that causes cardiovascular damage, but that vascular compromise itself is a driving factor in the pathogenesis of ovarian ageing (74).

Women with type 1 diabetes are at risk of premature morbidity and mortality from cardiovascular disease (178-181). We therefore hypothesized that premature vascular ageing due to type 1 diabetes precedes ovarian ageing, resulting in an early menopause. One study suggested that women with type 1 diabetes are at risk to experience early depletion of the ovarian follicle pool, resulting in menopause at a younger age compared to women without diabetes (182), although this observation was not supported by later reports (183;237).

Women with type 1 diabetes are reported to have a delayed age at menarche (238) and are at higher risk for menstrual irregularities (239;240) compared to women without diabetes of similar age. Combined with an earlier age at menopause, these women may be subjected to a 6-year reduction in reproductive years (182).

As all previous studies have important methodological shortcomings, it is urgently needed to confirm a possible early decay in ovarian reserve in women with type 1 diabetes.

In the present study we aimed to confirm the earlier reported difference in age at natural menopause in women with type 1 diabetes compared to women without diabetes.

RESEARCH DESIGN AND METHODS

Study design

A cross-sectional study was performed in 140 women with type 1 diabetes included in the OVarian Ageing in type 1 DIAbetes mellitus (OVADIA) study. The Prospect-Epic cohort of Dutch postmenopausal women was used as reference population of women without diabetes. This study has been registered at http://www.clinicaltrials.gov under NCT01665716.

Study population

Patients with type 1 diabetes were recruited from the department of Internal Medicine of several Dutch hospitals spread around the country. Invitation letters were sent until 140 women with type 1 diabetes agreed to participate. Also, patients were recruited through the Dutch Diabetes Society (advertisements in newsletter and on website). Caucasian women of at least 51 years old with type 1 diabetes who had experienced a natural menopause were considered eligible for the study. Exclusion criteria were an induced menopause, i.e. hysterectomy, ovarian surgery, chemo- or pelvic radiation therapy, endometrial ablation or perimenopausal use of hormones. A total of 590 potentially eligible women were identified. These women were sent a letter of invitation explaining the nature of the study. In total, 323 women (55%) responded of whom 80% was willing to consider participation. After sending these women the detailed study information, they were screened for in- and exclusion criteria by telephone. Overall, 45% did not fulfil the inclusion criteria, i.e. 16% had an induced menopause, 19% had an unknown menopausal status due to perimenopausal exogenous hormone use or could not recall age at menopause and 10% had not yet experienced the full twelve consecutive months of amenorrhea. As depicted in **Figure 1**, this resulted in 140 women who were sent the study questionnaire and consent forms.

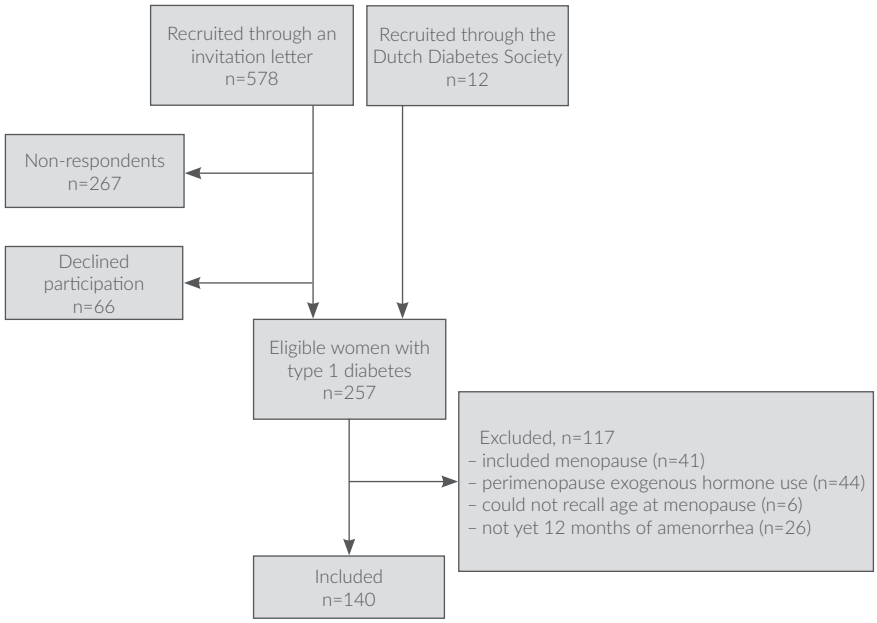

Figure 1. Study flowchart of women with type 1 diabetes.

Type 1 diabetes was defined according to the clinical criteria applied in that time. Approval was obtained from the Institutional Review Board of the University Medical Center Utrecht (UMCU) and all participants provided written informed consent prior to inclusion.

As a reference group of women without diabetes, the Prospect-Epic cohort was used. The design and rationale of this study has been described previously (241). In brief, the cohort comprises 17,357 Caucasian women living in the Netherlands, aged 49 – 70 years. Women were invited to participate in the study through the national breast cancer screening between 1993 and 1997. At enrolment, all participants underwent a physical examination and filled out detailed questionnaires about dietary, reproductive, and medical history. To ensure comparability with the diabetes group, for the current study we selected women of at least 51 years who experienced natural menopause. Excluded were 67% of the women who were premenopausal (n= 3497), had a surgical menopause (n= 4449), used hormones during the menopausal transition (n=2161), with an unknown menopausal status or age (n=1194), with diabetes mellitus (n=343) or who were younger than 51 years at inclusion (n=287), resulting in a total of 5426 women available for analysis.

Study parameters

Data on health issues, such as medication use and smoking history, and reproductive history were collected by a questionnaire. Women were asked about their age at menarche and subsequent menstrual cycle pattern, i.e. the time it took until the menstrual cycle became regular. Furthermore they reported the number of live born children, use of hormonal contraceptives and age at last menstrual period.
The primary outcome measure was age at natural menopause, defined according to the World Health Organization as amenorrhoea for at least 12 consecutive months without other obvious reasons.

Statistical analysis

Descriptive parameters and population characteristics were reported for women with and without diabetes as means ± SD and categorical data were expressed as percentages.
The distribution of age at menopause was depicted graphically in a boxplot for both women with and without diabetes. Linear regression analyses were used to compare mean age at menopause, with 95% confidence intervals (CI) between women with and without diabetes. Adjustments were made for age at questionnaire, smoking history (ever/never smoking) and nulliparity. To correctly model a possibly non-linear relationship between age at questionnaire and age at menopause, a restricted cubic spline with three knots was used.

In addition, a sensitivity analysis was performed restricting to women with diabetes diagnosed before age 35 year (n=96) to ensure sufficient duration of disease. The second sensitivity analysis was restricted to women who developed a normal regular menstrual cycle within 5 years after menarche (n=109 with diabetes and n=4469 without diabetes), in order to exclude women with possible polycystic ovary syndrome (PCOS) (53) who are suggested to reach menopause at a later age than women with regular menstrual cycles (242).
A difference in menopausal age of 1 year (SD ± 4.1 years) between women with and without type 1 diabetes was considered a relevant finding. To ensure the statistical significance of such a difference with a reference group of 5800 women, a total of 137 women with type 1 diabetes was needed, using an $\alpha<0.05$ and $\beta= 0.80$.
Data were analysed using SPSS for Windows, version 20.0 (SPSS INC., Chicago, IL) and R version 2.10.0 (http://www.r-project.org).

RESULTS

Population characteristics of the women with and without diabetes are presented in **Table 1**. Both groups were comparable in terms of age at questionnaire (59.9 years vs. 60.0 years). In addition, parity and smoking behaviour were comparable across both groups.

Table 1. Population characteristics of women with and without type 1 diabetes.

	With diabetes n=140	Without diabetes n=5426
Age at questionnaire in years	59.9 ± 6.9	60.0 ± 5.1
Age menarche in years	13.8 ± 3.6	13.5 ± 1.6
Nulliparity, n (%)	25 (18%)	789 (15%)
Ever smoker, n (%)	85 (61%)	3803 (52%)
Age diagnosis diabetes mellitus in years	28.0 ± 14.2	-

Data are presented in mean ± SD or number (%).

Age at menopause

Mean age at natural menopause was 49.8 ± 4.7 years in women with type 1 diabetes and 49.8 ± 4.1 in women without diabetes, as depicted in **Figure 2**. After adjustment for age, smoking history and parity, women with type 1 diabetes had a non-significant 0.34 years (95% CI -0.34, 1.01) higher age at menopause than women without diabetes (**Table 2**).

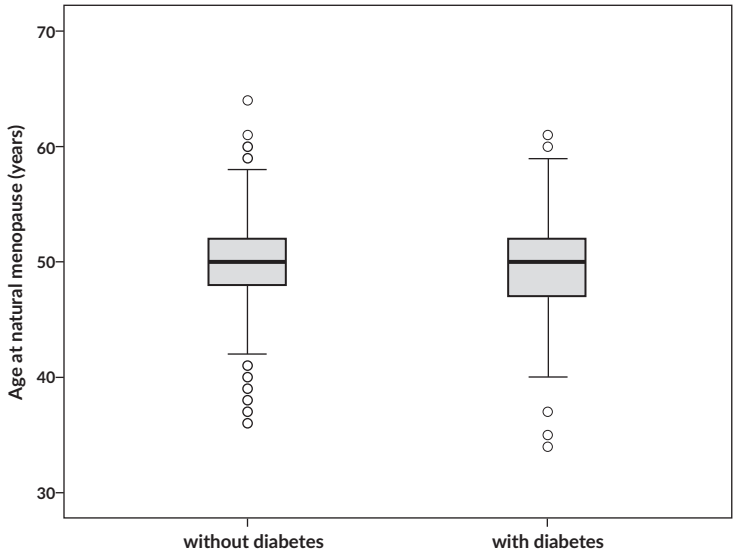

Figure 2. Boxplot distribution of age at natural menopause for women with and without type 1 diabetes.

Table 2. Linear regression analysis comparing age at menopause in women with and without diabetes.

	Difference in age at menopause (years)	95% CI
With diabetes vs. without diabetes		
unadjusted	-0.02	-0.72, 0.67
adjusted for age	0.27	-0.41, 0.95
adjusted for age and smoking	0.31	-0.37, 0.99
adjusted for age, smoking and nulliparity	0.34	-0.34, 1.01

Regression coefficient with 95% confidence interval (CI).

Sensitivity analyses exluding all women with diabetes diagnosed after 35 years did not materially change these results (difference in age at menopaue 0.41 years 95% CI -0.39, 1.24). The same was true for the sensitivity analyses excluding those women with a possible PCOS. Women with type 1 diabetes had a non-significant 0.05 years (95% CI -0.80, 0.71) earlier menopause than women without diabetes, after adjustment for confounders (**Table 3**).

Table 3. Sensitivity analyses: linear regression analysis comparing age at menopause.

Restricted to women with diabetes diagnosed before 35 years (n=96) and without diabetes (n=5426)		
With diabetes vs. without diabetes	Difference in age at menopause (years)	95% CI
unadjusted	0.08	-0.75, 0.91
adjusted for age	0.35	-0.47, 1.16
adjusted for age and smoking	0.40	-0.41, 1.22
adjusted for age, smoking and nulliparity	0.42	-0.39, 1.24
Restricted to women without possible PCOS (n=109 with diabetes, n=4469 without diabetes)		
With diabetes vs. without diabetes	Difference in age at menopause (years)	95% CI
unadjusted	-0.44	-1.21, 0.33
adjusted for age	-0.11	-0.87, 0.65
adjusted for age and smoking	-0.07	-0.82, 0.69
adjusted for age, smoking and nulliparity	-0.05	-0.80, 0.71

Regression coefficient with 95% confidence interval (CI).

DISCUSSION

The present study does not confirm an earlier observation that women with type 1 diabetes experience menopause earlier than women without diabetes. This implies that the current hypothesis that the process of ovarian ageing is accelerated in women with type 1 diabetes may have to be rejected.

The current study results are in line with a previous study, which also could not detect an early menopause in women with type 1 diabetes (183). Sjöberg et al. studied a large population based cohort of Finnish women with childhood-onset type 1 diabetes who were at least 40 years old when questioned about their age at last menstrual period and reported a median age at menopause of 52.5 years (183). Factors associated with an earlier menopause were the presence of end-stage renal disease and proliferative retinopathy. However, median age at menopause in the women with type 1 diabetes was compared to a population estimate, thereby lacking the adjustment for possible confounders in the comparison of menopausal age.

The Diabetes Control and Complication Trial/ Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) study is a multicentre, randomised controlled clinical trial, designed to compare the impact of intensive diabetes treatment on the development and progression of early microvascular complications in type 1

diabetes (237). In this study 240 women had reached natural menopause and mean age at menopause did not differ between the intensive treatment and conventional treatment groups. In secondary analyses, glycaemic control and microvascular complications were not related to age at menopause in this study, but increase in the required insulin dosage decreased risk of natural menopause (hazard ratio 0.91, 95% CI 0.75-0.98).

These results are all in contrast with one previous large cohort study, the Familial Autoimmune and Diabetes (FAD) study, reporting that women with type 1 diabetes had a two times higher risk to experience menopause compared to women without diabetes (182). In this study, adults with type 1 diabetes and controls, both related and unrelated, were included in 1981 to study mortality in type 1 diabetes. During follow-up rounds in 1990 and 1993 data on menstrual cycle characteristics and age at menopause were assessed by questionnaires.

Possible explanations for these conflicting results in comparison to our study may lie in the age at which menopause was assessed. In the FAD study, women had an average age of 42 years and therefore only a small proportion had reached menopause (~ 10%) (182). As a result, the distribution of menopausal age becomes left-skewed as only those who have experienced an early menopause are represented. Moreover, to accelerate the ovarian ageing process, in terms of an earlier age at menopause, prolonged poor glycaemic control and subsequent effects on vascular health have been proposed as the possible mechanism behind the link between ovarian ageing and type 1 diabetes. The improved glycaemic control during the last decades may have prevented vascular damage to occur to the extent that it would affect organ function. Indeed, microvascular complications have been suggested as the cause of the advanced ovarian ageing process in women with type 1 diabetes (183). Intensive glycaemic control, which is currently considered standard therapy, reduces both micro- and macrovascular complications in type 1 diabetes (243;244). As a result, an accelerating effect of type 1 diabetes on age at menopause onset may currently be prevented by treatment, and therefore not be observed anymore. However, the study of Kim et al. did not find an earlier menopause in women on conventional treatment as compared to intensive treatment (237).

The strength of this study lies in the direct comparison of the group with type 1 diabetes with a population based reference cohort without diabetes. Both groups consist of only postmenopausal women, which could lead to selection bias due to the exclusion of women who have not yet reached menopause. As the age at assessment of menopausal age is on average 60 years and comparable between the women with and without diabetes, we do not feel this could have led to an underestimation of the menopausal distribution as presented.

A limitation of the present study is the lack of information with regard to microvascular complications, which would have enabled us to investigate the possible association

between vascular health and menopausal age.
Nevertheless, the present findings are reassuring for reproductive health prospects in women with type 1 diabetes.
In conclusion, the present study demonstrates that women with type 1 diabetes are not at risk to experience an earlier menopause than normal.

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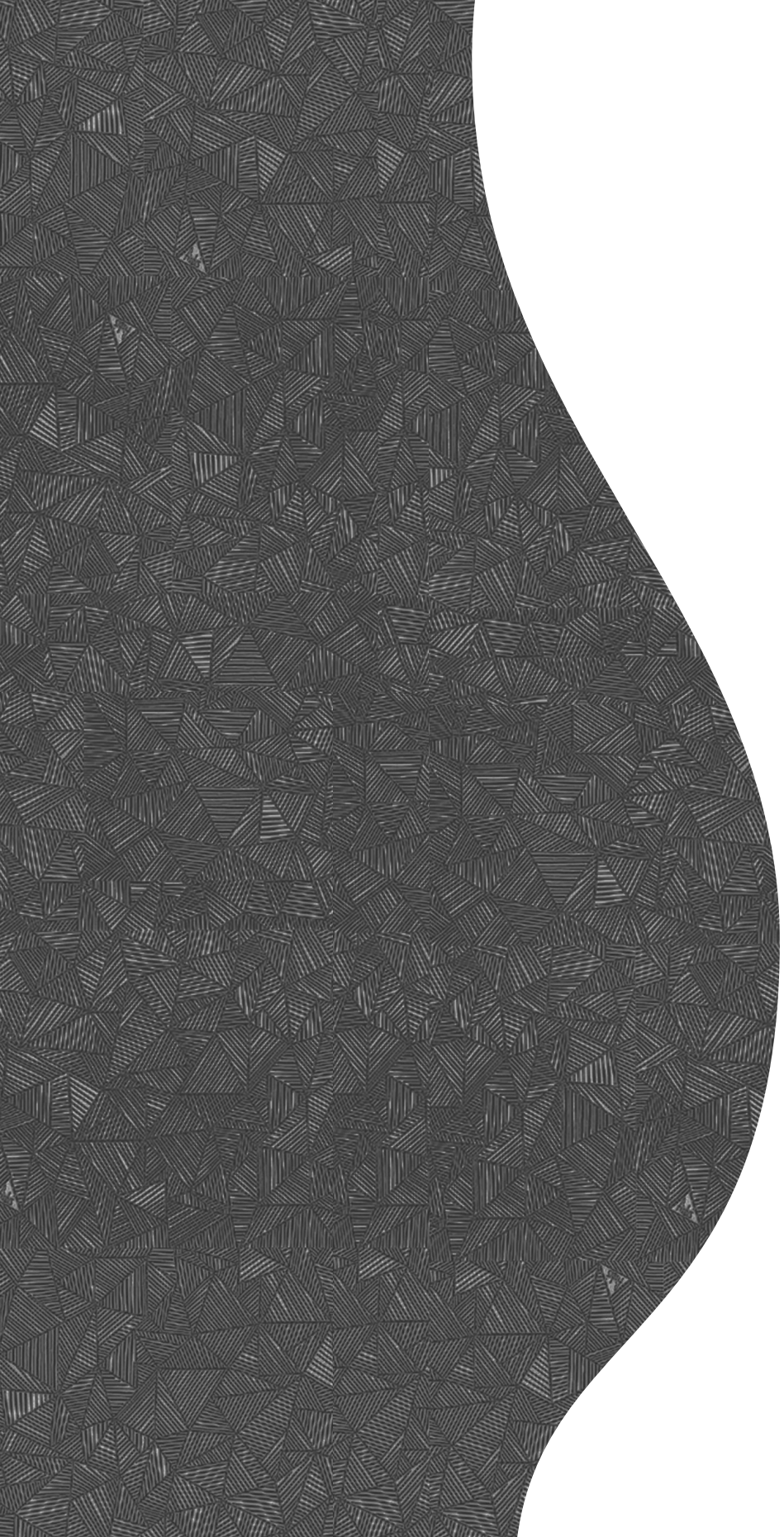

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General discussion

GENERAL DISCUSSION

Female reproductive ageing reflects the gradual decline of both quantity and quality of the primordial follicle pool, with menopause marking the end of this reproductive process.

Timing of menopause has great implications for female health and fertility. The normal process of ovarian ageing varies considerably among women. Insights in the cause and consequence of advanced ovarian ageing could have a great impact on the management of fertility and general health conditions such as colorectal cancer, osteoporosis and subfertility.

In this thesis we examined the relationship between ovarian ageing, fertility and vascular health. In the first part of the thesis we aimed to identify patient characteristics and ovarian reserve markers that forecast pregnancy in women with signs of advanced ovarian ageing. We systematically studied the literature about pregnancy prospects in poor responders in assisted reproductive techniques (ART), such as in vitro fertilisation (IVF). Moreover, it was investigated whether ovarian reserve markers could predict the occurrence of a live birth in subfertile women with advanced ovarian ageing, as expressed by elevated basal FSH levels.

In the second part of the thesis, we investigated whether unfavourable cardiovascular health is associated with advanced ovarian ageing. Three distinct groups of women were used as a model of compromised vascular health: women with a history of preeclampsia, women with type 1 diabetes and women with exposure to prenatal famine.

In this chapter, we discuss the meaning of the findings in this thesis, recommendations for future research are proposed and finally, we present the possible implications for clinical practice.

ADVANCED OVARIAN AGEING – FOCUS ON FERTILITY

Female reproductive lifespan is marked by four reproductive stages, which are believed to occur at fixed intervals. The variation in age at menopause is large and ranges between 40 and 60 years. Similarly, the variation in age at sterility is large, with some women already expressing signs of decreased fertility in their early twenties, while other women may remain fertile into their forties. The timing of the end of female fertility and the onset of menopause, approximately 20 years later, is the result of a complex interplay of follicle quantity loss together with decreasing oocyte quality. Although female age is generally considered a marker of quality, observations that biologic ageing does not always coincide with chronological ageing have taught that these processes are modulated by more than age alone.

Identifying women with advanced ovarian ageing is important as these women may be at risk of early infertility and may need counselling with regard to their fertility

prospects and preventive strategies such as oocyte vitrification. Current markers for ovarian reserve mainly express the quantity of follicles still present in the ovaries, and the significance of reduced quantity for the oocyte quality is a matter of continued debate.

The effect of limited quantity on remaining quality

It has been suggested that a direct relationship exists between follicle quantity and oocyte quality. This means that with a lower number of follicles present in the ovaries, the quality of the oocytes held within these follicles will become jeopardised. As advancing age induces a decrease in both the numbers and quality of remaining oocytes, the direct effect of quantity on quality is difficult to assess.

From animal studies it has been demonstrated that after the removal of one ovary in newborn mice, the oocytes in the remaining ovary showed signs of diminished quality. Besides an early occurrence of cycle irregularity, an increased rate of aneuploidy in the offspring was reported (245). This finding implicates that next to chronological age, quantity may directly affect oocyte quality. In humans, a diminished ovarian reserve, as expressed by a status after ovarian surgery or elevated basal FSH levels, has also been associated with an increased rate of chromosomal abnormalities (107;246-248) and interestingly, women with a history of trisomy 21 offspring at a young age expressed subtle signs of advanced ovarian ageing (249;250).

In line this, in chapter 3 it was demonstrated that in women with elevated FSH levels, AMH (a proven quantitative measure of ovarian reserve) was predictive of ongoing pregnancy (a proxy of oocyte quality) irrespective of female age.

Assisted reproduction technology, specifically IVF, offers a model to study the quantity-quality relation. Several studies in ART populations have revealed that AMH, as a marker for the antral follicle count, is a prognosticator of live birth and cumulative pregnancy rates independent of female age (121;142;251-254). However, any added value of AMH on top of female age in women conceiving naturally has not been clearly demonstrated (255;256), indicating that in such circumstances, other determinants of pregnancy have a much higher impact, such as semen quality (257).

Further evidence for a parallel decline in both oocyte quantity and quality comes from a study group observing that next to advanced female age, a poor response in IVF is independently associated with a higher risk of clinical miscarriage (258).

Although a direct relationship between quantitative and qualitative aspects of the oocyte pool sounds rather logical, several studies from IVF populations suggest otherwise. An array of studies have demonstrated that AFC and AMH, both proxies of follicle quantity, fail in the prediction of pregnancy and that female age remains the best prognosticator of the occurrence of pregnancy (131;134;139;259).

In addition, in chapter 2 we have demonstrated that young women with advanced ovarian ageing, as expressed by a poor response in IVF, have better pregnancy

prospects than older women with a similar response, thus suggesting that in women with a low quantity the quality of oocytes is clearly influenced by age. These results support the notion that there is not a one-to-one relationship between quantity and quality, but that this relation is highly modulated by female age. This is also illustrated in the study by Sunkara et al., who demonstrated that with increasing age, live birth rates following IVF decrease, but that within female age categories, the number of oocytes is predictive of live birth (109). Indeed, in embryology studies it has been observed that with increasing age the euploidy rate of both day 3 and day 5 embryo's decreases. Within age categories, however, the euploidy rate remained similar and irrespective of the cohort size, whereas the chance of having at least one normal embryo was clearly reduced with decreasing embryo numbers (18). These findings may explain the way quantity affects quality, as a limited number of embryos with a fixed proportion of euploidy, will lead to an increasing chance of having no normal embryos at all and thereby negatively affecting the chances of pregnancy.

The hypothesis that follicle quantity affects oocyte quality has become known as the limited oocyte pool hypothesis (260). During each menstrual cycle a small cohort of follicles develops of which, usually, one grows into dominance. With advancing age the number of developing follicles as well as the total follicle pool diminishes. The limited pool hypothesis captures the concept that in young women follicles with oocytes of suboptimal quality are not likely to develop into dominant follicles due to the presence of sufficient follicle-oocyte complexes of good quality. Older women with a smaller follicle pool, on the other hand, have a higher probability that follicles containing poor quality oocytes will develop further into dominance, merely due to the fact that the presence of normal oocytes has become severely diminished. As a result, an increased tendency of having chromosomal abnormalities will affect both pregnancy and miscarriage rates.

This means that next to female age, the quantity of the primordial follicle pool acts upon the remaining quality. Whether the driver in the development of aneuploidy conceptions is the numbers or the age remains under debate, as well as the mechanism by which these increased meiotic errors in the oocyte occur. Possible explanations for age-related quality demise include changes in the oocyte or its environment, such as a reduction in meiotic proteins or meiotic checkpoints as well as genetic alterations resulting in mitochondrial deletions (Warburton 2005). The way through which quantity affects quality may be explained by factors that implicate ovarian changes such as vascularisation (261), elevated basal FSH levels that induce meiotic errors (249) or through impaired signalling pathways between the oocyte and its surrounding somatic cells (262). Therefore, it seems that besides the absolute decline in the primordial follicle pool, a limited pool may also have a biological effect.

Clinical implications in women with advanced ovarian ageing

Poor responders in IVF are not a homogeneous group with respect to their pregnancy prospects. Female age and oocyte yield are both important prognosticators of a subsequent pregnancy. Therefore, in young poor responders, continuation of IVF treatment should not be brought to discussion, as based on a poor response alone it cannot be concluded that these women have pregnancy prospects close to zero. Especially in women with an unexpected poor response, based on ovarian reserve tests prior to IVF, it has been demonstrated that the pregnancy outcomes are superior to women with an expected poor response (39). In contrast, older women with a poor response, especially with low AMH levels, may be advised to refrain from treatment, as prospects for pregnancy may be considered poor.

The use of FSH as a marker for advanced ovarian ageing appears to be limited. Women with elevated basal FSH levels are also notably heterogeneous and not infertile by definition. Indeed, a considerable proportion of women with elevated basal FSH levels became pregnant spontaneously during the three years of follow-up (Chapter 3), supporting FSH being a poor predictor of limited quantitative ovarian reserve. In addition, a large variation in AMH was observed in women with elevated FSH levels. AMH expresses the number of antral follicles present that will be the potential source for an oocyte leading to a live birth. Therefore, AMH can be used to identify those women with elevated basal FSH levels that indeed have a very limited prognosis, and in whom the basal FSH level elevation correctly classified the patient as having an advanced ovarian age. According to chapter 3, an elevated basal FSH level together with an AMH level below 1 ng/ml indicated advanced ovarian ageing with reduced pregnancy rates. However, no added effect of female age was detected in the prediction of live birth.

AMH, as reproductive biomarker, is increasingly used in clinical practice to predict response to, and outcome after, controlled ovarian hyperstimulation in IVF treatment. Therefore, AMH is a promising tool for the identification of (poor) responders as well as those women with elevated FSH levels with still favourable pregnancy prospects. The real value of individualised stimulation dosing, however, still needs to be determined (263;264). In addition, the use of individual AMH results in clinical practice should be handled with caution. The stability and reproducibility of the current assay systems have been questioned recently (137). AMH may be influenced by storage temperature and complement interference and thereby may present a distorted reflection of the current ovarian reserve. Therefore, until automated assay system and an international laboratory standard become available, caution should be exercised with the regard to the interpretation of individual AMH levels (265).

Part one of this thesis confirms that there is a complex interplay between quantity and quality as was demonstrated in chapter 2 and 3. Future research will help us unravel the mechanism behind the individual variation of ovarian ageing. The assessment of a direct relationship between quantity and quality, however, is rather difficult to investigate. In animal models, this relation could be further elucidated by an interventional design as previously performed by Brook et al., who demonstrated that the removal of one ovary early in life results in an increased probability of trisomic pregnancy and an earlier onset of cycle irregularity (245). Proof for this concept in humans may be found in studies investigating whether expressions of limited quantity (poor responders in IVF, ovarian surgery, primary ovarian insufficiency) are associated to proxy's for limited quality (trisomic pregnancy, miscarriage) independent of female age (107).
Subsequently, challenges for future research may lie in increasing the oocyte yield in poor responders to ensure a higher probability that at least one normal embryo is present, which may be accomplished by using androgen suppletion (266), corticosteroid treatment (267) or by increasing the vascularization of the ovary (268).

ADVANCED OVARIAN AGEING – FOCUS ON VASCULAR HEALTH

It has often been argued that the hypo-oestrogenic state that accompanies menopause is responsible for an increase in cardiovascular disease. Interestingly, despite the fact that evidence for such a causative link is scanty, this has had clinical consequences to the extent that some physicians prescribe hormone replacement therapy to women with early menopause or primary ovarian insufficiency. Firstly, of all the studies upon which this theory is based are all very heterogeneous in size, design, type of menopause (artificial or natural), and study outcome. Secondly, they differ in their adjustment for possible confounders, and most importantly, they all showed only a very small inverse relationship between menopausal age and cardiovascular disease or mortality rates (57-61). We challenged this paradigm by hypothesising that variation in vascular health is causally related to variation in the ovarian ageing process, as expressed by the variation in the age at which women reach natural menopause. Such a reverse association may be plausible if we consider that irreversible vascular damage leads to compromised vascularisation of the ovarian tissue with increased loss or limited survival of primordial follicles as a result. This is supported by the inverse relationship between human ovarian microvascular density and primordial follicle apoptosis (269). Furthermore, accelerated ovarian ageing after chemotherapy was accompanied by vascular damage after doxorubicin treatment in both humans and mice (270). In addition, surgeries affecting the vascular supply of the reproductive organs, such as a salpingectomy, hysterectomy and uterine artery embolization, were associated with a reduced ovarian reserve

status (191;192). Studies into the quantification of unfavourable vascular function further question the direction of causality regarding the occurrence of menopause. Moreover, menopause with all its endocrine changes at the ovarian level does not promote the development of arterial stiffness (271;272).

In this thesis, the hypothesis that vascular ageing precedes and drives ovarian ageing is studied through models which can only portray associations between factors. A well-known limitation of cross-sectional studies, such as the ones used in chapters 4, 5 & 7, is, that although associations may be found, they will not provide information on the direction of the effect nor on the possible causality. Considering that it is rather difficult to design studies suitable for proving a causal link between vascular and ovarian ageing, it should be first investigated whether any conclusive associations exist prior to determining the necessity for interventional studies that can detect causality.

To investigate the relationship between vascular factors and ovarian reserve status, expressed by AMH or age at menopause, three phenotypes of compromised vascular health were studied; preeclampsia, prenatal famine exposure and type 1 diabetes.

Preeclampsia, and particularly early-onset preeclampsia is considered to be an early manifestation of impaired vascular health, and is associated with an increased risk for cardiovascular morbidity and events in the decades after pregnancy. In chapter 4 it was demonstrated that women with a history of preeclampsia have significantly lower AMH levels in comparison to women with normotensive pregnancies. In addition, an inverse association was found between AMH and the presence of hypertension as well as C-Reactive Protein (CRP) levels (two predictors of future cardiovascular disease). Together, these results support the hypothesis that vascular ageing and ovarian ageing are interrelated.

In line with this, a decreased ovarian reserve (expressed by higher stimulation doses and lower numbers of oocytes) was associated with an increased risk of developing preeclampsia in a subsequent pregnancy (152). However, an attempt to demonstrate that women with a poor response in IVF are more likely to develop vascular pregnancy complications in the subsequent pregnancy compared to women with a normal response, resulted in a non-significant trend (150). Moreover, the number of cardiovascular risk factors has been found to be higher among women with a reduced ovarian reserve, indicated by serum AMH levels. The association between a favourable ovarian reserve status and healthier cardio-metabolic profile was, however, reduced after controlling for body mass index (170). Further evidence comes from animal studies where atherosclerotic plaque sizes were shown to be inversely correlated with ovarian reserve status in premenopausal primates (75).

The hypothesis of vascular ageing preceding ovarian ageing was further studied by using type 1 diabetes as a model for vascular compromise, as diabetes is hallmarked by premature vascular complications (178-181). However, as described in chapter

5, no conclusive evidence was found for an advanced ovarian ageing process in women with type 1 diabetes. Out of all measured vascular factors, only systolic blood pressure and HDL-cholesterol were associated with markers of ovarian reserve. In addition, in the study described in chapter 7 we demonstrated that women with type 1 diabetes do not reach menopause earlier than women without diabetes. Together with the observation that ovarian reserve status was not diminished in type 1 diabetes (Chapter 5), we concluded that type 1 diabetes is not a determinant of an advanced ovarian ageing process. In view of the literature, only one study reported an early menopause in type 1 diabetes (182), whilst opposing results prevail (183;237).

Notably, the women with type 1 diabetes in our study were not as "vascularly compromised" as we had presumed according to their duration of diabetes. All measurements of vascular risk factors or vascular function tests were within the normal ranges. Due to the relatively young age of our study population, a longer duration of disease may have been necessary to cause vascular compromise to the extent in which it would affect ovarian reserve. In addition one may hypothesize that glycaemic control in these women, who were recruited from endocrinologists' clinics, may have been optimal. This in turn may have prevented vascularly mediated decline of ovarian reserve status, suggesting that meticulous diabetes care may prevent early vascular compromise to develop. In one study HbA1c levels were correlated with ovarian reserve in type 2 diabetes, however this could not be confirmed in our study (198).

The foetal origin of vascular disease has been previously described by Barker, indicating the importance of an adequate prenatal environment for the prevention of age-related diseases (80). Prenatal undernutrition has been associated with an increased risk of adult vascular disease, and therefore women with prenatal famine exposure serve as another model of possible early vascular compromise. In chapter 6 we provided evidence that intra-uterine factors may affect organ function at the ovarian level by demonstrating that prenatal famine exposure is associated with earlier occurrence of natural menopause. In addition, exposure to famine during early childhood has been linked to the occurrence of early menopause (83).

In summary, associations between vascular factors and ovarian reserve status were found in two out of three of the studied phenotypes, both direct in preeclampsia and indirect through prenatal factors, although with small effect sizes. The third phenotype, type 1 diabetes, was not related to advanced ovarian ageing. However, due to good glycaemic control and subsequent prevention of significant vascular compromise, the suitability of this model may be questioned.

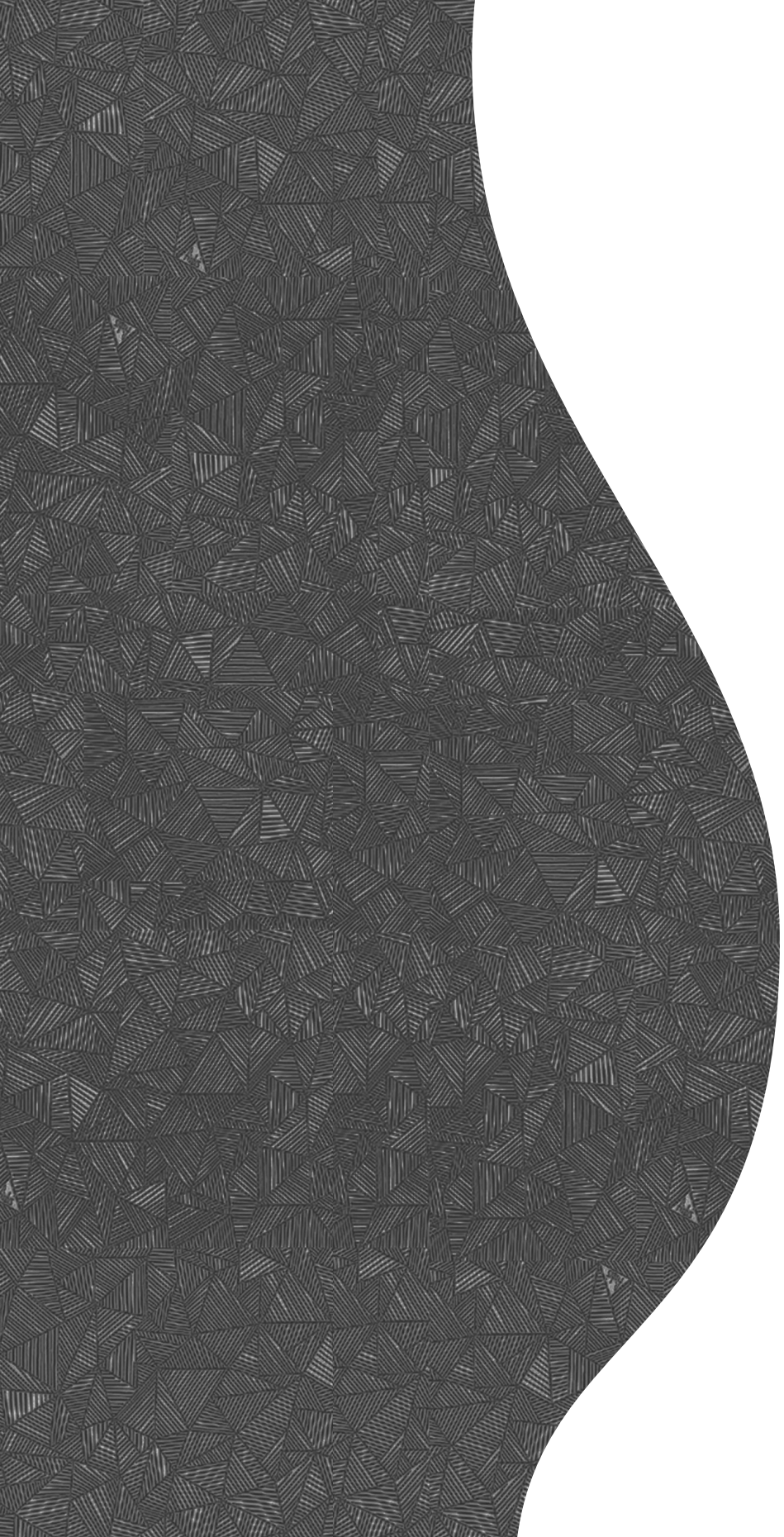
With respect to the literature, the association between vascular factors and ovarian reserve markers has not been clearly demonstrated. Previous studies have produced conflicting results with regard to the association of AMH with cardio-metabolic

markers such as insulin resistance, BMI, lipid profile and CRP levels (168;169;273-281). However, these results came from either small studies or with selected populations of women with PCOS or adolescent girls, and therefore not representing normal female reproductive ageing nor an extreme phenotype of vascular demise.
Support for a role for vascular factors in ovarian ageing, instead of the other way around, came from the observation that the changes in lipid profile, associated with the menopausal transition, already occur before the onset of irregular menses (282). In the adult male, AMH can also be detected in serum, although no function has been attributed to it yet. The role for AMH as a regulator of the cardiovascular system has recently been noted due to the observation that high AMH levels are associated with the absence of cardiovascular disease (283) as well as appear inversely related to aortic diameter on ultrasound (284). In females, this possible link between AMH as a hormonal factor and vascular health has become clear in the negative correlation between atherosclerotic plaque sizes and ovarian reserve status in premenopausal primates (75).
Finally, a recent study detected a link between diminished ovarian reserve and unfavourable cardiovascular health. In 100 women with an AFC below 5 or elevated FSH levels, markers of insulin resistance, CRP levels, triglyceride and HDL-cholesterol were independently associated with a diminished ovarian reserve compared to women with a normal ovarian reserve (285).
In summary, the scarce literature on the association between unfavourable vascular health and advanced ovarian ageing reveals no clear and consistent patterns, let alone any hint of a causal mechanism.

Future research proposals and concluding remarks

In part two of this thesis we were not able to elaborate on the role for vascular compromise as a causal factor in the ovarian ageing process. However, associations were detected between expressions of vascular compromise and reduced ovarian reserve status, although the effect sizes were small. Considering the challenges for future research, in humans the only way to study this association is to perform prospective cohort studies of sufficient sample size and in which markers of vascular health and ovarian reserve are collected from early adulthood onwards. However, this would only reconfirm associative relationships and not prove causality per se. Furthermore, for future studies we must carefully select the disease models in which ovarian ageing is studied. A possible explanation for the reason we could not find an association may lie in the relatively healthy vascular profile of our study models. For example early-onset chronic kidney disease (286) or inherited conditions characterised with premature atherosclerosis such as Pseudoxanthoma elasticum (287;288), may provide more suitable models for vascular compromise. The next step, after confirming an explicit association between vascular and ovarian ageing, would be the investigation of a causal relationship. Our understanding would also

benefit from an intervention studies to elucidate whether vascular compromise accelerates ovarian ageing. Obviously, investigating such a causal relationship will be a challenge, and will require animal models in which the effect of induced vascular damage on subsequent reproduction and ovarian ageing can be examined.
Another possibility is that advanced general ageing rather than vascular compromise dictates the ovarian ageing. This notion is supported by the association between a variant of the APO-E gene and longevity, atherosclerosis, but also with age at natural menopause (78;79). Moreover, a number of loci associated with age at menopause have shown to be located in gene regions involved in processes like DNA repair, cell death and immune system maintenance (67;68), indicating the possibility that general health and ageing patterns drive the ageing process in the ovaries.
In conclusion, the knowledge resulting from the studies in this thesis and the available evidence from the literature, the association between vascular health and ovarian ageing has not been firmly established. Future studies in humans or animals may unravel the contribution of vascular compromise to ovarian ageing. Until that time the question “Vascular ageing - cause or consequence?“ will remain unanswered.



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Summary / Samenvatting

SUMMARY

This thesis aims to identify patient characteristics and ovarian reserve markers that forecast pregnancy in women with advanced ovarian ageing and to investigate whether unfavourable cardiovascular health is associated with advanced ovarian ageing.

Chapter 1 addresses the concept of female reproductive ageing. The gradual decline in both oocyte quantity and quality will lead to four reproductive milestones, i.e. subfertility, sterility, menopausal transition and menopause. Female age has been identified as an important modulator of the reproductive ageing process, although the large inter-individual variation in normal ovarian ageing varies considerably, suggesting other factors may play a role.
Women with a poor response in IVF or with elevated basal FSH levels, expressions of limited oocyte quantity, are suggested to have poor pregnancy prospects (an expression of poor oocyte quality), although reasonable pregnancy prospects have been published. The possible inconsistency between the quantity and quality of the primordial follicle pool as observed in women with advanced ovarian ageing evokes the question whether Anti-Müllerian Hormone (AMH) or patient characteristics can identify those women who still have acceptable pregnancy prospects.
The large variation in normal ovarian ageing is also expressed by a considerable variation in age at normal menopause, covering a range from 40 to 60 years of age. Early menopause is associated with an increased risk of cardiovascular disease. However, recently it has been suggested that vascular health may act as cause instead of a consequence of menopause.
This thesis investigates whether markers of vascular ageing are associated with advanced ovarian ageing. Elucidation of these associations will create more understanding about the variation of the reproduction ageing process.

Chapter 2 is a systematic review of existing literature on patient characteristics and ovarian reserve tests in the prediction of pregnancy in poor responders. In total nineteen studies were included. Ten studies were found reporting on age and indicated that older poor responders have unfavourable pregnancy rates compared to younger poor responders. Four studies showed that the number of oocytes retrieved modulated the pregnancy prospects, with better prospects when more oocytes were retrieved. Five studies demonstrated that pregnancy rates in subsequent cycles were more favourable in women with an unexpected poor response and when at least 2 oocytes were retrieved. These results demonstrate that poor responders are not a homogeneous group of women with regards to pregnancy prospects.

Chapter 3 presents the results of a prospective observational cohort study which investigates the role of serum AMH as a predictor of live birth and reproductive stage in 96 subfertile women with elevated basal FSH levels. After a median follow-up of 3.3 years, AMH was associated with live birth up till an AMH level of 1 microgram/L. In addition, AMH was associated with the occurrence of menopausal transition or menopause during follow-up. FSH level, antral follicle count (AFC), female age, duration of infertility, pack years smoking or BMI appeared of limited value in the prediction of live birth.

Chapter 4 focusses on the hypothesis that an unfavourable vascular profile could act as a causative mechanism in early ovarian ageing by investigating whether women with a history of preeclampsia (PE) have a diminished ovarian reserve status, expressed by AMH levels, compared to women with normotensive pregnancies. In a retrospective cohort study of 336 women with a history of PE and 329 women after a normotensive pregnancy it was demonstrated that after an average follow-up of ten years, AMH levels in women with a history of PE were significantly lower. This translates into an advancement of reproductive age by approximately 1.5 years. As PE is considered a manifestation of impaired vascular health, these results support the hypothesis that compromised vascular health could act as a causative mechanism in early ovarian ageing.

Chapter 5 is a cross-sectional study which investigates whether type 1 diabetes is a determinant of advanced ovarian ageing. One hundred fifty premenopausal, regularly cycling women with type 1 diabetes were recruited to investigate the association between vascular health and ovarian reserve status, indexed by AMH and AFC. Systolic blood pressure was negatively correlated with both serum AMH and AFC. No associations were detected between lipids, C-reactive protein, HbA1c or vascular function tests reflecting endothelial function, vascular stiffness and subclinical atherosclerosis and serum AMH or AFC. In addition, AMH levels in women with type 1 diabetes were compared with a reference population of 177 healthy fertile women, revealing no difference in AMH after adjustment for confounders.

Chapter 6 explores the Barker hypothesis of foetal origin for adult disease hypothesis, by investigating whether acute prenatal famine exposure is associated with subsequent reproductive performance and age at menopause. A cohort of 1070 men and women born around the time of the Dutch Famine of 1944-1945 as well as their siblings was studied. Prenatal famine exposure was not associated with nulliparity, age at birth of first child, difficulties conceiving or pregnancy outcome in men and women. It was, however, demonstrated that natural menopause occurs earlier after prenatal famine exposure independent of birth weight.

Chapter 7 examines whether women with type 1 diabetes express a more advanced pattern of ovarian ageing, resulting in an early age at natural menopause. A cross-sectional study including 140 postmenopausal women with type 1 diabetes and 5426 postmenopausal women without diabetes was performed. Age at menopause appeared comparable between both groups, with an average age at menopause of 49 years, thereby concluding that no evidence was found that type 1 diabetes is associated with the occurrence of early menopause.

Chapter 8 discusses the results and conclusions that can be drawn from this thesis. In this thesis it was confirmed that there is a complex interplay between ovarian reserve quantity and quality. Female age plays an important role in the modulation of this interaction. Women with advanced ovarian ageing, as expressed by a poor response in IVF or elevated basal FSH levels, appear to be a heterogeneous group in which ovarian reserve tests, next to female age, may identify those women with reasonable pregnancy prospects. Future research will help us unravel the mechanisms behind the individual variation of ovarian ageing. Furthermore, no conclusive evidence was found for an association between vascular health and the ovarian ageing process, using women with a history of preeclampsia, women with type 1 diabetes and women with exposure to prenatal famine as a model of compromised vascular health. To elucidate whether vascular compromise accelerates ovarian ageing, future studies would benefit from animal studies with an interventional design.

SAMENVATTING

Het doel van dit proefschrift is het evalueren van de waarde van patiëntkarakteristieken en ovariële (eierstok) reserve testen in het voorspellen van de kans op een zwangerschap in vrouwen met een verminderde eierstokreserve. Daarnaast wordt onderzocht of een ongunstig vasculair (bloedvat) profiel geassocieerd is met een verminderde eierstokreserve.

In **hoofdstuk 1** wordt het concept van ovariële veroudering besproken. De geleidelijke afname van zowel de kwantiteit als de kwaliteit van de eicellen leidt tot vier reproductieve mijlpalen, namelijk verminderde vruchtbaarheid, onvruchtbaarheid, menopauzale transitie en menopauze (overgang). De leeftijd van de vrouw wordt gezien als een belangrijke factor in het reproductieve verouderingsproces. De variatie in eierstokveroudering tussen verschillende vrouwen verschilt echter dusdanig, dat naast leeftijd andere factoren een rol moeten spelen.
Van vrouwen met een slechte respons op ovariële stimulatie tijdens een IVF behandeling of met verhoogde basale FSH waarden, beide uitingen van een verminderde eierstokreserve, wordt gedacht dat zij verminderde zwangerschapskansen hebben (marker voor eicel kwaliteit). Echter er zijn ook studies gepubliceerd die redelijke zwangerschapskansen laten zien in deze groepen vrouwen. De mogelijke inconsistentie tussen het aantal en de kwaliteit van de eicellen in vrouwen met een verminderde eierstokreserve roept de vraag op of het Anti-Müllers hormoon (AMH), als marker voor eierstokreserve, of patiëntkarakteristieken, kunnen helpen bij het identificeren van juist die vrouwen die nog een goede prognose hebben.
Naast de grote variatie in het verloop van eierstokveroudering, is er ook een aanzienlijk verschil in menopauze leeftijd tussen vrouwen, met een spreiding van 40 tot 60 jaar. Een vroege menopauze wordt geassocieerd met een verhoogd risico op het ontwikkelen van hart- en vaatzieken. Er zijn echter ook aanwijzingen dat deze vaatziekten niet het gevolg, maar juist de oorzaak van de menopauze zijn. In dit proefschrift wordt onderzocht of markers van vaatveroudering geassocieerd zijn met een versneld ovarieel verouderingsproces. Het aantonen van dit verband draagt bij aan de kennis rondom de individuele variatie in reproductieve veroudering.

Hoofdstuk 2 is een systematische review van de bestaande literatuur over patiënt karakteristieken en ovariële reserve testen in het voorspellen van zwangerschap in vrouwen met een slechte response op ovariële stimulatie. In totaal werden 19 studies geselecteerd. Tien studies rapporteerden over leeftijd en lieten zien dat oudere vrouwen met een slechte respons slechtere zwangerschapskansen hadden dan jongere vrouwen met een slechte respons. Vier studies lieten zien dat het aantal verkregen eicellen bij een IVF behandeling bepalend is voor de kans op zwangerschap, waarbij de kansen toenemen naarmate er meer eicellen verkregen worden.

Vijf studies toonden dat in de opvolgende IVF cycli de zwangerschapskansen gunstiger waren wanneer er sprake was van een onverwachte slechte respons en als minstens twee eicellen verkregen werden. Deze resultaten laten zien dat vrouwen met een slechte respons op ovariële stimulatie geen homogene groep zijn met betrekking tot zwangerschapskansen.

In **Hoofdstuk 3** worden de resultaten van een prospectieve observationele cohort studie gepresenteerd waarin wordt onderzocht of serum AMH waarden de kans op een levendgeborene alsmede het reproductief stadium kunnen voorspellen in 96 vrouwen met verhoogde basale FSH waarden. Na een mediane vervolgduur van 3.3 jaar waren AMH waarden tot 1 microgram/L geassocieerd met de kans op een levendgeborene. Daarnaast was de hoogte van het AMH geassocieerd met het optreden van de menopauzale transitie of menopauze gedurende de studie. FSH waarden, antrale follikel telling (AFC), leeftijd van de vrouw, duur van de onvruchtbaarheid, aantal pakjaren roken of de body mass index bleken van weinig waarde in het voorspellen van een levendgeborene.

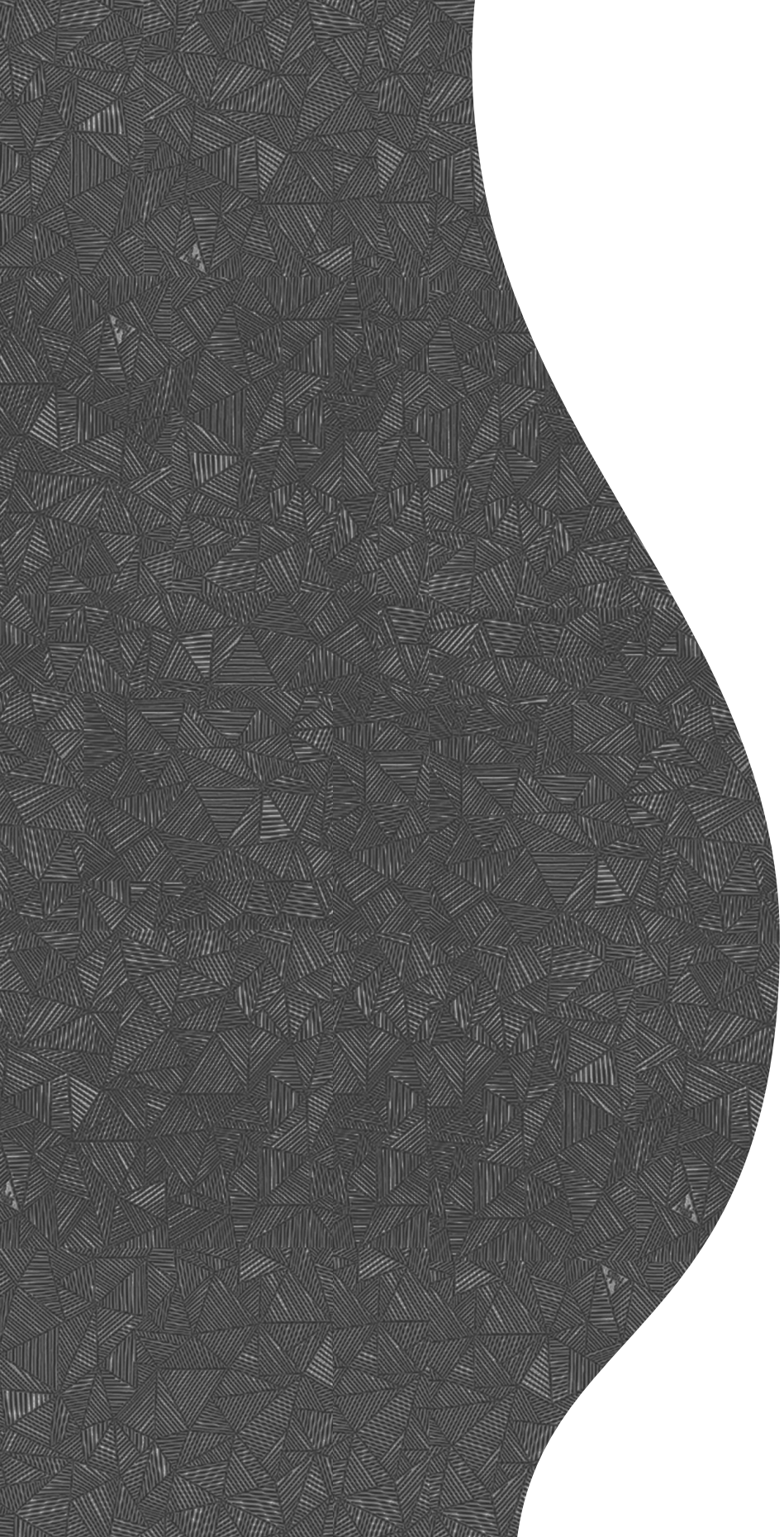
Hoofdstuk 4 focust zich op de hypothese dat een ongunstig vasculair profiel een causale rol speelt in vroege eierstokveroudering door te onderzoeken of vrouwen met een voorgeschiedenis van preeclampsie (PE, zwangerschapsvergiftiging) een verminderde eierstokreserve hebben, uitgedrukt in AMH waarden, vergeleken met vrouwen met een normotensieve zwangerschap in de voorgeschiedenis. In een retrospectieve cohort studie van 336 vrouwen met PE en 329 vrouwen met een normotensieve zwangerschap hebben we laten zien dat na een gemiddelde vervolgduur van tien jaar, vrouwen met PE significant lagere AMH waarden hebben. Dit zou omgerekend een versnelling in reproductieve veroudering van 1,5 jaar betekenen. Aangezien PE wordt beschouwd als een manifestatie van verminderde vaatgezondheid ondersteunen deze resultaten de hypothese dat een ongunstig vasculair profiel een causale rol kan spelen in vroege eierstokveroudering.

Hoofdstuk 5 is een cross-sectionele studie waarin wordt onderzocht of diabetes type 1 (suikerziekte) een determinant is van versnelde eierstokveroudering. Hiervoor zijn 150 premenopauzale vrouwen met diabetes type 1 en een regelmatige menstruatie cyclus gerekruteerd om te onderzoeken of er een associatie is tussen vaatgezondheid en eierstokreserve, uitgedrukt in AMH en AFC. Systolische bloeddruk bleek negatief gecorreleerd te zijn met zowel het AMH als de AFC. Er werden geen associaties gevonden tussen lipiden, CRP, HbA1c of vasculaire functietesten die een reflectie geven van endotheel functie, vaatstijfheid en subklinische aderverkalking aan de ene kant en serum AMH waarden en de AFC aan de andere kant. Bovendien bleken, na correctie voor confounders, AMH waarden in vrouwen met diabetes type 1 niet te verschillen van AMH waarden van 177 gezonde, vruchtbare vrouwen uit een referentie populatie.

Hoofdstuk 6 exploreert de Barker hypothese dat er een foetale oorsprong is voor ziekten op de volwassen leeftijd door te onderzoeken of acute prenatale ondervoeding geassocieerd is met reproductie en menopauze leeftijd. Een cohort van 1070 mannen en vrouwen samen met hun broers en zussen, geboren rondom de Nederlandse Hongerwinter van 1944-1945, werd bestudeerd. Prenatale ondervoeding bleek niet geassocieerd te zijn met nulliparitеit (kinderloosheid), leeftijd van de moeder bij de geboorte van het eerste kind, vruchtbaarheidsproblemen of zwangerschapscomplicaties. Echter, natuurlijke menopauze trad eerder op in vrouwen die blootgesteld waren aan prenatale ondervoeding, onafhankelijk van het geboortegewicht.

Hoofdstuk 7 onderzoekt of vrouwen met diabetes type 1 een versneld ovarieel verouderingsproces doormaken en derhalve op jongere leeftijd de menopauze bereiken. Een cross-sectionele studie werd uitgevoerd in 140 postmenopauzale vrouwen met diabetes en 5426 postmenopauzale vrouwen zonder diabetes. Beide groepen waren vergelijkbaar met betrekking tot menopauze leeftijd met een gemiddelde menopauze leeftijd van 49 jaar. Hieruit werd geconcludeerd dat er geen bewijs is dat diabetes type 1 geassocieerd is met het optreden van een vroege menopauze.

Hoofdstuk 8 bediscussieert de resultaten en conclusies die getrokken kunnen worden uit dit proefschrift. We hebben bevestigd dat er sprake is van een complex samenspel tussen de kwantiteit en de kwaliteit van de eierstokreserve. De leeftijd van de vrouw speelt een belangrijke rol in het moduleren van deze interactie. Vrouwen met een verminderde eierstokreserve, uitgedrukt in een slechte respons op ovariële stimulatie of verhoogde basale FSH waarden, blijken een heterogene groep te zijn waarbij ovariële reserve testen, naast leeftijd van de vrouw, mogelijk die vrouwen kunnen identificeren met nog redelijke zwangerschapskansen. Toekomstige studies kunnen ons helpen om het mechanisme achter de individuele variatie in eierstokveroudering te ontrafelen. Daarnaast kon in dit proefschrift geen overtuigend bewijs gevonden worden voor een associatie tussen vasculaire gezondheid en eierstokveroudering, met vrouwen met PE, diabetes type 1 of prenatale ondervoeding als model voor gecompromitteerde vaatgezondheid. Om de hypothese dat een ongunstig vaatprofiel voorafgaat aan eierstokveroudering verder te onderzoeken, kunnen toekomstige studies baat hebben bij dierstudies met een interventie design.



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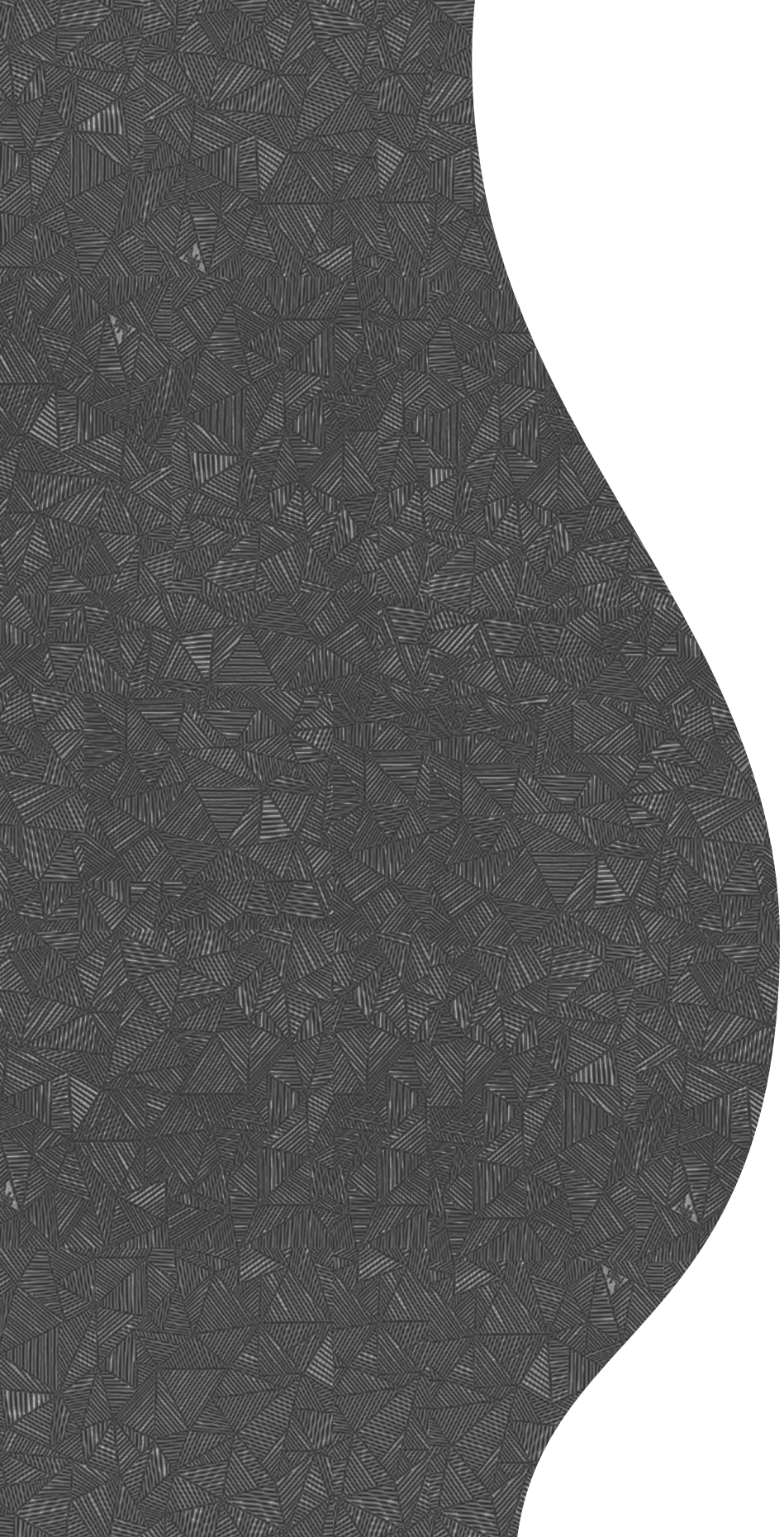

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About the Author
Dankwoord

Felicia Yarde was born in Eindhoven, the Netherlands on January first, 1985. She grew up in Eindhoven with her parents and brother and attended high school at the van Maerlant College from which she graduated in 2003.
With a predisposition for scientific research, Felicia first chose to do a year of Biomedical Sciences before deciding upon- and being accepted into- Medical School at the University Medical Center Utrecht. During medical school Felicia developed a fervent fascination for life abroad: she did a gynaecology internship in (Paarl) South Africa which seeded her interest in gynaecology. She further did an ophthalmology internship in (Kuala Lumpur) Malaysia, a tropical medicine internship in (Sumbawanga) Tanzania, and finally a research project in Southampton's DOHAB laboratory (about microvascular dysfunction in animals and humans).
In the fifth year of medical school Felicia did research with prof dr. C.H. van de Vaart about pelvic floor prolapse, and through this she came into contact with prof. dr. F.J.M. Broekmans. Her scientific research placement in year six resulted in an offer for a PhD position with prof. dr. F.J.M. Broekmans about the vascular factors of ovarian ageing at the department of reproductive medicine in the University Medical Center Utrecht. After graduating from medical school in 2011, she gladly accepted this offer. Since July 2014 Felicia has been working as a gynaecology resident at the St. Antonius Hospital Nieuwegein which is preparing her for her long awaited adventure, which is to work at the Obstetrics and Gynaecology department of the Benedictine hospital in Nongoma, Kwazulu-Natal, South Africa starting in January 2015.

Joehoe – het boek is af!

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